

TECH TIP #209

HotStart-IT®: A Novel Hot Start PCR Method Based on Primer Sequestration

Introduction

A common problem with the polymerase chain reaction is the formation of non-specific products, especially primer-dimers. These unwanted products interfere with generating the desired amplicons. A major cause for this lack of specificity occurs during reaction set-up and before the initial denaturation step. At these lower temperatures before denaturation, primers can bind to non-target sequences with incomplete homology, such as other primers⁽¹⁾. These non-specific hybrids can be extended by the polymerase, even at lower temperatures, which creates competing targets during subsequent PCR cycling (e.g. primer-dimers). Hot start PCR methods provide a solution to this lack of specificity by reducing or eliminating non-specific product formation before high-temperature cycling⁽²⁾.

Current hot start methods target the polymerase by muting its activity before the initial denaturation step, most commonly with a blocking antibody or chemical modification. Problems with these methods are two-fold: 1) the antibody is from hybridoma cells which can contaminate reactions with mammalian DNA; and 2) removal of the chemical blocking group on the polymerase typically requires initial denaturation times of

greater than 10 minutes which causes heat-damage to DNA samples. An ideal solution is a hot start method that eliminates the risk of contaminating mammalian nucleic acids while also allowing shorter initial denaturation times.

To address these issues, USB Corporation has developed an alternative hot start strategy called HotStart-IT®. This novel method uses a single-stranded DNA binding protein to sequester primers at lower temperatures, making them unavailable for extension by the polymerase. Following the initial denaturation step, the binding protein is inactivated and the primers are free to participate in the amplification reaction (Fig. 1). This technique solves the problems that arise from other hot start methods because the binding protein is produced in *E. coli* and is heat-labile so that initial denaturation times are 2 minutes or less. Thus, there is no chance of mammalian genomic DNA contamination and less chance of heat-induced DNA damage. Another advantage is that because the method targets the primers and not the polymerase, it is portable to a variety of thermostable polymerases. USB has demonstrated that the “primer-sequestration” technique effectively blocks non-specific product formation before thermal-cycling and enhances end-point and real-time PCR reactions.

USB HotStart-IT® Method: Primer Sequestration

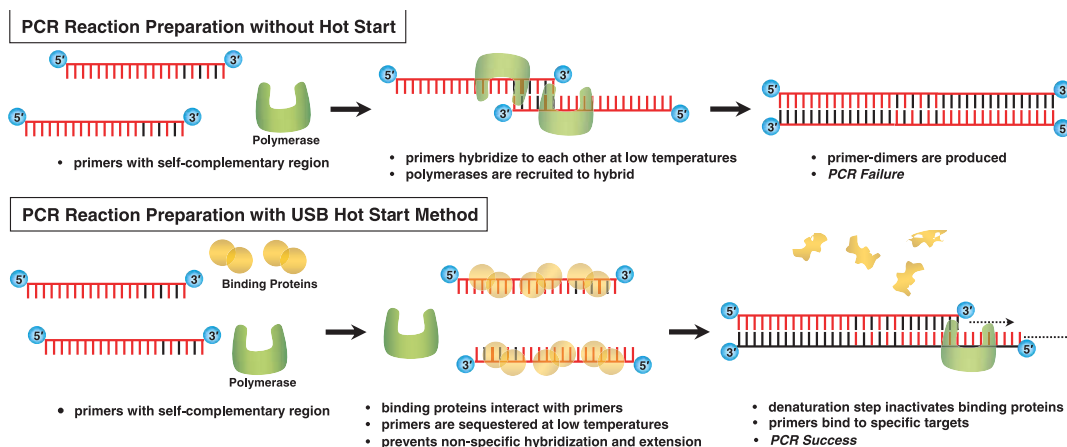


Fig. 1. HotStart-IT® method.

Top Panel: Non-specific products can be generated at low temperatures which causes PCR reaction failure.

Bottom Panel: HotStart-IT® binding protein blocks non-specific product formation at low temperatures which results in successful PCR reactions.

