

TaqBead™ Hot Start Polymerase

INSTRUCTIONS FOR USE OF PRODUCT M5661.

TaqBead™ Hot Start Polymerase

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1. Description

Hot-start PCR protocols increase amplification specificity and amplicon yield by creating conditions that minimize the possibility of nonspecific priming, primer-dimer formation or other reactions, which can occur at low temperatures once all the PCR reaction components are mixed.

Unwanted side reactions occurring during the PCR process usually begin at room temperature. Examples of several such reactions are illustrated in Figure 1. In general, hot-start techniques limit the availability of one essential reaction component until a higher temperature (>60°C) is reached. This can be done manually by the addition of the critical component when the reaction mixture reaches the higher temperature (1); however, this method is tedious and can increase the chances of contamination. Other techniques incorporate the critical substance in a wax bead, which melts at the higher temperature, releasing the missing component (2,3). Another method involves use of an antibody to the polymerase, which at lower temperatures binds the enzyme and prevents polymerization. At higher temperatures, the antibody binding is reversed, releasing a functional polymerase (4).

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*TaqBead*TM Hot Start Polymerase^(a) facilitates hot-start PCR by keeping the enzyme sequestered in paraffin wax until the reaction temperature reaches approximately 60°C. This technique increases PCR specificity by keeping the polymerase separate from the rest of the reaction components until a critical temperature is reached, thus decreasing the probability of amplifying products that are the result of nonspecific binding of primers to each other or to template DNA.

The physical characteristics of *TaqBead*TM Hot Start Polymerase are listed in Table 1.

Table 1. Characteristics of *TaqBead*TM Hot Start Polymerase.

Source	<i>Thermus aquaticus</i> DNA polymerase
Appearance	White, spherical
Wax	Paraffin
Weight	12.5mg/bead
Diameter	3mm
Performance	PCR performance test
Melting Temperature	60°C

Note: *TaqBead*TM Hot Start Polymerase wax beads **do not** form a vapor barrier in a 0.5ml reaction tube.

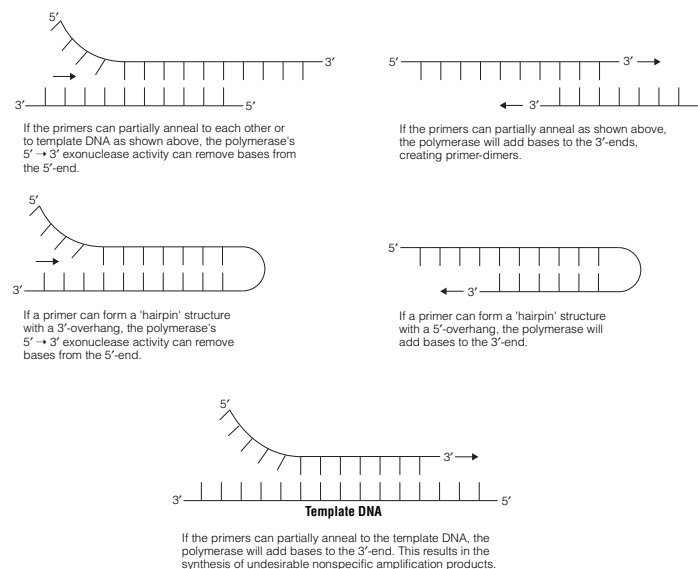


Figure 1. Examples of unwanted reactions that occur at lower temperatures prior to PCR amplification.

2. Product Components and Storage Conditions

Product	Size	Cat.#
<i>TaqBead</i> TM Hot Start Polymerase	100 beads	M5661

Includes:

- 100 *TaqBead*TM Hot Start Polymerase wax beads (1.25u/bead*)
- 750µl Thermophilic DNA Polymerase 10X Reaction Buffer, Mg-Free
- 750µl 25mM Magnesium Chloride Solution

*To prepare *TaqBead*TM Hot Start Polymerase, *Taq* DNA Polymerase is incorporated into wax beads. In the process of bead formation, approximately 1.25 units of enzyme are incorporated into each bead. We have therefore assigned a nominal value of 1.25 units to each bead and recommend using this nominal value in applications using *TaqBead*TM Hot Start Polymerase.

Storage Conditions: Store at -20°C.

3. *Taq*Bead™ Hot Start Polymerase PCR Protocol

Materials to Be Supplied by the User

- dNTPs (10mM each)
- template DNA
- downstream oligonucleotide primer
- upstream oligonucleotide primer
- nuclease-free light mineral oil (e.g., Sigma-Aldrich Cat.# M5904). **Do not autoclave**
- 0.5ml thin-walled reaction tubes
- Nuclease-Free Water

Note: The use of aerosol resistant tips is strongly recommended.

