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3B BlackBio Biotech India

3B DNA POLYMERASE 1 U/µl

PRODUCT	FORMAT	REF.
3B DNA Polymerase (1U/µl) – Standard Reaction Buffer	500 U	3B001
3B DNA Polymerase (1U/µl) – Standard Reaction Buffer	1000 U	3B002
3B DNA Polymerase (1U/µl) – Buffer $MgCl_2$ Free	500 U	3B005
3B DNA Polymerase (1U/µl) – Buffer $MgCl_2$ Free	1000 U	3B006
3B DNA Polymerase (1U/µl) – Standard Reaction Buffer + dNTP mix	500 U	3B019
3B DNA Polymerase (1U/µl) – Standard Reaction Buffer + dNTP mix	1000 U	3B020
3B DNA Polymerase (1U/µl) – Buffer $MgCl_2$ Free + dNTP mix	500 U	3B024
3B DNA Polymerase (1U/µl) – Buffer $MgCl_2$ Free + dNTP mix	1000 U	3B025

1. GENERAL CONSIDERATIONS

3B BlackBio Biotech DNA Polymerase is a modified thermostable recombinant DNA polymerase from the thermophilic bacterium Thermus sp. expressed in E. coli. The general characteristics of 3B DNA Polymerase make the enzyme suitable for applications requiring a highly thermostable and processive enzyme capable of synthesizing DNA strands at elevated temperatures in amplification or similar reactions (e.g. primer extension), thus resolving the most complex secondary structures.

Due to its processivity and accuracy 3B DNA Polymerase allows the generation of long templates with a base misincorporation rate (1-10 x 10^{-6} bp) lower than most commercial Taq DNA polymerases.

The procedure employed for the purification of thermostable enzymes is proprietary of 3B BlackBio Biotech India Limited. It involves a simple and non chromatographic procedure which renders a top high yield and quality enzyme.

*The enzyme is supplied at a **concentration of 1 U/µl** in a storage buffer. This concentration allows accurate pipetting of small amounts.*

Product applications:

- Standard PCR
- Multiplex PCR
- In situ PCR
- DNA sequencing
- Other reactions requiring DNA synthesis at high temperature

2. ENZYME FEATURES

Concentration:	1 U/µl
Performance:	
Working concentration	20-25 mU/µl
pH	8-9
Elongation temperature	72ºC
$MgCl_2$ concentration	2 mM
Size of PCR products:	Up to 5 Kb
PCR cloning:	T/A
Endonuclease activity:	No
Reverse transcriptase activity:	No
5´→3´exonuclease activity:	Yes
3´→5´exonuclease activity:	No
Nicking activity	No

Note:
*This enzyme is **not** recommended for certain experiments dealing with amplification of sequences homologous to those found in E. coli.*

3. STORAGE CONDITIONS

Store package components at -20ºC in a constant temperature freezer. Under these conditions enzyme activity remains unaltered over a **24 months** period. . The glycerol present in the storage buffer prevents the enzyme from freezing at -20ºC. If the enzyme is frozen, its activity is not altered.
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

4. PRODUCT SPECIFICATIONS

Unit Definition- One unit is defined as the amount of enzyme which incorporates 10 nanomoles of dNTPs into acid-insoluble DNA within 30 min at 72 ºC.

Storage Buffer- 10 mM Tris-HCl (pH 8.0); 50 mM KCl; 1 mM EDTA; 0.1% Triton X-100; 50% glycerol (v/v).

10X Reaction Buffer- 750 mM Tris HCl (pH 9.0), 500 mM KCl, 200 mM $(NH_4)_2SO_4$. The **10X STANDARD REACTION BUFFER with $MgCl_2$ includes 20 mM $MgCl_2$** in its composition.

5. GENERAL ASPECTS OF REACTION COMPONENTS

Enzyme Concentration

As an initial guide we recommend employing the following enzyme units/rxn.

Final reaction volume	Recommended enzyme units
100 µl	Up to 2.5 U
50 µl	1-1.25 U
25 µl	0.5-0.625 U

The addition of higher quantities of enzyme generally does not produce significant yield increase. Only for certain applications or when working on long DNA fragment amplifications (longer than 2 Kb from genomic DNA) it might be necessary to increase the concentration.

DNA Template

The quality and quantity of the DNA template affects both the sensitivity and efficiency of the amplification. High amounts of DNA usually increase the amplification of nonspecific PCR products.

