Produced by:
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BIOTOOLS
BIOTOOLS B&M LABS.S.A.

BIOTOOLS HOTSPLIT DNA POLYMERASE (5 U/µl)

REF.	FORMAT	CONTENT
10.562	500 U	**BIOTOOLS HotSplit DNA Polymerase (5 U/µl)** 10X Reaction Buffer MgCl2 FREE
10.563	1000 U	**BIOTOOLS HotSplit DNA Polymerase (5 U/µl)** 10X Reaction Buffer MgCl2 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 HotSplit DNA Polymerase is a hot start polymerase with enhanced efficiency and fidelity. The technology behind *BIOTOOLS HotSplit DNA Polymerase* is based on the use of thermolabile blocking groups acting over the amino-acids residues involved in enzyme polymerization. The polymerase activity is inhibited at low temperatures and the full activity is restored during the initial denaturation step, when amplification reactions are heated at 94-95 °C for 5 minutes.

HotSplit DNA Polymerase is advantageous for some amplification targets because it may eliminate or minimize primer-dimer and nonspecific products. Additionally, hot-start polymerase may improve reaction yields.

*The enzyme is supplied at a **concentration of 5 U/µl** in a storage buffer.*

Applications:

- High sensitivity PCR
- High specificity PCR
- Standard PCR
- Multiplex PCR
- qPCR

2. PRODUCT ESPECIFICATIONS

Concentration:	5 U/µl
Performance:	
Working concentration	20-40 mU/µl
pH	8-9
Extension temperature	72ºC
Concentration of $MgCl_2$	2 mM
Amplified PCR products:	Up to 5 Kb
Cloning:	T/A
Endonuclease activity:	No
Reverse Transcriptase Activity:	No
5´→3´Exonuclease Activity:	Yes
3´→5´Exonuclease Activity	No
Nicking activity	No

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** (i.e. do not use frost-free freezers)**.** Under these conditions enzyme activity remains stabilised until its expiration date printed 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, 1 mM EDTA, 0.1% Triton X-100 and 50% glycerol.

10X Reaction Buffer $MgCl_2$ FREE: 750 mM Tris HCl (pH 9.0), 500 mM KCl, and 200 mM $(NH_4)_2SO_4$.

5. GENERAL ASPECTS OF REACTION COMPONENTS

Enzyme concentration

As initial guide it is recommended to employ the following enzyme units per reaction. Addition of higher quantities of enzyme generally does not produce significant yield increase, only for certain application it might be necessary to increase the amount of enzyme used.

Final reaction volume	Recommended enzyme units
100 µl	2.0-3.0 Units
50 µl	1.0-1.5 Units
25 µl	0.5-0.75 Units

DNA Template

The quality and quantity of the DNA template affects both the sensitivity and efficiency of the amplification. Biotools recommends the use of Speedtools kits for extraction and purification of different DNAs.

High amounts of DNA usually increase the amplification of nonspecific 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 DNA included in the amplification reaction, or repurify it 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 HotSplit 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 buffer provided with the kit (10X Reaction Buffer $MgCl_2$ FREE) has been specially formulated to facilitate the amplification of any PCR product. It creates the appropriate stringent conditions for primer-annealing over a wide range of temperatures. The Reaction buffer is free of any detergent.

PCR Additives

The technology developed for the enzyme is not compatible with certain PCR additives such as DMSO. Before employing an additive of the PCR is advisable to check it in a control reaction.

$MgCl_2$ Concentration

The optimal concentration of $MgCl_2$ in the reaction needs to be determined experimentally by the users; the recommended range is 1.5-4 mM.

High concentration of $MgCl_2$ may promote low enzyme fidelity and non specific products, whereas low concentrations reduce the yield of the desired amplification product. 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 5´end of a primer may contain mismatches between the primer and template, whereas this is not recommended at the 3´end.

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 optimal quantity of template and primers must be determined empirically for each combination of template and primer. The recommended concentration range of each primer is 0.1-1.0 µM. The range 0.2 µM-0.5 µM works for most amplification; use 0.2 µM of each primer as starting point of optimisation.

6. STANDARD PROTOCOL

Reaction conditions should be optimised for each experiment.

Keep the enzyme Biotools HotSplit DNA polymerase under refrigerated conditions at all the times.

Proceed to the Reagent Preparation Area in a laminar flow cabinet.

1.- Thaw and preserve reagents in ice during their manipulation. 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.

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
$MgCl_2$ (50 mM)*	1.5-4 mM	1.5-4 µl	0.6-1.6 µl
dNTPs Mix	200 µM each	x µl	x µl
Primers	0.1-1.0 µM	x µl	x µl
***HotSplit* DNA Polymerase (5U/µl)**	20-40 mU/µl	0.2-0.4 µl	0.08-0.16 µl
Nuclease-free water	-	Up to 50 µl	Up to 20 µl
Template DNA	**Variable**	**Variable**	**Variable**

* Optimise the concentration of $MgCl_2$ good results have been obtained with 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. Program the thermal cycler according to Table 2. Place the vial in a thermal cycler and perform the selected program.

TABLE 2. Standard Amplification Program

STEP	Nº CYCLES	TEMPERATURE	TIME
Initial Denaturation*	1	94ºC	**5-6 min***
Denaturation Annealing Extension	25-35**	94ºC XºC 72ºC	5-60 sec 30-60 sec 60 sec/1 kb
Final Extension	1	72ºC	5-15 min
Cooling	∞	4ºC	∞

**Full enzyme activity is regained during the initial denaturation step.*

***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 inefficiency first amplification cycle and poor PCR yield. **A 5-minute initial denaturation step at 94-95 °C is required to activate the BIOTOOLS HotSplit DNA Polymerase**. Templates rich in G+C content require longer initial denaturation (up to 10 min).

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- 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 primers have a high Tm, a two-step cycling without annealing step is recommended.

Extension Step- The annealed primers must be extended at 70-74ºC. The extension time depends on the size of the expected product. Recommended extending time for *Biotools HotSplit DNA polymerase* is approximately 1 min/ 100 bp 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 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.

Final Extension Step- After the last PCR cycle the sample should be incubated at 72ºC for 5-15 min. The *BIOTOOLS HotSplit 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	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.
	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.
	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 parameters of the PCR program (see Point 7): **Denaturation-** A 5-minute initial denaturation step at 94-96 °C is required to activate the enzyme. **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 decrements of 0.1 µM.
	Excess of DNA template	Reduce the volume of template DNA in the reaction, or dilute the template DNA prior to addition.
	Enzyme concentration too high	Decrease enzyme units, in 0.2 U decrements.
	$MgCl_2$ 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	
	10.562	**10.563**
Biotools HotSplit DNA Polymerase (5 U/µl)	500 U	2 x 500 U
10X Reaction Buffer $MgCl_2$ FREE	2 x 1.8ml	3 x 1.8ml
50 mM $MgCl_2$ Solution	2 x 1.8ml	2 x 1.8ml