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3B DNA POLYMERASE GEL FORM

PRODUCT	FORMAT	REF.
3B DNA Polymerase Gel Form p96 Plates (2U/well)	10 plates	3B074
3B DNA Polymerase Gel Form p96 Plates (2U/well)	20 plates	3B075
3B DNA Polymerase Gel Form Vials –12x8 strip vials (2U/vial)	96 vials	3B078

1. PRODUCT DESCRIPTION

Aliquoted vials/plates with 3B DNA Polymerase in gel form, dNTPs and Reaction Buffer in its composition. In this way, only primers and DNA template to a final volume of 50 µl have to be added, minimising the handling steps and risk of contaminations.

3B DNA Polymerase in gel form* represents a step forward in respect to its liquid counterpart because it is stored at 4ºC, and can be shipped and handle at room temperature. Two different formats are available: "Ready-to-Use" Vials (96 x 0.2 ml vials) and "Ready-to-Use 96 Well Plate".

TABLE 1. Advantages of gelification technology

GEL FORM	
One tube one reaction	√
Stable at 4ºC	√
Shipping at room temperature	√
Reaction set-up at room temperature	√
Saving in consumables (vials, tips, etc.)	√
Allow open field experiments	√
Less handling steps	√
Non user expertise required	√

3B DNA Polymerase is a recombinant, modified form of the enzyme from a thermophilic bacterium that belongs to *Thermus* genus expressed in *E. coli* (see Note 1). It is a highly thermostable DNA polymerase suitable for applications requiring a thermostable and processive enzyme capable of synthesising DNA strands at elevated temperatures in DNA amplification reactions or similar (e.g. primer extension), thus resolving the most complex secondary structures.

****Gelification technology is covered by an international 3B B&M Labs. patent***

Product applications are:

- High throughput PCR

To perform PCR, only the addition of a template and primers is required. The 3B DNA Polymerase gel form can be used with conventional PCR machines.

- End-Point PCR

Amplified products can be purified with any commercial Clean-Up Kits for purification of PCR products (Speedtools PCR Clean-Up Kit Cat. No. 21.201/2).

2. ENZYME PROPERTIES

Enzyme concentration:	1 U/rxn
Optimal *Elongation temperature*	72ºC
Size of PCR products:	Up to 5 Kb
PCR cloning:	T/A cloning
Endonuclease activity:	No
Reverse transcriptase activity:	No
5´→3´exonuclease activity:	Yes
3´→5´proofreading activity:	No
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.*

3. STORAGE CONDITIONS AND PRODUCT STABILITY

Product is stable until the expiration date shown in the main product label. Store "ready-to-use" vials/plates at 4ºC. Shipping as well as reaction setup can be performed at room temperature without the need of using ice.

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 72ºC.

5. GENERAL ASPECTS OF REACTION COMPONENTS

DNA Template

The quality and quantity of DNA template affects both the sensitivity and efficiency of the amplification.High amounts of DNA usually increase the yield 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 trace amounts of inhibitors reduce or dilute the volume of the DNA template included in the amplification reaction, or repurify the template by ethanol precipitation and several washing steps.

Reaction Buffer and dNTPS Concentration

The buffer provided with the kit 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. Reaction buffer includes $MgCl_2$ to a final concentration of **2 mM**.

The purity and quality of the dNTPs give a confidence in the performance of the enzyme. The final concentration of dNTPs is **200 µM**.

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), 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 have 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 lower Tm. As starting point use an annealing temperature bellow 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 with more than 25 bases is necessary to use specialised computer programs accounting for the interaction between bases, salt concentration and other factors.

Regarding the concentration of the primers is recommended starting with a 0.2 µM concentration, in case of poor PCR efficiency increase the concentration in 0.1 µM increments. The concentration range is 0.1-1 µM.

PCR Additives

In certain cases the presence of DMSO, betaine, formamide or any other PCR additive might be necessary for optimization of difficult amplification reactions. The enzyme and buffer provided with this kit are compatible with most PCR additives. When calculating the annealing temperature for the PCR cycling program, it should be noted that certain additives may decrease the melting temperature of the primers.

6. PROTOCOL OF USE

Optimal conditions vary among experiments and therefore must be determined for each individual experimental system.

Wear disposable gloves and make use of sterile, DNase- and RNase-free pipette tips in order to avoid contaminations and false negative results. It is advisable to work with the primers in the reagent preparation area and with the template in a separate area for DNA. Thaw primers and DNA template on ice. After complete thawing mix well by gentle vortexing, spin down in a bench-top centrifuge and keep on ice.

1.- Calculate the number of needed reactions do not forget to include a negative control (without template DNA). **Add primers and DNA template** at the appropriate concentration to each vial/well. **Complete with bidistilled sterile water to 50 µl final reaction volume** (*gel volume can be discarded for volume calculations).*

Final reagents concentration is as follows: 200 µM dNTP, 1 X Reaction Buffer, 1 Units for the vials/ 1 Unit in each well plate. The concentration of $MgCl_2$ for the Standard Reaction Buffer is 2 mM.

2.- To perform Hot Start reaction **do not resuspend the vial/well content**. The reagents will be suspended during the initial denaturation step 94 ºC for 5-10 min**.**

3.- For thermal cycler without heated lid overlay a mineral oil layer. Close the amplification vials.

Proceed to the Amplification Area

4.- Program the thermal cycler according to the guide to 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	5-10 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	∞

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

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. However, the denaturation must be keep as short as possible in order to avoid enzyme inactivation. For most samples 94ºC for 5-10 min initial denaturation is satisfactory. Templates rich in G+C content require longer initial denaturation.

Denaturation Step-The PCR product synthesized in the amplification cycling is shorter than the template DNA and therefore need a short denaturation step. A denaturation step of 5-60 sec at 94ºC is sufficient.

Primer Annealing Step-In general for primers < 20 bases the optimal annealing temperature is equal to the T_m of the lower T_m primer. To find the optimal annealing temperature a temperature gradient can be used. Start using an annealing temperature 5ºC bellow T_m of the primers.

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 DNA Polymerase is 1 min for each 1000 bp of expected product.

Number of PCR Cycles-Cycling program usually consists of 25-35 cycles. This parameter depends on the amount of starting material in the reaction mix and on 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. Determine experimentally 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 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
No amplification product or low yield	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 for possible degradation. If the template is difficult e.g. rich in GC sequences it is recommended to add DMSO to the enzyme mixture. Repeat the PCR with a new dilution or starting, or with a new template 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.
	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 (see section 6). To increase the specificity perform a touchdown or stepdown PCR. Extensión 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.
	Mg^{2+} concentration not optimal	Repeat the PCR with different Mg2+ concentration from 1.5-4 mM in 0.25 increments.
Multiple non specific amplification products or background smear	Annealing temperature too low	Increase the annealing temperature by increments of 1ºC.
	Problems with the primers	Revise the primer design and the primer condition. Both primers must have the same concentration. Titrate 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.
	Carry-over contamination	Always prepare a negative control PCR (no template). If the NC shows a PCR product or a smear exchange all the reagents.
	Enzyme concentration too high	Optimise the concentration of enzyme in your experiment.
	Low specificity	Perform a touchdown or stepdown PCR. Cycling program with too many cycles. Reduce the number of cycles.
	Mg^{2+} concentration not optimal	Repeat the PCR with different Mg2+ concentration from 1.5-4 mM in 0.25 increments.
PCR products in negative control	Carryover contamination	Exchange all the reagents.