



An Introduction to ThermaStop™ from ThermaGenix, Inc.

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ABSTRACT

This paper introduces ThermaStop™, an innovative reagent from ThermaGenix, Inc. (<https://thermagenix.com>) that suppresses PCR mis-priming errors at low temperatures. ThermaStop™ functions like a hot-start that binds and inhibits the activity of DNA polymerases, but unlike antibody and chemical hot-start reagents, ThermaStop™ renatures upon cooling and resumes its functions whenever the reaction temperature is lowered during or after amplification. ThermaStop™ is also better than an aptamer because it not enzyme specific, in fact all 33 of the Type A and Type B DNA polymerases that we have tested are inhibited by ThermaStop™ at low temperatures. Thus, ThermaStop™ delivers High-Precision PCR by promoting more specific and efficient primer use, better multiplexing, and improved monoplex and multiplex amplification sensitivity and quantitative accuracy. And, combination of ThermaStop™ with traditional hot-start antibodies keeps complete master mixes, even those containing DNA primers and templates, inactive for months prior to PCR amplification when stored at 4°C or for days when stored at room temperature.

KEYWORDS: High-Precision PCR • ThermaStop™ • Hot-Start • PCR Additive • PCR Mispriming • PCR Multiplexing • Type A and Type B DNA Polymerases • Primer Dimers • PCR Master Mix • Master Mix Stability

INTRODUCTION

The Polymerase Chain Reaction (PCR) was invented by Kary Mullis in 1983 and quickly became a mainstay in the tool kit of molecular biologists. Since that time, many investigators and biotechnology companies have improved the basic chemistry of PCR with thermocyclers, reagent kits, and refined enzymes and buffers designed to reduce the time needed to design/optimize primers, probes, and reaction conditions. Despite these efforts, the problem of mis-priming prior to amplification remained. To address this issue, hot-start antibodies were invented by Scalice *et al.* in 1992 [1] “to reduce or eliminate the formation of non-specific products in polymerase chain reaction methods”. More than two decades later, however, Stevens *et al.* [2] demonstrated that 12 out of 17 hot-start enzymes failed a stringent test for polymerase activity at room temperature prior to the start of amplification. The only enzymes that passed their test were chemically blocked. Today, the basic problem of mis-priming is frequently ignored, mis-interpreted, or smooth away with mathematical formulae embedded in hidden software, as extensively discussed by Bustin and Nolan [3]. Mis-priming before, during, and after amplification needs to be solved at the molecular level to achieve the quality of PCR and RT-PCR results needed for many applications including NGS of whole genomes, single cell genomics, early detection of drug resistance, quantitative accuracy in digital PCR, and exquisitely sensitively detection of cancer biomarkers in liquid biopsy [4].

The greatest risk of mis-priming occurs at low temperature prior to amplification because: 1) all of the reactants are present in the master mix; 2) the length of time required to set-up the master mix is measured in minutes rather than seconds; 3) non-hot-start DNA polymerases are active at these temperatures; 4) set-up temperatures are well below the melting temperatures of the primers and their targets, making it possible

for partially homologous sequences to hybridize and extend at their 3' ends; 5) most hot-start antibodies are “leaky” at low temperature and, as pointed out by Steven’s *et al.* “Although enzyme suppliers provide evidence of tests for polymerase activity in documents accompanying their products, there is no evidence that they test for polymerase activity prior to thermal activation” [2]. Multiplexing increases all the above risks. For instance, a PCR reaction designed to simultaneously amplify ten targets, such as the one described below, contains twenty primers. As a result, there are at least four hundred possible ways in which the 3’end of one primer could interact with the sequence of itself or another primer, and there are many more ways in which the primers could incorrectly interact with the target DNA.