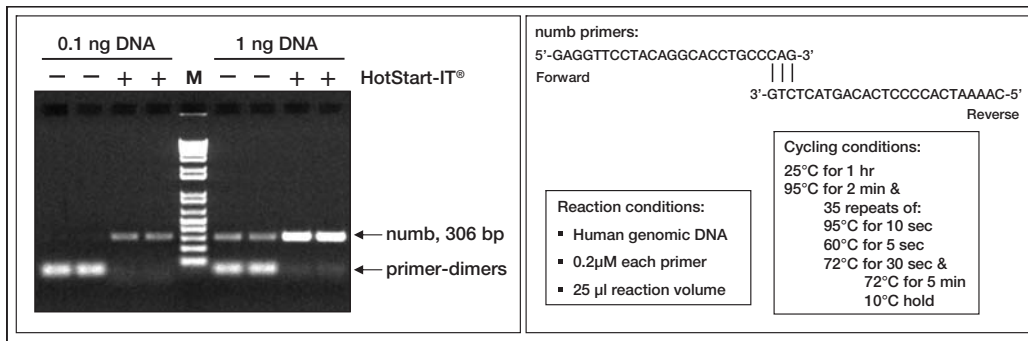


Fig. 2. HotStart-IT® validation test.

Left Panel: USB hot start method results in a shift from primer-dimers to the desired target.
Right Panel: Description of reaction conditions for the experiment.

Method Validation

To validate the USB HotStart-IT® method, a 306 base pair target from the single-copy numb gene was amplified from human genomic DNA with USB Taq PCR Master Mix (without hot start) or with HotStart-IT® Taq PCR Master Mix. The standard Taq Master Mix does not contain any binding protein and, thus, has no hot start feature. Primers were designed with 3 bases of complete homology at their 3'-ends to favor primer-dimer formation. To provide the most extreme testing conditions, the reactions were set-up at room temperature and were also pre-incubated at 25°C for one hour prior to thermal-cycling to facilitate primer binding and low-temperature synthesis. As seen in Figure 2, the results of this “failure-by-design” experiment demonstrated that when no hot start method was used, the primer-dimer created in the pre-incubation step competed with the diluted template for preferential amplification. This problem intensified as less template was used. When HotStart-IT® was used, a shift from mainly primer-dimers to the desired product occurred, which enhanced the overall specificity and yield of the reaction.

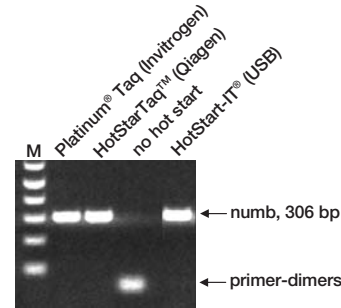


Fig. 3. HotStart-IT® method comparison.

“Failure-by-design” experiment with 1 nanogram of human genomic DNA as template. Results demonstrate that the USB hot start method is comparable to both antibody and chemical methods.

To demonstrate the functional equivalence of USB HotStart-IT® compared to current hot start methods, the previous assay was performed versus two alternative products using 1 nanogram of human genomic DNA as template. As seen in Figure 3, all three hot start methods caused a shift from primer-dimers to the specific target compared to a reaction which used no hot start method. These data confirm that the USB hot start technique is as effective as methods that use a blocking antibody (Platinum® Taq) and chemical modification with (HotStarTaq™) without the risk of mammalian DNA contamination or DNA damage.

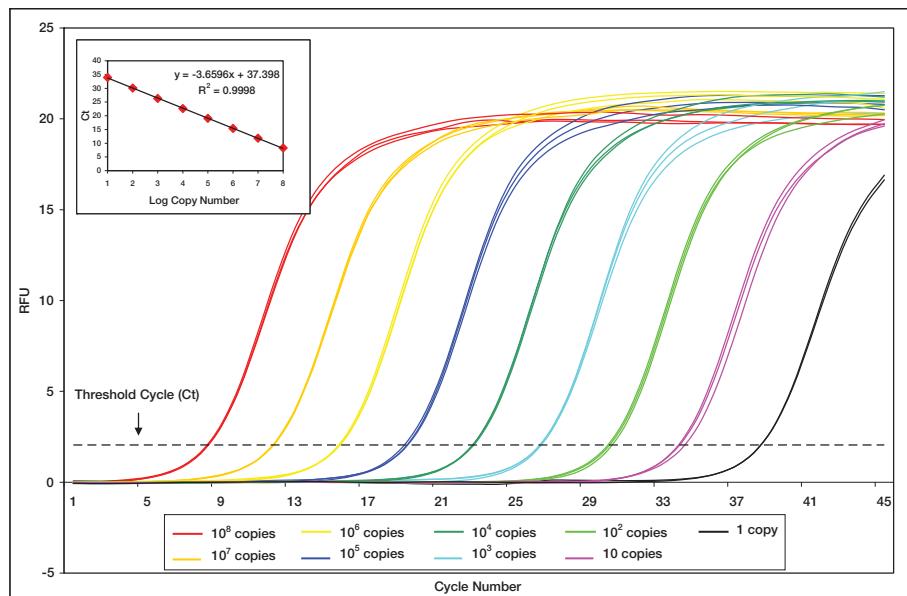


Fig. 4. GAPDH assay using HotStart-IT® SYBR® Green qPCR Master Mix with dUTP and UDG (PN 75760).

