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3B BlackBio Biotech India

3B NATIVE DNA POLYMERASE 1 U/µl

PRODUCT	FORMAT	REF.
3B Native DNA Polymerase 1U/µl-Standard reaction Buffer	100U	3B055
3B Native DNA Polymerase 1U/µl-Standard reaction Buffer	250U	3B056
3B Native DNA Polymerase 1U/µl-Reaction Buffer $MgCl_2$ Free	100U	3B059
3B Native DNA Polymerase 1U/µl- Reaction Buffer $MgCl_2$ Free	250U	3B060

1. DESCRIPTION

3B Native DNA Polymerase is a thermostable enzyme from *Thermus thermophilus* HB-27, which is also over-expressed, isolated and purified from *T. thermophilus*. In the presence of magnesium chloride this polymerase catalyses DNA synthesis, in the 5´→3´ direction, with a processivity and fidelity similar to that of the *Taq* DNA Polymerase.

This enzyme is highly purified using a proprietary procedure that guarantees a highly pure enzyme free of contaminating bacterial DNA. Our Native DNA Polymerase avoids the frequent PCR false positives resulting from bacterial DNA.

The ability of **3B Native DNA Polymerase** to replicate DNA at elevated temperatures (above 70 °C) minimises the problems encountered with strong secondary structures in DNA. Higher temperatures also result in increased specificity of primer hybridization and extension.

Standard Applications:

- Especially recommended for PCR from bacterial DNA
- Amplification of DNA targets with secondary structures
- Standard PCR

2. ENZYME CHARACTERISTICS

Concentration:	1 U/µl
Performance:	
Working concentration	20-25 mU/µl
pH	8-9
Extension temperature	> 70ºC
Concentration of $MgCl_2$	2 mM
Amplified PCR products:	Up to 5 Kb
Cloning:	T/A
Endonuclease activity:	No
Reverse Transcriptase Activity:	Not detectable
5´→3´Exonuclease Activity:	Yes
3´→5´Exonuclease Activity	No
Nicking activity	No

3. PRODUCT SPECIFICATIONS

Unit Definition- One unit is defined as the amount of enzyme that incorporates 10 nmol of total nucleotide into acid-precipitable form in 30 minutes at 72 ºC

Storage Conditions- Store all components of the kit at **-20ºC**, in a constant temperature freezer (do not use frost-free freezers). Under these conditions enzyme activity remains stable for **24 months**. The glycerol present in the storage buffer prevents the enzyme from freezing at -20ºC. If the enzyme is frozen, its activity is not altered.

Storage Buffer- 20 mM Tris-HCl (pH 8.0); 50 mM KCl; 0.25% NP40; and 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 $MgCl_2$ also includes 20 mM of $MgCl_2$ in its composition.

Contaminating Activity Assays- 3B Native DNA Polymerase is free of detectable non-specific DNase and RNase activities. This enzyme is also **free of contaminating bacterial DNA.**

4. GENERAL ASPECTS OF AMPLIFICATION REACTION

Enzyme Concentration

3B Native DNA Polymerase is suitable for standard and specialised PCR applications. As an initial guide we recommend employing the following enzyme units per reaction.

Final reaction volume	Recommended enzyme units
100 µl	Up to 2.5 U
50 µl	1-1.25 U
25 µl	0.5-0.75 U

The addition of higher quantities of enzyme generally does not produce significant yield increase. Only for certain applications (e.g. PRIND or Primed In Situ Synthesis) or when working with 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. 3B recommends the use of Speedtools kits for extraction and purification of different DNAs.

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 DNA template contains traces of inhibitors, reduce the amount of DNA included in the amplification reaction, or repurify it by ethanol precipitation and several washing steps.

dNTPs Concentration

The premixing of the four dNTPs at equal concentrations prevents problems of misincorporation, which could occur if an unbalanced dNTP mix is used. For 3B Native DNA Polymerase, a concentration range of 50-500 µM per each dNTP is recommended for optimal product yield (200 µM each is the most commonly used).

The concentration of dNTPs may be decreased (e.g. when unspecific amplification occurs), inreased (e.g. for long amplifications), or even unbalanced in favour of any of the particular dNTPs (e.g. in vitro mutagenesis experiments).

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.

3B Native 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.

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) which facilitates the use of the enzyme.

$MgCl_2$ 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.

Primers 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), the G+C content and length has to be selected accordingly. The C and G nucleotides should be distributed uniformly. More than three G or C at the 3´ end should be avoided. Primers with a T at the 3´end are prone to mismatch.

Whereas the 5´ end of a primer may content mismatches between the primer and template, the 3´ end has to include complementary bases. Thus when working with degenerated primers avoid degeneracy at the 3´end.

