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3B HOTSPLIT DNA POLYMERASE 1 U/µl

PRODUCT	FORMAT	REF.
3B Hot Split DNA Polymerase (1U/µl)	250 U	3B041
3B Hot Split DNA Polymerase (1U/µl)	500 U	3B042
3B Hot Split DNA Polymerase (1U/µl)	1000 U	3B043

1. GENERAL CONSIDERATIONS

3B BlackBio Biotech HotSplit DNA Polymerase has been designed and developed to **overcome the problems due to nonspecific priming, primer-dimer formation or other unwanted reactions occurring at low temperature** during the PCR process.

Different approaches to hot start techniques have been successfully employed in commercial PCR kits. The most common is the use of antibodies which binds to the enzyme in a reversible temperature manner. At low temperature enzyme activity is prevented due to binding of the antibody to the enzyme, whereas at high temperature the antibody is released and enzyme activity is recovered. Another method consists of entrapping the enzyme in a bead which melts at high temperature. Other techniques rely on a chemical modification of the enzyme.

The technology behind 3B HotSplit DNA Polymerase is based on the use of thermolabile bocking groups acting over the amino-acids residues involved in enzyme polymerization. Full enzyme activity is recovered after a **short activation time during the initial denaturation step**.

This formulation provides the following features to our HotSplit DNA Polymerase:

- Enhanced enzyme specificity and sensitivity
- Increase the obtained yield
- Reduce mispriming and primer-dimer formation

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

Applications:

- Standard PCR
- Multiplex PCR
- DNA labelling
- Difficult PCR because the target sequence has a low copy number
- Problematic PCR reactions due to primer-dimers

2. PRODUCT ESPECIFICATIONS

Concentration: ..... 1 U/µl
Performance:
Working concentration ..... 20-40 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: ..... 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** (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, 50% glycerol (v/v).

10X Reaction Buffer $MgCl_2$ FREE: Recommended reaction buffer without Mg^{2+}. Its composition is as follows: 750 mM Tris HCl (pH 9.0), 500 mM KCl, 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	Up to 2.5 Units
50 µl	1-1.25 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. 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 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 1.5-3 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. 3B recommends start with a concentration of 1.5-2mM $MgCl_2$. 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.

6. STANDARD PROTOCOL

Reaction conditions should be optimal for each experiment.

Note:
Keep the enzyme 3B HotSplit DNA polymerase under refrigerated conditions at all the times.

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
$MgCl_2$ (50 mM)*	1.5-3 mM	1.5-3 µl	0.6-1.2 µl
dNTPs Mix	200 µM each	x µl	x µl
Primers	variable	x µl	x µl
***HotSplit* DNA Polymerase (1U/µl)**	20-40 mU/µl	1-2 µl	0.4-0.8 µl
Sterile bidestilled water	-	Up to final volume	Up to final volume
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. 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

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 the time, the temperature and the number of cycles of the PCR.*

7. GUIDE TO AMPLIFICATION PROGRAM

Initial Denaturation- Incomplete denaturation of the PCR reaction results in an inefficiency first amplification cycle and poor PCR yield. Keep the denaturation as short as possible in order to avoid enzyme inactivation. **For *3B HotSplit DNA Polymerase* 94ºC for 5-6 mins initial denaturation is satisfactory**. Templates rich in G+C content require longer initial denaturation.

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 HotSplit DNA polymerase* is approximately 1 min/ 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.

Final Extension- After the last PCR cycle the sample should be incubated at 72ºC for 5-15 min. The *3B 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	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. 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 3 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 and/or the time of the step. **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. **Nº 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 3 mM, in 0.25 mM increments
PCR products in negative control	Carryover contamination	Repeat the PCR exchanging all the reagents.