This paper introduces ThermaStop™, an innovative additive that helps solve the problem of mis-priming at low temperatures prior to and during PCR amplification. The reagent consists of a chemically-modified single-stranded oligonucleotide that forms a secondary configuration below 60°C. In this configuration ThermaStop™ interacts with and inhibits DNA polymerase activity making it less likely that mis-priming will occur during set-up. Unlike conventional hot-start antibodies and enzyme alkylation, ThermaStop™ is not irreversibly inactivated by heating. Instead, it is active whenever the temperature of a reaction is low enough for it to reassume its secondary structure. Unlike an aptamer ThermaStop™ is a fully optimized reagent that works for both Type A and Type B DNA polymerases. Use of ThermaStop™ results in High-Precision PCR because both monoplex and multiplex reactions use primers more efficiently, exhibit higher yields of intended products with improved the sensitivity and quantitative accuracy.

MATERIALS AND METHODS

For All Experiments Described Here:

The order in which ThermaStop™ and reaction components are assembled into a master mix is important. Specifically, regardless of whether a hot-start or a non-hot-start enzyme is used, or whether the master mix is assembled on ice or at room temperature, ThermaStop™ should always added to the enzyme prior to the addition of the primers and the target DNA. ThermaStop™ was supplied by ThermaGenix, Inc. (Natick, MA).

For electrophoresis of samples PCR products (9µl) were visualized on an E-Gel™ EX 2% agarose gel with E-Gel® Low-Range DNA ladder. See Figure Legends for detailed descriptions of the methods employed for individual experiments.

RESULTS

Functionalities of ThermaStop™: The primers and amplification conditions described by Stevens *et al.* [2] were used to test the activities of many hot start Type A and Type B DNA polymerases from various manufacturers prior to amplification in the absence or presence of ThermaStop™. In agreement with Stevens *et al.* [2], every antibody based hot-start enzyme tested was “leaky” during set-up in the absence of ThermaStop™, *i.e.*, they generated primer dimers, see Figure 1 for representative results for four Type A DNA polymerases from four manufactures. Addition of an equivalent number of units of ThermaStop™ to the recommended number of units of each of these enzymes in their own buffer prevented primer-dimer amplification, Figure 1. A list of all 33 enzymes tested thus far without and with ThermaStop™ can be found at <https://www.thermagenix.com/thermastop-test-data.html> . These data demonstrate that the hot-start

activity of ThermaStop™ is not enzyme-specific and that ThermaStop™ works in a variety of PCR reaction conditions. Furthermore, the synergistic effect of ThermaStop™ with hot-start antibody (see below) indicates that ThermaStop™ and hot-start antibodies inhibit DNA polymerase activity by different mechanisms.