1. Using sterile forceps or a sterile needle, carefully add one bead to a thin-walled 0.5ml reaction tube. Use one bead for each 50µl reaction.
2. Combine the following components in each reaction tube:

Reaction Component	Volume	Final Concentration
Thermophilic DNA Polymerase 10X Reaction Buffer	5µl	1X
dNTP mix (10mM of each dNTP)	1µl	0.2mM each
25mM MgCl ₂	3µl	1.5mM
downstream primer	50pmol*	1µM
upstream primer	50pmol*	1µM
template DNA	yµl**	—
Nuclease-Free Water to final volume	50µl	

* A general formula for calculating the number of nanograms of primer equivalent to 50pmol is: ng of primer = 50pmol × 0.33 × b; where b is the number of bases in the primer.

** If possible, start with >10⁴ copies of the target sequence to obtain a signal in 25–30 cycles (see Table 2), but keep the final DNA concentration of the reaction at ≤10ng/µl. Less than 10 copies of a target can be amplified, but more cycles may be required to detect a signal by gel electrophoresis. Additional cycles may increase nonspecific amplification, evidenced by smeared bands upon gel electrophoresis.

Note: If working with multiple samples, prepare a master mix consisting of appropriate multiples of the listed reaction components (except template) and add the appropriate volume such that, after template addition, the final volume is 50µl. Use individual pipette tips for all additions, being careful not to cross-contaminate the samples. See Section 4.E for more information.

3. When melted, the *Taq*Bead™ Hot Start Polymerase wax beads do not form a complete barrier over the reaction surface in a 0.5ml tube. Consequently, some evaporation and condensation may occur. **We recommend that the reaction be overlaid with one or two drops of light mineral oil unless your thermal cycler has a heated lid.**
4. Place the tubes into the preheated, 95°C block of the thermal cycler and proceed with the thermal cycling profile chosen for your reactions.
5. Chill the completed reactions to 4°C; the mineral oil/paraffin wax overlay will solidify.
6. Using a sterile pipette tip, make a hole in the center of the mineral oil/paraffin wax overlay. Use a fresh pipette tip for each tube to prevent cross-contamination of samples.
7. Insert a fresh pipette tip through the hole in the mineral oil/wax overlay to remove the PCR reaction products.

To facilitate optimization, troubleshooting and validation of PCR amplifications, we strongly recommend including both positive and negative (no target) control reactions.

4. General Considerations for Successful PCR

4.A. Magnesium Concentration

Magnesium concentration is a crucial factor affecting the performance of *Taq* DNA Polymerase. Reaction components, including template DNA, chelating agents (e.g., EDTA or citrate), dNTPs and proteins all affect the amount of free magnesium in the PCR reaction mixture. In the absence of adequate free magnesium, *Taq* DNA Polymerase is inactive (5). Conversely, excess free magnesium reduces enzyme fidelity (6) and may increase the level of nonspecific amplification (7,8). For these reasons, it is important to empirically determine the optimal magnesium concentration for each reaction. To do so, prepare a reaction series containing 1.5–3.0mM Mg²⁺ in 0.5mM increments by adding 3, 4, 5 or 6µl of the 25mM MgCl₂ stock to 50µl reactions.

*Taq*Bead™ Hot Start Polymerase wax beads are supplied with a magnesium-free 10X Reaction Buffer and a tube of 25mM MgCl₂. This allows adjustments of the Mg²⁺ concentration to optimal levels for each reaction.

Notes: We have identified two important steps for use of MgCl₂ solutions. These two steps, though seemingly simple, eliminate the source of many failed experiments.

1. **It is important to completely thaw the Magnesium Chloride solution prior to use.**
2. **Vortex the Magnesium Chloride solution for several seconds prior to pipetting.** Magnesium Chloride solutions form a concentration gradient when frozen and vortexing is required to obtain a uniform solution.

4.B. Primer Design

PCR primers generally range in length from 15–30 bases and are designed to flank the DNA sequence of interest. Primers should contain 40–60% G+C and care should be taken to avoid sequences that produce internal secondary structure. The 3'-ends of the primers should not be complementary to avoid the production of primer-dimers in the PCR reaction. Avoid three G or C nucleotides in a row near the 3'-end of the primer. Ideally, both primers should anneal at the same temperature. The annealing temperature is dependent upon the primer with the lowest melting temperature (T_m).

