

primer that can partially anneal to the second primer, or to template DNA, may result in 5' → 3' exonuclease removal of bases (**Panel A**) or addition of bases at the 3'-end (**Panel B**). Likewise, similar polymerase activities may occur to a primer that can self-hybridize forming a 'hairpin' structure (**Panels C and D**). **Panel E** illustrates the formation of a primer-dimer when two primers with complementary 3'-ends anneal.

Manual hot start methods require the researcher to withhold a critical component, usually magnesium or the polymerase, until the reaction has been heated. The withheld component then is added to initiate the reaction. This method requires extra manipulations of the sample and increases the risk of contamination.

A second method, barrier hot start, uses a physical barrier (e.g., wax) to separate a critical component from the template and primers (2-4). Most barrier methods use wax and require a two-step approach. The wax bead is melted and allowed to cool, forming a barrier of solidified wax over the aqueous phase. The critical component is then added to the surface of the solidified wax layer. Upon reheating, the wax barrier melts, allowing the final component to mix with the pre-added components in the aqueous phase.

Finally, a third method of hot start amplification is reversible polymerase inactivation. The polymerase is reacted with an antibody (5) or oligonucleotide aptamer (6) that binds to the polymerase's nucleotide binding domain, rendering the polymerase inactive. Upon heating, the compound dissociates from the polymerase, restoring enzyme activity.

Promega's *TaqBead*TM Hot Start Polymerase combines aspects of the barrier and polymerase inactivation techniques (7,8) by providing *Taq* DNA Polymerase sequestered in a paraffin wax bead. **Figure 2** illustrates the ease of using the *TaqBead*TM Hot Start Polymerase for PCR amplification. In this manner, the polymerase can be held separate from, but in the same tube as, the remaining reaction components. Upon heating, *Taq* DNA Polymerase is released from the wax and is able then to mix with the remaining reaction components. Each wax bead contains sufficient polymerase for a 50µl amplification reaction in a 0.5-0.6ml tube.

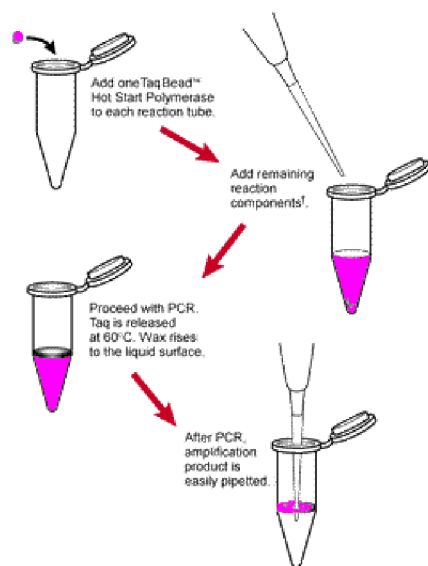


Figure 2. PCR using *TaqBead*TM Hot Start Polymerase. Performing hot start PCR in this manner is more convenient and reduces the chances for contamination as the polymerase is added at the same time as the remaining reaction components.

*TaqBead*TM Hot Start Polymerase

Primer-dimer formation during PCR amplification can occur due to poor primer design or failure to perform a hot start. Primer pairs that are complementary at their 3'-ends can anneal to each other (Figure 1E). The annealing is favored by the high primer concentrations, typically 50pmol of each primer (1µM each in a 50µl reaction). Primer extension from the 3'-ends creates a small double-stranded primer-dimer.

The formation of primer-dimers consumes primers and dNTPs, frequently reducing the yield of the desired amplification product (**Figure 3**). The primer-dimer migrates more slowly than the primers on an agarose gel. Because the product is double-stranded, it stains more intensely with intercalating agents (e.g., ethidium bromide) than the single-stranded primers. Hot start amplification facilitated by the *TaqBead*TM Hot Start Polymerase significantly reduces the amount of primer-dimer, as compared to a conventional "cold start" reaction.

