Ed.20- April 11

3B BlackBio Biotech India

7-C, Industrial Area, Govindpura
Bhopal-462 023

Tel. +91-755-4077847
Fax +91-755-4282659
E-mail: info@3bblackbio.com,
orders@3bblackbio.com
Website: www.3bblackbio.com

3B BlackBio Biotech India

3B *Pfu* DNA POLYMERASE 1 U/µl

PRODUCT	FORMAT	CAT Nº
3B Pfu DNA Polymerase (1U/µl) – Standard Reaction Buffer	100 U	3B047
3B Pfu DNA Polymerase (1U/µl) – Standard Reaction Buffer	250 U	3B048
3B Pfu DNA Polymerase (1U/µl) – Reaction Buffer $MgCl_2$ Free	100 U	3B051
3B Pfu DNA Polymerase (1U/µl) – Reaction Buffer $MgCl_2$ Free	250 U	3B052

1. GENERAL CONSIDERATIONS

3B *Pfu* DNA Polymerase is a highly termostable polymerase with proof-reading activity. It is a recombinant protein from the hiperthermophilic bacterium *Pyrococcus furiosus* expressed in *E. coli* (see Note 1).

3B *Pfu* DNA polymerase is suitable for applications which require a proof-reading, highly thermostable and processive enzyme capable of synthesising DNA strands at elevated temperatures in amplification reactions or similar (e.g. primer extension). *Pfu* DNA polymerase has an error rate 10-fold lower than non proof-reading DNA polymerases.

The enzyme is free of unspecific endonuclease activity, as well as nicking activities. It does not exhibit nucleotidyl terminal transferase activity so its amplification products can be directly used for cloning in blunt-ended vectors.

*The enzyme is supplied at a **concentration of 1 U/µl** in a storage buffer. This concentration allows accurate pipetting of small amounts of the DNA polymerase, so that further dilutions are not necessary.*

Product Applications:

- Standard PCR and other reactions requiring DNA synthesis at high temperature.
- Multiplex PCR
- In situ PCR

2. ENZYME FEATURES

Concentration:	1 U/µl
Optimal activity:	
Enzyme concentration	20-40 mU/µl
pH	8-9
Elongation temperature	72ºC
$MgCl_2$ concentration	2 mM
Size of PCR products:	Up to 5 Kb
PCR cloning:	Blunt ends
Endonuclease activity:	No
Reverse transcriptase activity:	No
5´→3´exonuclease activity:	No
3´→5´exonuclease activity:	Yes
Nicking activity:	No

Note 1:

*This enzyme is **not** recommended for certain experiments dealing with amplification of sequences homologous to those found in E. coli* or amplifications with very low annealing temperatures (*e.g. RAPDs, Random Amplified Polymorphic DNAs)*

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, its activity is not altered.**

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 minutes at 72 ºC.

Storage buffer- 20 mM Tris-HCl (pH 8.0); 50 mM KCl; 0.25 % NP 40; 0.25 % Tween 20; 40 % 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 $MgCl_2$ in its composition.**

5. GENERAL ASPECTS OF REACTION COMPONENTS

Enzyme Concentration

3B *Pfu* DNA Polymerase is suitable for standard and specialized 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 Units
50 µl	1-1.25 Units
25 µl	0.5-0.75 Units

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

dNTPs Concentration

Generally equal concentrations of all the four dNTPs are used. The concentration of each dNTP should be 50-500 µM, being 200 µM the most commonly used concentration.

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).

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 *Pfu* DNA Polymerase is suitable for its use with modified dNTPs (e.g. radioactively or fluorescein labelled) as substrate. 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* 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.

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 has to be selected accordingly. 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 by gentle vortexing, 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.

Adding the Pfu DNA polymerase at the end is recommended to avoid oligonucleotide degradation because of its 3'-5' exonuclease activity (maximum is added in the absence of dNTPs).

TABLE 1. Master Mix preparation

COMPONENTS	Final Concentration	50 µl rxn	20 µl rxn
Master Mix			
10X REACTION BUFFER	1X	5 µl	2 µl
50 mM $MgCl_2$*	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
Pfu DNA Polymerase (1 U/µl)	20-40 mU/µl	1-2 µl	04-0.8 µl
Sterile bidistilled water	-	To final volume	To final volume
Template DNA	**Variable**	**Variable**	**Variable**

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

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 section 6). 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	5-30 sec 30-60 sec 2 min/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 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 Step-The PCR product synthesized in the amplification cycling is shorter than the template DNA and therefore needs a short denaturation step; 5-30 seconds 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 5 ºC bellow T_m of the primers. If the 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. Recommended extending time for 3B *Pfu* DNA Polymerase is 2 min for each Kp 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 Step-After the last PCR cycle the sample should be incubated at 72ºC for 5-15 min. The *Pfu* DNA polymerase does not add extra adenine nucleotides to the 3´ends of the PCR products.

8. TROUBLESHOOTING

Problem	Cause	Recommendation
Low yield or no amplification product	Missing reagent or pipetting error	Check the concentration and storage conditions of dNTPs, primers, etc. Repeat the PCR..
	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 primers design and the primers 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.
	Mg^{2+} concentration	Repeat the PCR with a different Mg^{2+} concentration (1.5-4 mM). Try with 1.5-4 mM in 0.25 mM increases.
	Low enzyme concentration	Increase the enzyme concentration in 0.2 U increments.
	Incorrect PCR cycling conditions	Check the following parameters of the amplification program: **Denaturation-**increase the temperature and length of initial denaturation. **Annealing-** optimise the annealing temperature and time . To increase the specificity perform a touchdown or step-down PCR. **Extension time-** in case the extension time is short increase the extension time by increments of 30 sec. **Cycle number-**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.
	Primer design	Review the primer design and primer condition. Both primers must have the same concentration. Titrate the primer concentration. Repeat PCR with different primer concentration from 0.1-0.5 µM in 0.1 increments. Check primer degradation on a denaturing polyacrylamide gel.
	Too much DNA template	Use a serial dilution of the template.
	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	Optimise the concentration of enzyme in your experiment.
	Low specificity	Perform a touchdown or step-down PCR. Reduce the number of cycles
	Mg^{2+} concentration *	Repeat the PCR with a different Mg^{2+} concentration (1.5-4 mM). Try with 1.5-4 mM in 0.25 mM increases.
PCR products in negative control	Carryover contamination	Exchange all the reagents.