Numerous formulae exist to determine the theoretical T_m of nucleic acids (9,10). These may serve as a useful starting point for optimizing annealing conditions. It is best to optimize the annealing conditions by performing the reaction at several temperatures, starting approximately 5°C below any calculated T_m . Visit our web site for a melting temperature calculator (www.promega.com/biomath) or use the formula below to estimate the melting temperature for oligonucleotides:

$$T_m = 81.5 + 16.6 \times (\log_{10}[\text{Na}^+]) + 0.41 \times (\%G + C) - \frac{675}{n}$$

where $[\text{Na}^+]$ is the molar salt concentration, $[\text{K}^+$ or $\text{Na}^+]$; and n = number of bases in the oligonucleotide.

Example:

To calculate the melting temperature of a 22mer oligonucleotide with 60% G+C in 50mM KCl:

$$T_m = 81.5 + 16.6 \times (\log_{10}[0.05]) + 0.41 \times (60) - \frac{675}{22}$$

$$T_m = 81.5 + 16.6 \times (-1.30) + 24.60 - 30.68 = 53.84^\circ\text{C}$$

The sequence of the primers can also include regions at the 5'-ends that are useful for downstream applications. For example, restriction enzyme sites can be designed at the 5'-end of primer pairs if the desired PCR product is to be subsequently cloned. Caution should be used, however, since all restriction enzymes may not cut efficiently close to the end of DNA fragments (11).

Regardless of primer choice, the final concentration of primers in the reaction must be optimized. We recommend adding 50pmol of primer (1 μ M final concentration in a 50 μ l reaction) as a starting point for optimization.

4.C. Template Considerations

Successful amplification of the region of interest is dependent upon the amount and quality of the template DNA. Reagents commonly used to purify nucleic acids (e.g., salts, guanidine, proteases, organic solvents and SDS) are potent inhibitors of DNA polymerases. Spiking a positive control DNA fragment and the appropriate primer pair into a PCR reaction containing the target DNA preparation is a useful means of verifying the purity of a particular DNA sample. A final ethanol precipitation of the nucleic acid sample will eliminate most inhibitory agents.

The amount of template required for successful amplification is dependent upon the complexity of the DNA sample. For example, in a 4kb plasmid containing a 1kb insert, 25% of the input DNA is the target of interest. Conversely, a 1kb gene in the human genome (3.3×10^9 bp) represents approximately 0.00003% of the input DNA. Consequently, approximately 1,000,000-fold more human genomic DNA is required to maintain the same number of target copies per reaction. Two common mistakes are the use of too much plasmid DNA or too little genomic DNA. If possible, start with $>10^4$ copies of the target sequence to obtain a signal in 25–30 cycles, but keep the final DNA concentration of the reaction at ≤ 10 ng/ μ l.

Table 2. Number of Molecules of Template Derived from 1 μ g of Various Common Sources of DNA and RNA.

Source	Number of Molecules
1 μ g of 1kb dsDNA	9.12×10^{11} molecules
1 μ g of pGEM [®] Vector DNA	2.85×10^{11} molecules
1 μ g of lambda (λ) DNA	1.9×10^{10} molecules
1 μ g of <i>E. coli</i> genomic DNA	2×10^8 molecules
1 μ g of human genomic DNA	3.04×10^5 molecules
1 μ g of 1kb RNA	1.77×10^{12} molecules

4.D. Cycle Parameters

Primer sequences are a major consideration in determining the temperature of PCR amplification cycles. For primers with a high T_m , it may be advantageous to increase the annealing temperature. The higher temperature minimizes nonspecific primer annealing, increasing the amount of specific product produced and reducing the amount of primer-dimer formation.

During the extension step, allow approximately 1 minute for every 1kb of amplicon (minimum extension time = 1 minute). Generally, 25–40 cycles are sufficient for most PCR reactions.

4.E. Nucleic Acid Cross-Contamination

It is important to take great care to minimize the potential for cross-contamination between samples and to prevent carryover of DNA from one experiment to the next. Use separate work areas and pipettes for pre- and post-amplification steps. Use positive displacement pipettes or aerosol resistant tips to reduce cross-contamination during pipetting. Wear gloves and change them often.

Use UNG^(b) (12) or another contamination control technique to prevent DNA carryover to subsequent reactions.

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: techserv@promega.com

Symptoms	Causes and Comments
Low yield or no amplification product	Suboptimal reaction conditions. Optimize Mg ²⁺ concentration, annealing temperature and extension time. Verify that primers are present in equal concentrations.
	Insufficient number of cycles. Return reactions to thermal cycler for five more cycles.
	Template degraded. Verify the integrity of the DNA by electrophoresis.
	Thermal cycler programmed incorrectly. Verify that times and temperatures are correct. Use step cycles, not hold segments.
	Temperature too low in some positions in the thermal cycler. Perform a set of control reactions to determine if certain positions in the thermal cycler give low yields.
	Top of thermal cycler open. The top must be closed for correct heating and cooling.
	Inhibitor present in the reaction. Reduce the volume of sample DNA in the reaction. Ethanol precipitate template DNA to remove inhibitors.
	Improper reaction conditions. Reduce the annealing temperature and/or allow longer extension times.
Missing reaction component. Check the reaction components and repeat the reaction.	