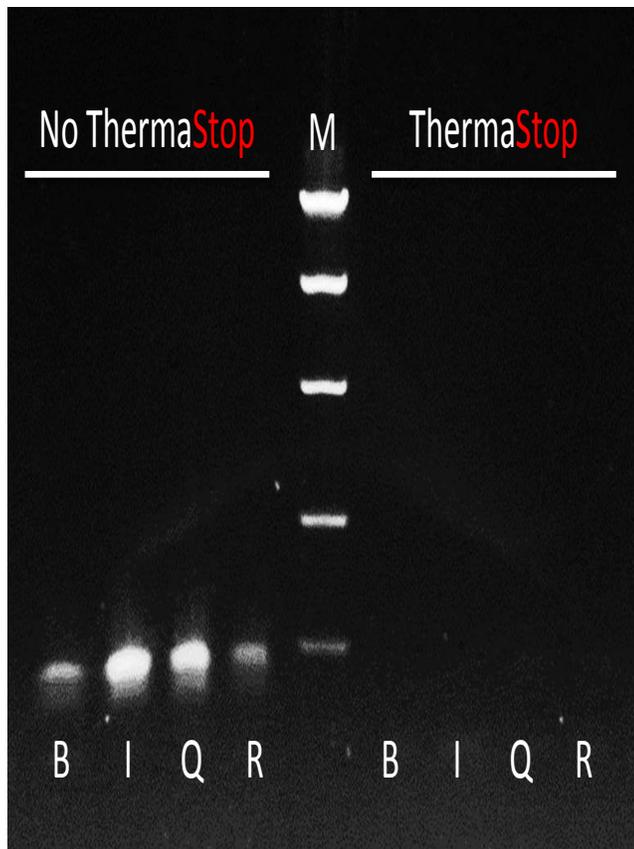


Figure 1 Legend: ThermaStop™ Prevents Primer-Dimer Formation From “Leaky” Hot Start DNA Polymerases. Four different manufactures of hot start Taq DNA polymerases were tested using the Stevens *et. al.* hot start DNA polymerase test [2]. Primer dimer formation were visualized on an E-Gel™ EX 2% agarose gel with E-Gel® Low-Range DNA ladder (Lane M - size range: 2000, 800, 400, 200, and 100 base pairs; Thermo Fisher Scientific, Waltham, MA). Samples without ThermaStop™ are to the left of the DNA marker while samples to the right contained equivalent units of ThermaStop™ based on manufacturer recommendations of DNA polymerase concentration. All four DNA polymerases produced primer dimers without ThermaStop™ whereas, samples with ThermaStop™ showed no primer dimer formation. (B: MyTaq™ HS DNA polymerase, Bioline USA, Taunton, MA; I: Platinum™ Taq DNA polymerase, Invitrogen, Carlsbad, CA; Q: AccuStart II Taq DNA polymerase, Quantabio, Beverly, MA; R: FastStart™ Taq DNA polymerase, Roche CustomBiotech, Indianapolis, IN). 30µl reactions contained 1X PCR buffer, MgCl₂, dNTPs (Bioline USA, Taunton, MA), hot-start DNA polymerase, primers at the concentrations recommended by each manufacturer, and either an equivalent volume of 10mM Tris-Cl, pH 8.3 (lanes labeled “No ThermaStop™”) or an equivalent units of ThermaStop™ to Taq DNA polymerase units per reaction (lanes labeled “ThermaStop™”) added directly to the reaction mix. Cycling conditions were one cycle at 95°C for 2 min; 10 cycles at 95°C, 15 sec; 65°C, 15 sec; 72°C, 45 sec; 25 cycles at 95°C, 15 sec; 60°C, 15 sec; 72°C, 45 sec; then a final extension at 72°C for 5 min in an Aria Mx Real-Time PCR System (Agilent, Santa Clara, CA).

Monoplex Reactions:

ThermaStop™ is a robust hot-start reagent on its own under standard set-up conditions. Figure 2 shows the results of a test of a non-hot start Type A DNA polymerase with a pair of primers known to form primer-dimers in the absence of template DNA when incubated at 25°C for 30 minutes in the absence of

