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BIOTOOLS B&M LABS.S.A.

BIOTOOLS *Pfu* DNA POLYMERASE (1 U/µl)

REF.	FORMAT	CONTENT
10.501	100 U	**BIOTOOLS *Pfu* DNA Polymerase (1 U/µl)** 10X Standard Reaction Buffer with $MgCl_2$
10.502	250 U	**BIOTOOLS *Pfu* DNA Polymerase (1 U/µl)** 10X Standard Reaction Buffer with $MgCl_2$
10.511	100 U	**BIOTOOLS *Pfu* DNA Polymerase (1 U/µl)** 10X Reaction Buffer $MgCl_2$ FREE
10.512	250 U	**BIOTOOLS *Pfu* DNA Polymerase (1 U/µl)** 10X Reaction Buffer $MgCl_2$ FREE

Store at -20ºC

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. GENERAL CONSIDERATIONS

BIOTOOLS *Pfu* DNA Polymerase is a highly thermostable polymerase with proof-reading activity. In addition to 5'-3' DNA polymerase activity, it also possesses 3'-5' exonuclease (proofreading) activity. It is a recombinant protein isolated from *Pyrococcus furiosus* expressed in *E. coli*.

BIOTOOLS *Pfu* DNA polymerase is recommended for use in PCR and primer extension reactions that require high fidelity. *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:

- High-fidelity PCR
- Long and complex amplifications
- Gene cloning
- Mutation analysis
- Standard PCR
- In situ PCR

2. ENZYME FEATURES

Concentration:	1 U/µl
Optimal activity:	
Enzyme concentration	20-50 mU/µl
pH	8-9
Elongation temperature	72-75 ºC
$MgCl_2$ 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

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

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

Biotools *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	2.0-2.5 Units
50 µl	1.0-1.25 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

The concentration of each dNTP in amplification reactions 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.

Biotools *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

The proofreading activity of *Pfu* DNA Polymerase may degrade the amplification primers resulting in nonspecific amplification and reduced product yield. To overcome primer degradation, longer primers (20-35 bases) with maximized G+C content can be used. Primers can also be protected by introducing a single phosphorothioate bound at their 3´end.

To avoid primer-dimer and hairpin formation the primers should not be self-complementary or complementary to any other primer present in the reaction. The annealing temperature of the primers should be similar (< 5ºC variation).

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 complex amplifications the presence of DMSO, betaine, formamide or any other PCR additives might be necessary. 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.

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

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

Note 1: 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
Nuclease-free water	-	To 50 µl	To 20 µl
Pfu DNA Polymerase (1U/µl)**	20-50 mU/µl	1.0-2.5 µl	0.4-1.0 µl
Template DNA	**Variable**	**Variable**	**Variable**

*not necessary for 10X Standard Reaction Buffer, it includes $MgCl_2$
**See Note 1

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 Table 2. 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	∞

Depending on the template (see Point 7).
**Optimize time; temperature; and number of cycles (see Point 7).*

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- To find the optimal annealing temperature, you can use a temperature gradient. Start using an annealing temperature 5 ºC below the 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. Due to the proofreading activity of the *Pfu* DNA Polymerase, it requires more time for extension as compared to other polymerases. An extension time of 2 min for every 1 kb to be amplified should be used.

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 assays, 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 each 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 concentration and storage conditions of dNTPs, primers, etc. Repeat the PCR including all reagents.
	DNA template problems	Check the concentration and quality of starting material. Repeat the PCR with a new dilution of template or with a new DNA purification. If the template is difficult e.g. rich in G+C sequences we recommend adding DMSO to the master mix.
	Problems with primers	Revise the primers design and the primers storage condition. Check primer degradation on a denaturing polyacrylamide gel. 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. It is critical to add the *Pfu* DNA Polymerase to the amplification reaction last, particularly following the addition of dNTP (see Note 1).
	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 (see point 7): **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.
Nonspecific amplification products or background smear	Problems with primers	Check primer degradation on a denaturing polyacrylamide gel. 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.
	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	Increase the annealing temperature in increments of 1ºC and/or reduce the time of this step. Reduce the number of cycles.
PCR products in negative control	Carryover contamination	Exchange all reagents.

9. ORDERING INFORMATION

Components	References			
	10.501	**10.502**	**10.511**	**10.512**
Biotools *Pfu* DNA Polymerase (1 U/µl)	100 U	250 U	100 U	250 U
10X Standard Reaction Buffer with $MgCl_2$	1.8ml	1.8ml		
10X Reaction Buffer $MgCl_2$ FREE			1.8ml	1.8ml
50 mM $MgCl_2$ Solution			1.8ml	1.8ml