Symptoms	Causes and Comments
Low yield or no amplification product (continued)	Mineral oil problem. If your thermal cycler does not have a heated lid, the reaction must be overlaid with high-quality, nuclease-free light mineral oil. Do not use autoclaved mineral oil.
	Reaction tubes. Autoclaving tubes eliminates contaminants that inhibit amplification.
	Poor primer design. Make sure primers are not self-complementary or complementary to each other. Try a longer primer.
	Incorrect primer specificity. Verify that the primers are complementary to the appropriate DNA sequences.
Multiple nonspecific amplification products	Primer concentration too low. Verify primer concentration in the reaction. Increase if necessary.
	Nucleotides degraded. Store nucleotides frozen at -20°C in aliquots, thaw quickly and keep on ice once thawed. Avoid multiple freeze-thaw cycles.
	Target sequence not present in template DNA. Redesign experiment or try other DNA templates.
	Poor primer design. Make sure primers are not self-complementary or complementary to each other, especially near the 3'-ends. Try a longer primer. Avoid using three G or C nucleotides in a row at the 3'-end of a primer.
	Primer concentration too high. Verify primer concentration in the reaction. Try a lower concentration.
	Contamination by another target DNA. Use positive displacement pipettes or aerosol resistant tips to reduce cross-contamination during pipetting. Use separate work areas and pipettes for pre- and post-amplification procedures. Wear gloves and change them often. Use UNG (12) or another contamination control technique to prevent DNA carryover to subsequent reactions.
	Multiple target sequences exist in template DNA. Design new primers with higher specificity to target sequence.

6. Composition of Thermophilic DNA Polymerase 10X Reaction Buffer

500mM	KCl
100mM	Tris-HCl (pH 9.0 at 25°C)
1.0%	Triton® X-100

7. Related Products

DNA Purification

Product	Size	Cat.#
Wizard® SV Gel and PCR Clean-Up System	50 preps	A9281
	250 preps	A9282

For Laboratory Use.

dNTPs

Product	Size	Cat.#
dATP, 100mM	40µmol	U1201
dCTP, 100mM	40µmol	U1221
dGTP, 100mM	40µmol	U1211
dTTP, 100mM	40µmol	U1231
dUTP, 100mM	40µmol	U1191
dATP, dCTP, dGTP, dTTP, each at 100mM	40µmol of each	U1240
	10µmol of each	U1330
dUTP, dCTP, dGTP, dATP	10µmol of each	U1335

For Laboratory Use.

Thermostable DNA Polymerases

Product	Size	Cat.#
GoTaq® Green Master Mix	100 reactions	M7112 ¹ , M7122 ²
	1,000 reactions	M7113 ¹ , M7123 ²
GoTaq® Colorless Master Mix	100 reactions	M7142 ¹ , M7132 ²
	1,000 reactions	M7143 ¹ , M7133 ²

GoTaq® Master Mixes are premixed solutions containing GoTaq® DNA Polymerase, GoTaq® Reaction Buffer (Green or Colorless), dNTPs and Mg²⁺.

¹Cat.#s M7112, M7113, M7142 & M7143 are available in Europe or through Distributors supported by Promega European Branch Offices.

²Cat.#s M7122, M7123, M7132 & M7133 are available in all other countries, including the United States. For Laboratory Use.

Product	Size	Cat.#
GoTaq® DNA Polymerase	100u	M3171 ¹ , M3001 ²

Available in additional sizes.

¹Cat.# M3171 is available in Europe or through Distributors supported by Promega European Branch Offices.

²Cat.# M3001 is available in all other countries, including the United States. For Laboratory Use.

Product	Size	Cat.#
GoTaq® Flexi DNA Polymerase	100u	M8301 ¹ , M8291 ²

GoTaq® Flexi DNA Polymerase includes 5X Green GoTaq® Flexi Buffer, 5X Colorless GoTaq® Flexi Buffer and Magnesium Chloride Solution, 25mM. Reaction buffers are magnesium-free. Available in additional sizes.

¹Cat.# M8301 is available in Europe or through Distributors supported by Promega European Branch Offices.

²Cat.# M8291 is available in all other countries, including the United States. For Laboratory Use.

Product	Size	Cat.#
<i>Tth</i> DNA Polymerase	100u	M2101
<i>Tfi</i> DNA Polymerase*	100u	M1941
<i>Tli</i> DNA Polymerase*	50u	M7101

*For Laboratory Use. Larger sizes are available.

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