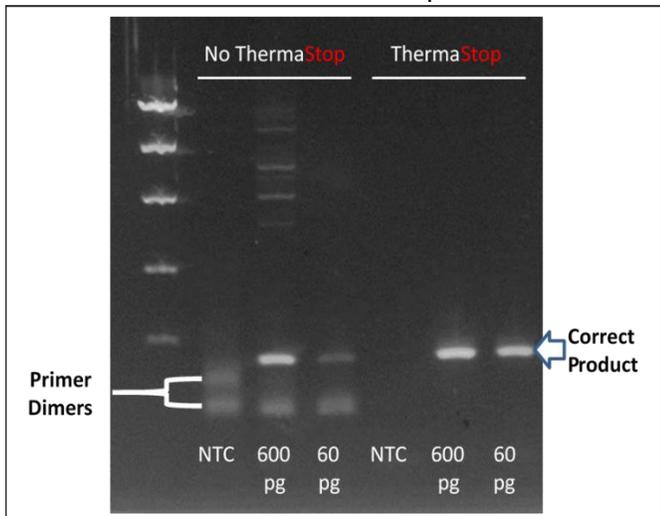


Figure 2 Legend: ThermaStop™ Is an Effective Hot-Start for Type A DNA Polymerases from Different Manufacturers. 25µl reactions contained 1X PCR buffer, 3mM Mg²⁺, 250nM dNTPs, 300nM, of forward and reverse primers for a portion of the alpha subunit of the human β-N-acetylhexosaminidase gene responsible for Tay-Sachs disease (8), either an equivalent volume of 10mM Tris-Cl, pH 8.3 (lanes labeled “No ThermaStop™”) or an equivalent units of ThermaStop™ to Taq DNA polymerase units per reaction (lanes labeled “ThermaStop™”) added directly to the reaction mix, and 1.25 units of Syd Taq DNA polymerase (Syd Labs, Natick, MA). All reactions were pre-incubated at 25°C for 30 minutes to promote formation of primer dimers followed by thermacycling for one cycle at 95°C for 3 min; 40 cycles at 95°C, 10 sec; 60°C, 15 sec; 72°C, 30 sec, then a final extension at 72°C for 5 min. in an Agilent Stratagene Mx Pro 3005 Real-Time PCR instrument (Santa Clara, CA).

ThermaStop™. In the presence of target DNA alone, these primers amplified the correct product but the efficiency of product synthesis was poor because primer-dimer formation still occurred. Addition of ThermaStop™ entirely blocked primer-dimer formation and increased the yield of the correct product for large (600 pg) and small (60 pg) amounts of starting target DNA, equivalent to 100 and 10 human genomes, respectively. These results illustrate the hot start activity of ThermaStop™ and show that primer-dimer formation reduces product yield thus impacting reaction sensitivity and quantitative accuracy.

Multiplex Reactions:

The hot-start capacity of ThermaStop™ was further demonstrated by constructing a 10 multiplex reaction. First, two large sets of primer pairs were chosen from two unrelated papers in the literature, strictly on the basis of amplicon product size. One set was comprised of six pairs of primers for sequences in the lambda phage genome [6]. A second set was comprised of four pairs of primers for sequences in the mouse genome [7]. None of the twenty primers were checked for compatibility with the other primers and some primer pairs had mismatched melting temperatures, Table 1.

Organism	Fragment Size (base pair)	Primer Name	Sequence 5' to 3'	Taq (°C)*	SuperFi (°C)*	Phusion (°C)*
LAMBDA	139	Lam139F	ACGATCTGGTACGCAATAACG	55.5	59	63.3
		Lam139R	AAGGCACGGGCTTCTTCC	59.6	61.1	66.1
MOUSE	185	Mou185F	GCGGGTCTCCTTCTCTACTATCC	62.3	64.5	68.2
		Mou185R	CAGCTCAGTCTTCATGTGGTCTCC	60.6	63.6	67.8
MOUSE	214	Mou214F	TCGACTGCATGGTGTGAACGG	61.1	63.9	71.1
		Mou214R	TCCCTTATCAACTGCCCTAACTTCC	59.7	63	67.4
MOUSE	293	Mou293F	GAGGGTCGTGGGCGGTGTG	65.7	66.3	73
		Mou293R	TGGCTCTGGCTGGCTTGTATGG	63.4	65.9	71.7
LAMBDA	431	Lam431F	CGTCGTGGCTCTAATTC	53.9	55.9	60.5
		Lam431R	GGCAATCAGTTCATCTTTCG	52.3	55.6	61.8
MOUSE	515	Mou515F	AGGGAACCACAACCTCAGCAAATACC	60.3	64	68.3
		Mou515R	ACACACCATGACCAAAGGCAACC	61.3	64.8	70.4
LAMBDA	600	Lam600F	TCCGGATGCGGAGTCTTATCCGTG	64.4	66.8	74.7
		Lam600R	GGCCTGTTATTAGCTCAGTAATGT	55.2	59.3	60.9
LAMBDA	715	Lam715F	GTCACCGCCAGTTAATCC	54.3	56.2	60.2
		Lam715R	ATCAGCACATCATCTTCAGG	52.4	56	59.9
LAMBDA	860	Lam860F	CGATAGATAGCCACGGACTTC	55.8	58.3	62.1
		Lam860R	GCTCTCGGAATATCAATGAAGG	54.1	57.3	63.2
LAMBDA	962	Lam962F	ATCAGAAACGAAACGCATCATCAAGT	57.5	62.1	68.7
		Lam962R	GCCTCGCATATCAGGAAGCAC	60	62.1	68.3