For primers < 20 bases the annealing temperature is determined by that of the primer with the lowest Tm. As a starting point use an annealing temperature of less than 5ºC of the calculated Tm. The following equation can be used to estimate the melting temperature for primers < 25 bases.

$$Tm = 2\,(A+T) + 4\,(G+C)$$

For primers > 25 bases it is necessary to use specialised computer programs accounting for the interaction between bases, salt concentration and other factors.

Regarding the concentration of the primers we recommend starting with 0.2 µM. In case of poor PCR efficiency increase the concentration in 0.1 µM increments. The concentration range is 0.1-1 µM.

5. STANDARD PROTOCOL

Reaction conditions should be optimal for each experiment.

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 and preserve them in ice during their manipulation. Reaction tubes should also be kept in ice before thermal cycling.

2.- Prepare a Master Mix for the appropriate number of samples to be amplified (see Table 1). The inclusion of positive and negative amplification controls is recommended.

TABLE 1. Preparation of Master Mix

COMPONENT	Final Concentration	Final Volume 50 µl /rxn	Final Volume 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
NATIVE DNA POLYMERASE (1 U/µl)	20-25 mU/µl	1-1.25 µl	0.4-0.5 µl
Sterile bidistilled water	-	Up to final volume	Up to final volume
DNA Template	**Variable**	**Variable**	**Variable**

* not necessary for 10X Standard Reaction Buffer because it includes $MgCl_2$ to give a final concentration of 2 mM

3. Aliquot the Master Mix into PCR tubes

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

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

Proceed to the Amplification Area

5. Perform PCR using optimised cycling conditions (see Table 2). Place the vial in a thermal cycler and perform the selected PCR program.

TABLE 2. Standard Amplification Program

CYCLE STEP	Nº CYCLES	TEMPERATURE	TIME
Initial Denaturation	1	94ºC	3-5 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	∞

**Optimise time,temperature and number of cycles.*
***Depending on template*

6. GUIDE TO AMPLIFICATION PROGRAM

Initial Denaturation- 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 mins should be satisfactory. Templates rich in G+C content often require longer initial denaturation and the length of this step can be extended up to 10 minutes.

Denaturation-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.

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

Extension-The annealed primers must be extended at 70-75ºC. The extension time depends on the size of the expected product. Recommended extending time for 3B Native DNA Polymerase is approximately 1 min per 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 of cycles leads to an increase in nonspecific products and consequently to a decrease in the yield of specific product. You should experimentally determine the optimal number of cycles for your experiment.

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

8. TROUBLESHOOTING

Problem	Cause	Solution
Little or no amplification detected	Missing reagent or pipetting error	Check the concentration and storage conditions of dNTPs, primers, etc. Repeat the PCR and make sure that no pipetting errors or missing reagents occur.
	Problems with the template DNA	Check the concentration and quality of the starting material. If the template is difficult e.g. rich in GC 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 the primers	Revise the primer design and primer condition. Avoid any designs prone to the formation of primer dimmers. Repeat PCR with different primer concentration from 0.1-0.5 µM in 0.1 µM increments. Check primer degradation on a denaturing polyacrylamide gel.
	Concentration of $MgCl_2$	Perform reactions with varying concentration of Mg^{2+} starting at 1.5 mM up to 4 mM, in 0.25 mM increments
	Low enzyme concentration	Increase the enzyme-concentration in 0.2 U increments
	Cycling parameters	Check the following parameters of the amplification program: **Denaturation-** Increase the temperature of initial denaturation up to 95°C. Increase the length of initial temperature denaturation up to 5 minutes. **Annealing-** Lower the annealing temperature in 2ºC increments. To increase the specificity perform a touchdown or step-down PCR cycling. **Extension time-** Increase the extension time by increments of 30 sec. **Number of cycles-** Perform additional cycles in increments of 5 Verify or include a **final elongation** step.
Multiple products or a smear detected	Primer design	Review the primer design and primer condition. Both primers must have the same concentration. Titrate concentration of primers. Repeat PCR with different primer concentrations, from 0.1-0.5 µM in 0.1 µM increments. Check primer degradation by electrophoresis in a denaturing acrylamide gel
	Too much DNA template	Check the concentration of DNA by agarose gel electrophoresis or fluorimetry. Use a serial dilution of the template. Organic extraction followed by ethanol precipitation may remove some amplification inhibitors
	Carryover contamination	Always prepare a negative control PCR (no template). If the NC shows a PCR product or a smear exchange all reagents.
	Enzyme-concentration too high	Decrease the amount of enzyme added to the reaction
	Cycling parameters	Increase annealing temperature in 2°C increments. Decrease number of cycles in decrements of 5 Perform touchdown or step-down PCR cycling.
	Concentration of $MgCl_2$	Perform reactions with varying concentration of Mg^{2+} starting at 1.5 mM up to 4 mM, in 0.25 mM increments
PCR products in negative control	Carryover contamination	Repeat the PCR exchanging all the reagents.