The PCR is inhibited by various compounds e.g. ionic detergents, phenol, gel loading dyes, etc. If the template contains traces of inhibitors, reduce the amount of the DNA included in the amplification reaction, or repurify the template by ethanol precipitation and several washing steps.

dNTPs Concentration

Generally equal concentrations of all four dNTPs are used. The concentration of each dNTP should be 50-500 µM, being 200 µM the most commonly used concentration. 3B offers equimolar mixes of dNTPs (10 mM and 25 mM each).

The concentration of dNTPs may be decreased (e.g. when unspecific amplification occurs), increased (e.g. for long amplifications), or even unbalanced in favour of any of the particular dNTPs (e.g. in vitro mutagenesis experiments). 3B DNA Polymerase is suitable for its use with modified dNTPs (e.g. radioactively or fluorescein labelled) as substrates. It can also be used with dUTP and other analogues.

The dNTPs behave as potent Mg^{2+} chelating agents reducing therefore the availability of free Mg^{2+} for polymerase activity. Thus an increase in dNTPs should be accompanied by an increase in $MgCl_2$ concentration.

Reaction Buffer

The provided buffer has been specially formulated to facilitate the amplification of any PCR products. It creates the appropriate stringent conditions for primer-annealing over a wide range of temperatures. Moreover, the *Standard Reaction Buffer* with $MgCl_2$ includes Mg^{2+} at the optimal concentration for most experiments (final concentration: 2 mM).

$MgCl_2$ Concentration

The optimal $MgCl_2$ concentration may vary depending on the primer and template that are used and must be determined by experimentation. In most cases, a final concentration of $MgCl_2$ at 2 mM in the reaction mix works well.

High concentrations of $MgCl_2$ may promote low enzyme fidelity and non specific amplification products; whereas low concentrations should reduce the yield of the desired amplification products. If the samples contain any chelating metal agents such as EDTA, the concentration of $MgCl_2$ should be increased accordingly.

Primer Design

PCR primers are usually 15-30 nucleotides in length with a content of 40-60% G+C residues. To avoid primer-dimer and hairpin formation the primers should not be self-complementary or complementary to any other primer present in the reaction mixture. The annealing temperature of the primers should be similar (< 5ºC variation). Length and G+C content of primers are used to predict their annealing temperature to the template DNA. The 5´end of a primer may contain mismatches between the primer and template, whereas this is not recommended at the 3´end.

PCR Additives

In certain cases the presence of DMSO, betaine, formamide or any other PCR additives might be necessary for optimized complex PCR reactions. The provided enzyme and buffer are compatible with most PCR additives. When calculating the annealing temperature for the PCR cycling program, it is important to take into account that certain additives may decrease the melting temperature of the primers.

6. STANDARD PROTOCOL

Optimal conditions must be determined for each individual experimental system.

Proceed to the Reagent Preparation Area in a laminar flow cabinet. Wear disposable gloves and use sterile and nuclease free plastic material in order to avoid contaminations and false negative results.

1. Thaw reagents at room temperature or on ice. After complete thawing, mix the reagents well, spin down in a bench-top centrifuge and keep on ice.

2. Prepare a master mix in a sterile microcentrifuge tube according to Table 1. For each experiment include at least one negative control (without template DNA). To ensure sufficient volume for all desired reactions include additional reactions in the calculations.

TABLE 1. Master Mix preparation

COMPONENT	Final Concentration	50 µl rxn	20 µl rxn
Master Mix			
10X REACTION BUFFER	1X	5 µl	2 µl
50 mM $MgCl_2$ solution*	1.5-4 mM	1.5-4 µl	0.6-1.6 µl
dNTP Mix 10 mM each	200 µM of each	1 µl	0.4 µl
Primers	variable	variable	variable
DNA Polymerase (1 U/µl)	20-25 mU/µl	1-1.25 µl	0.4-0.5 µl
Sterile bidistilled water	-	Up to 50 µl	Up to 20 µl
Template DNA	**Variable**	**Variable**	**Variable**

*not necessary for 10X Standard Reaction Buffer because it includes $MgCl_2$

3. Mix the master mix thoroughly and keep on ice. Distribute the appropriate volume into each vial.

Proceed to DNA Purification Area separate from other sources of DNA.