Initially each pair of primers was individually tested without and with ThermaStop™ using two different non-hot-start Type A DNA polymerases in the absence of target DNA. The results with the two enzymes were similar. As shown for EconoTaq from Lucigen (Middleton, WI), each pair of primers generated primer-dimers in the absence of both target DNA and ThermaStop™, Figure 3A. In contrast, in presence of the reagent there was a complete absence of primer-dimer formation, Figure 3B. Each pair of primers was then individually tested with either 500 genomic copies of lambda DNA or 500 genomic copies of mouse DNA. In the absence of ThermaStop™ each pair of primers generated some amount of its expected product, but also generated primer-dimers and additional non-specific products, Figure 3C, particularly for primer pairs 4 and 6. In

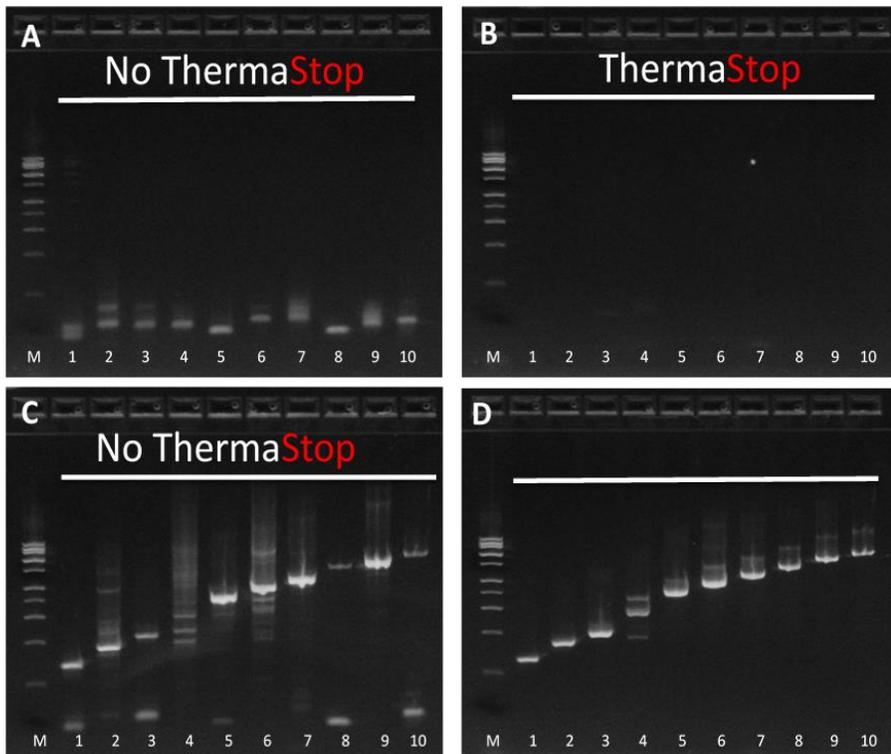
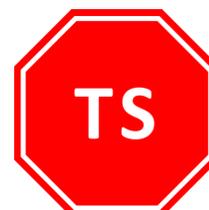
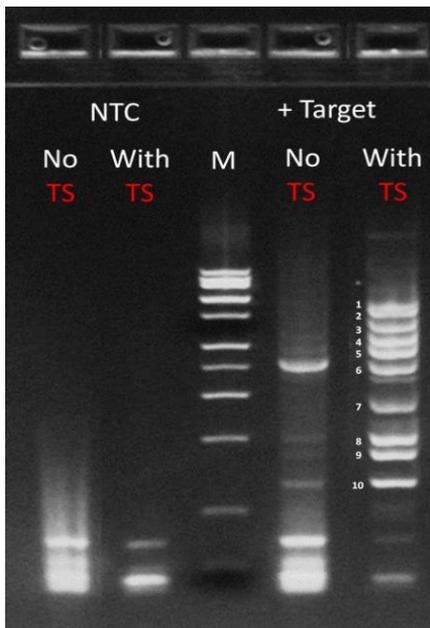