Triplicate reactions were performed on a cloned region of the human GAPDH gene as template using an ABI 7500 Fast instrument. The non-specific dsDNA binding dye, SYBR® Green I, was used to detect a 122 bp amplicon. ROX was used as the passive reference dye. The amplification process was linear over eight orders of magnitude (see inset) and a single copy of the target could be reliably detected. The no template control reaction generated no measurable fluorescence (not shown).

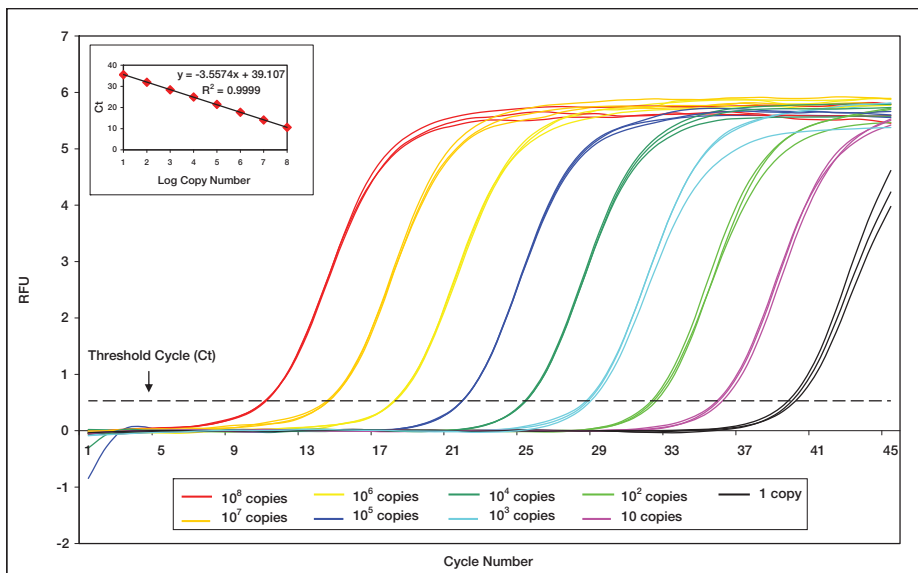


Fig. 5. GAPDH assay using HotStart-IT® Probe qPCR Master Mix with dUTP and UDG (PN 75764).

Triplicate reactions were performed on a cloned region of the human GAPDH gene as template using an ABI 7500 Fast instrument. A TaqMan® probe with FAM as the reporter fluorophore and BHQ-1® as the quencher was used to detect the 122 bp amplicon. ROX was used as the passive reference dye. The amplification process was linear over eight orders of magnitude (see inset) and a single copy of the target could be reliably detected. The no template control reaction generated no measurable fluorescence (not shown).

Applications

In addition to standard end-point PCR, the HotStart-IT® method has been extended to other applications. HotStart-IT® has been added to USB Fidelity™, which is a mixture of Taq DNA polymerase and a minor amount of a proofreading polymerase. This long and accurate enzyme combination allows up to 6-fold higher fidelity than Taq alone and extremely long amplification of targets in excess of 20 kb.

The HotStart-IT® technology has also been combined into a variety of real-time PCR master mixes. For SYBR® Green I and probe-based (e.g. TaqMan®) detection methods, master mixes are available with or without dUTP and a heat-labile uracil-DNA glycosylase (UDG) for carry-over contamination prevention. These universal mixes come with separate tubes of the passive reference dyes ROX™ and fluorescein and are compatible on most PCR instruments. Both the SYBR® Green I (Fig. 4) and probe-based (Fig. 5) mixes have superb sensitivity as they typically detect fewer than 10 target copies and are linearly quantitative over 7 to 8 orders of magnitude with correlation coefficients in excess of 0.95. Master mixes are also available for real-time, one-step RT-PCR (reverse transcription-PCR) using either SYBR® Green or probes as the detection chemistries. One-step RT-PCR simplifies RNA quantification by combining conversion of RNA to cDNA by MMLV-RT and amplification with Taq DNA Polymerase in a single, sequential reaction.

For custom applications in which a certain thermostable polymerase is preferred or required, the HotStart-IT® binding protein may be used separately by adding it directly to the polymerase to provide hot start capability. This binding protein, which is the active component of the HotStart-IT® method, is also useful in multiplex situations. Since one microgram of binding protein will sequester about 5 pmol of primers, users may tailor the amount of protein based upon their multiplexing demands to achieve the best results.