4. Add the template DNA to each reaction vial. Close the vials and mix gently. For thermal cycler without heated lid overlay a mineral oil layer.

Proceed to the Amplification Area

5. Program the thermal cycler according to the guide of the amplification program (see Table 2 and Section 7). Place the vials in the thermal cycler and perform the selected PCR program.

TABLE 2. Standard Amplification Program

CYCLE STEP	Nº CYCLES	TEMPERATURE	TIME
Initial Denaturation	1	94ºC	3-10 min**
Denaturation Annealing Extension	25-35*	94ºC T_m-5ºC 72ºC	5-60 sec 30-60 sec 60 sec/1 Kb
Final Extension	1	72ºC	5-15 min
Cooling	∞	4ºC	∞

**Optimize the time, the temperature and the number of cycles of the PCR.*
***Depending on the template*

7. GUIDE TO AMPLIFICATION PROGRAM

Initial Denaturation Step-Incomplete denaturation of the PCR reaction results in an inefficient first amplification cycle and low amplification yield. However, the denaturation must be kept as short as possible in order to avoid inactivation of the enzyme. For most samples 94ºC for 3-5 min should be satisfactory; templates rich in G+C often require a longer step (up to 10 minutes).

Denaturation Step-The PCR product synthesized in the amplification cycling is shorter than the template DNA and therefore needs a short denaturation step; 5-60 sec of denaturation at 94ºC should be sufficient.

Primer Annealing Step-In general for primers < 20 bases the optimal annealing temperature is equal to the T_m of the lowest T_m primer. To find the optimal annealing temperature, you can use a temperature gradient. Start using an annealing temperature 5ºC below T_m of the primers. If primers have a high T_m a two step cycling is recommended.

Extension Step-The annealed primers must be extended at 70-75ºC. The extension time depends on the size of the expected product. For 3B DNA Polymerase we recommend 1 min for each Kb of expected product.

Number of PCR Cycles-Cycling program usually consists on 25-35 cycles. This parameter depends on the amount of starting material and the expected yield. In certain experiments, increasing the number leads to an increase in nonspecific products. You should experimentally determine the optimal number of cycles.

Final Extension Step-After the last PCR cycle the sample should be incubated at 72ºC for 5-15 min. The DNA polymerase fills the protruding ends of the newly synthesized PCR products and adds extra adenine nucleotides to the 3´ends of the PCR product.

8. TROUBLESHOOTING

Problem	Cause	Recommendation
Low yield or no amplification product	Missing reagent or pipetting error	Check concentration and storage conditions of dNTPs, primers, etc. Repeat the PCR.
	DNA template problems	Check the concentration and quality of starting material. If the template is difficult e.g. rich in G+C sequences we recommend adding DMSO to the master mix. Repeat the PCR with a new dilution of template or with a new DNA purification.
	Problems with primers	Revise the primers design and the primers storage condition. Avoid any design prone to the formation of primer dimmers. Repeat PCR with different primer concentration from 0.1-0.5 µM in 0.1 increments. Check primer degradation on a denaturing polyacrylamide gel.
	Enzyme concentration too low	Increase enzyme concentration in 0.2 U increments.
	$MgCl_2$ concentration	Optimise $MgCl_2$ concentration of the PCR if necessary (1.5-4 mM)
	Incorrect PCR cycling conditions	Check the following parameters of the PCR program: **Denaturation-** Increase time and temperature of initial denaturation. **Annealing-** Optimise the annealing temperature and time. **Extension time-** Increase extension time by increments of 30 sec. **Number of cycles-**Perform additional cycles by increments of 5 cycles. Verify the final elongation step.
Nonspecific amplification products or background smear	Annealing temperature too low	Increase the annealing temperature in increments of 1ºC.
	Problems with primers	Design alternative primers. Both primers should be present at the same concentration (0.1-0.5 µM). Decrease primer concentration by increments of 0.1 µM . Check primer degradation on a denaturing polyacrylamide gel.
	Excess of DNA template	Use dilutions of your template.
	Enzyme concentration too high	Optimise polymerase concentration of the PCR if necessary
	$MgCl_2$ concentration	Optimise $MgCl_2$ concentration of the PCR if necessary (1.5-4 mM)
	Incorrect PCR cycling conditions	To increase the specificity you can perform a touchdown or step-down PCR. Reduce the number of cycles.
PCR products in negative control	Carryover contamination	Exchange all reagents.