Figure 3 Legend: Impact of ThermaStop™ Addition on PCR Amplification With Individual Primer Pairs Used In The 10-Plex Reaction Shown In Figure 4. Panels A & B: No template 25µl reactions contained 1X EconoTaq PCR buffer (Lucigen, Middleton, Wisconsin), 3mM MgCl₂, 400nM dNTPs, 200nM of all 10 individual primer-pairs listed in Table 1, either an equivalent volume of 10mM Tris-Cl, pH 8.3 (Panel A labeled “No ThermaStop™”) or an equivalent units of ThermaStop™ to Taq DNA polymerase units per reaction (Panel B labeled “ThermaStop™”), and 2.5 units of EconoTaq DNA polymerase (Lucigen, Middleton, Wisconsin). Panels C & D: 25µl reactions with genomic DNA contained 1X EconoTaq PCR buffer containing 3mM Mg²⁺, 400nM dNTPs, and 200nM of all 10 individual primer-pairs listed in Table 1, either an equivalent volume of 10mM Tris-Cl, pH 8.3 (Panel C labeled “No ThermaStop™”) or an equivalent units of ThermaStop™ to Taq DNA polymerase units per reaction (Panel D labeled “ThermaStop™”) added directly to the reaction mix, 2.5 units of EconoTaq DNA polymerase (Lucigen, Middleton, Wisconsin) and 500 genomes of lambda DNA (Thermo Scientific™ Lambda DNA, Waltham, MA) plus 500 genomes of mouse DNA (EMD Millipore™ Novagen™ Mouse Genomic DNA, Burlington, MA). In all cases cycling conditions were one cycle at 95°C for 3 min; 40 cycles at 95°C, 10 sec; 60°C , 15 sec; 72°C , 30 sec; then extension at 72°C for 5 min in an Agilent Aria Mx Real Time PCR Cycler

Figure 4 Legend: ThermaStop™ Enables Multiplex PCR Amplification. 25µl reactions contained EconoTaq 1X PCR buffer, 3mM Mg²⁺, 400nM dNTPs, and 200nM of all 10 primer-pairs listed in Table 1, either 3.75 units ThermaStop™ (lanes labeled “With TS”) or an equivalent volume of 10mM Tris-Cl, PH 8.3 (lanes labeled “No TS”), 1.25 units of EconoTaq DNA polymerase, and either 500 genomes of lambda DNA plus 500 genomes of mouse DNA (lanes labeled “+ Target”) or an equivalent volume of 10mM Tris-Cl, PH 8.3 (lanes labeled “NTC”). Cycling conditions were 95°C for 3 min; 40 cycles at 95°C, 10 sec; 60°C, 15 sec; 72°C, 30 sec; then a final extension at 72°C for 5 min. in an Agilent Aria Mx Real Time PCR Cycler.



contrast, in the presence of 1 unit of EconoTaq DNA polymerase plus 1 unit of ThermaStop™ all reactions were virtually free of spurious side products, Figure 3D. As a result, the collection of amplification products formed a ladder of size fragments from 139 to 962 base pairs in length, as intended.

Figure 4 shows what happened when the complete 10-plex reaction was assembled in the absence or presence of ThermaStop™ as well as in the absence or presence of 500 copies of lambda DNA plus 500 copies of mouse DNA. In the absence of ThermaStop™ only a very pronounced smear of primer-dimers was generated in both the no template controls (NTC) and the plus-DNA samples. In contrast, in the presence of ThermaStop™ only a fainter primer-dimer band was observed in the NTC and all ten correct amplicons were generated in the plus-DNA reaction together with a minor primer-dimer band. In addition, the intensities of all amplicons bands in the plus-DNA reaction were roughly equivalent, demonstrating that each primer pair worked efficiently. These results show that ThermaStop™ inhibition of Taq DNA polymerase activity during PCR set-up prevented a host of mis-priming errors that occurred in its absence.