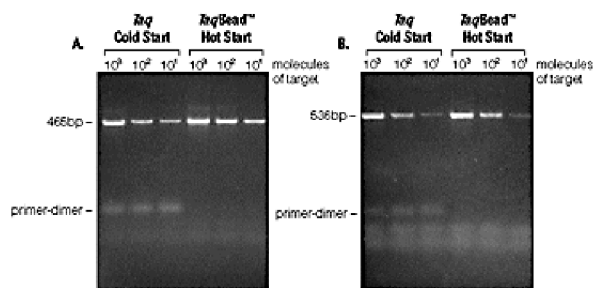


Figure 3. Hot start amplification reduces the yield of primer-dimers. Genomic DNA (1,000, 100 or 10 molecules of target) was amplified by PCR using Promega's *Taq* DNA Polymerase (Storage Buffer B) or *TaqBead*TM Hot Start Polymerase in Promega Reaction Buffer supplemented with 2mM MgCl₂. Both sets of reactions were assembled at room temperature and placed in a thermal cycler at room temperature. The thermal cycler temperature was ramped to 94°C as rapidly as possible and the samples were denatured at 94°C for 2 minutes prior to cycling. The reaction products were analyzed on a 4% gel (3% NuSieve[®] GTG[®] agarose/1% agarose; FMC Corporation) and visualized by staining with ethidium bromide. Lanes 1-3, PCR products amplified with *Taq* DNA Polymerase; Lanes 4-6, PCR products amplified with *TaqBead*TM Hot Start Polymerase. **Panel A:** A 465bp 16S ribosomal RNA gene product amplified from bacterial genomic DNA. **Panel B:** A 536bp β-globin gene product amplified from human genomic DNA.

At reduced temperatures, the high concentration of primers can result in primers annealing to the template at positions that are not fully complementary. *Taq* DNA Polymerase can extend these mismatched primers if there is sufficient complementarity at the 3'-end. This primer extension, which can occur at low temperatures, stabilizes the complex, allowing it to remain annealed even after the reaction temperature is increased. Primer extension from misprimed sites is responsible for the smear frequently observed on an agarose gel (Figure 4). The *TaqBead*TM Hot Start Polymerase sequesters the polymerase until the reaction reaches 60°C. At this temperature, most of the mismatched primers melt off the template and are unavailable for primer extension, significantly reducing the level of nonspecific amplification.

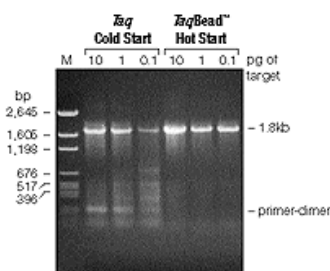


Figure 4. Hot start amplification reduces the yield of nonspecific amplification products. Aliquots of pGEM^{®(d)}-*luc* Vector (10, 1 or 0.1pg) were diluted in 30ng of human genomic DNA. A 1.8kb luciferase gene product was amplified by PCR using Promega's *Taq* DNA Polymerase (Storage Buffer B) or *TaqBead*TM Hot Start Polymerase in Promega Reaction Buffer supplemented with 2mM MgCl₂. Both sets of reactions were assembled and denatured as described in Figure 3. The reaction products were analyzed on a 1.2% agarose gel and visualized by staining with ethidium bromide. Lane M, pGEM[®] DNA Markers (Cat.# G1741); Lanes 1-3, PCR products amplified with *Taq* DNA Polymerase; Lanes 4-6, PCR products amplified with *TaqBead*TM Hot Start Polymerase.

^(d)U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

Additional considerations

Primer design, magnesium concentrations and cycling conditions all can affect significantly the quality of the amplification reaction. Effective PCR primers generally range in length from 18-30 bases, contain 40-60% G+C and contain sequences that avoid producing internal secondary structure. Primers should demonstrate minimal complementarity at their 3'-ends to minimize the potential for primer-dimer formation (see Figure 1E). Also, when designing primers, avoid placing three G or C nucleotides in a row at the 3'-end of the sequence; GC-rich regions at the 3'-end can stabilize mismatched primers, increasing the level of nonspecific amplification products.

Optimization of the magnesium concentration frequently results in increased product yield and minimal nonspecific amplification. Reaction components, including template DNA, chelating agents (e.g., EDTA or citrate), dNTPs, primers and proteins all affect the

amount of free magnesium in the PCR mixture. In the absence of adequate free magnesium, *Taq* DNA Polymerase is inactive (9). Conversely, excess free magnesium may increase the level of nonspecific amplification (10,11). *TaqBead*TM 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 magnesium concentration to optimal levels for each reaction.

High annealing temperatures tend to minimize nonspecific primer annealing, thereby increasing the amount of specific product and decreasing primer-dimer formation. Design primers with a melting temperature (T_m) greater than 50°C, and avoid excessive rounds of amplification. A total of 25-30 cycles usually is adequate for the visual detection of the sequence of interest.

Summary

Promega's *TaqBead*TM Hot Start Polymerase facilitates hot start PCR by keeping the enzyme sequestered in a paraffin wax bead until the reaction temperature reaches approximately 60°C. This technique increases PCR specificity and yield of the desired product by keeping the polymerase separate from the rest of the reaction components until a critical temperature is reached. This greatly decreases the probability of amplifying products which are the result of binding of primers to each other or to nonspecific template DNA.

References

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Ordering Information

Product	Cat.#
<i>TaqBead</i> TM Hot Start Polymerase, 1.25 units/bead, Nonbarrier	M5661

This product includes beads for 100 amplification reactions.

Editor's Note: *TaqBead*TM Hot Start Polymerase wax beads **do not** form a vapor barrier in 0.5-0.6ml reaction tubes.

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