Summary

Hot start PCR methods provide a solution to the problem of non-specific product formation that can occur before high-temperature cycling. The USB HotStart-IT® method is a novel approach that uses a binding protein which traps or sequesters primers before thermal cycling and releases them after heat-inactivation. Like current methods, the advantages of using hot start methods are: 1) room temperature reaction assembly; 2) high specificity; and 3) high sensitivity. Unlike current methods, the advantages of the USB hot start method are: 1) the binding protein is produced in *E. coli* which eliminates potential mammalian DNA contamination; and 2) no extensive initial denaturation step is necessary which could damage DNA samples. In addition, because the method is directed toward the primers and not the polymerase, it is portable to any thermostable polymerase.

USB offers the HotStart-IT® technology in a wide variety of products ranging from standard or end-point PCR, to high fidelity PCR, to real-time or quantitative PCR. Each product line is available in a 2X Master Mix format which provides for maximum convenience, ease-of-use, and reproducibility. These master mixes require only template, primers and water and are designed to be compatible on most PCR instruments. It is recommended to use USB HotStart-IT® products for all demanding PCR applications.

References

- Gillam, S. *et al.*, (1975) *Nucleic Acids Research* **2**(5), 625-634.
- Chou, Q. *et al.*, (1992) *Nucleic Acids Research* **20**(7), 1717-1723.

Related Products

<i>Product Name</i>	<i>Part Number</i>	<i>Application</i>
Taq DNA Polymerase	71160	End-Point PCR
Taq PCR Master Mix	71162	End-Point PCR
RubyTaq™ DNA Polymerase	71190	End-Point PCR: Direct Gel Loading
RubyTaq™ PCR Master Mix	71191	End-Point PCR: Direct Gel Loading
HotStart-IT® Binding Protein	71194	Custom PCR Requirements
HotStart-IT® Taq DNA Polymerase	71195	End-Point PCR
HotStart-IT® Taq PCR Master Mix	71196	End-Point PCR
FideliTaq™ DNA Polymerase	71180	Long and Accurate PCR
FideliTaq™ PCR Master Mix	71182	Long and Accurate PCR
HotStart-IT® FideliTaq™ DNA Polymerase	71155	Long and Accurate PCR
HotStart-IT® FideliTaq™ PCR Master Mix	71156	Long and Accurate PCR
HotStart-IT® SYBR® Green qPCR Master Mix with UDG	75760	Real-time PCR & Contamination Prevention
HotStart-IT® SYBR® Green qPCR Master Mix	75762	Real-time PCR
HotStart-IT® Probe qPCR Master Mix with UDG	75764	Real-time PCR & Contamination Prevention
HotStart-IT® Probe qPCR Master Mix	75766	Real-time PCR
Fluorescein Passive Reference Dye	75767	Real-time PCR: Passive Reference
ROX™ Passive Reference Dye	75768	Real-time PCR: Passive Reference
RT Script Kit	78360	First Strand cDNA Synthesis
One-Step RT-PCR Kit	78350	One-Step RT-PCR: Individual Components
RT-PCR Master Mix	78370	One-Step RT-PCR: Master Mix Format
FideliTaq™ RT-PCR Master Mix	71185	High Fidelity One-Step RT-PCR
HotStart-IT® SYBR® Green qRT-PCR Master Mix	75770	Real-time, One-Step RT-PCR
HotStart-IT® Probe qRT-PCR Master Mix	75772	Real-time, One-Step RT-PCR



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Taq DNA Polymerase – sold under licensing arrangements with Applied Biosystems. Purchase is accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) process in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Perkin-Elmer or as purchased, i.e., an authorized thermal cycler. The Polymerase Chain Reaction (PCR) is covered by patents owned by Roche Molecular Systems and F. Hoffmann-La Roche Ltd. Uracil-DNA Glycosylase (UDG) – Purchase of this product is accompanied by a limited license under U.S. Patent Nos. 5,035,996; 5,683,896; 5,945,313; 6,518,026 and 6,287,823 and corresponding foreign patents. SYBR is a registered trademark of Molecular Probes, Inc. and is provided under an agreement with Molecular Probes, Inc. TaqMan is a registered trademark of Roche Molecular Systems, Inc. Platinum is a registered trademark of Invitrogen. HotStarTaq is a trademark of Qiagen. ROX is a trademark of Applied Corporation or its subsidiaries in the U.S. and certain other countries. BHQ-1 is a registered trademark of Biosearch Technologies, Inc.