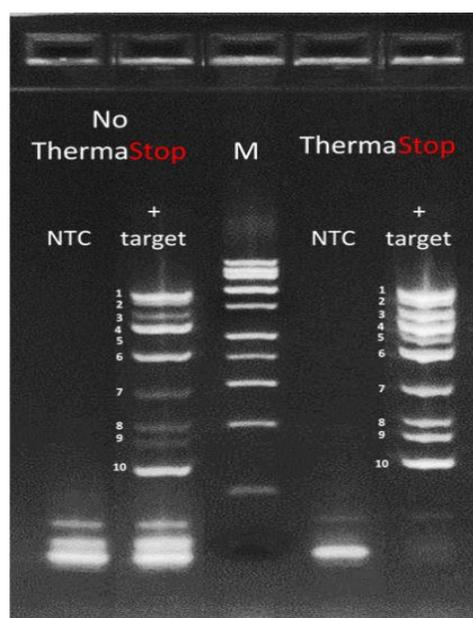


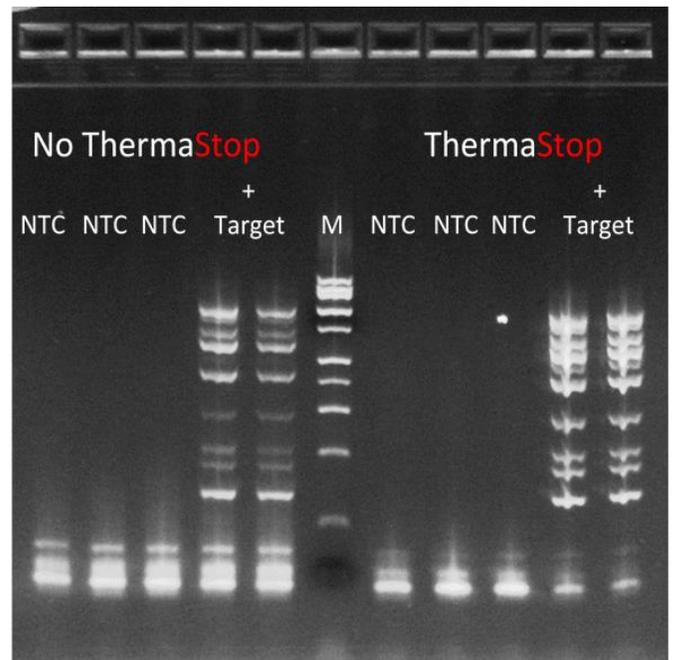
Figure 5 Legend: ThermaStop™ Combined with Hot-Start Antibody Enhanced Long Term Stability of Complete PCR Master Mixes for 48 Hours at 22°C. 25µl reactions contained 1X PCR buffer recommended by the DNA polymerase manufacturer, 300nM of each primer, 0.4mM dNTPs, 3mM Mg⁺⁺, 0.24X SYBR Green (Thermo Fisher Scientific, Waltham, MA), either 3.75 units ThermaStop™ (lanes labeled “ThermaStop™”), or an equivalent volume of 10mM Tris-Cl, pH. 8.3 (lanes labeled “No ThermaStop™”), 1.25 units of MyTaq™ HS polymerase (Bioline USA, Taunton, MA), and either 500 genomes of lambda DNA plus 500 genomes of mouse DNA (lanes labeled “+ Target”) or an equivalent volume of 10mM Tris-Cl, PH 8.3 (lanes labeled “NTC”). All samples were incubated for 48 hours at 22°C prior to PCR amplification. Cycling conditions were one cycle at 95°C for 3mins; 40 cycles at 95°C, 10 sec, 60°C, 15sec, and 72°C, 30 sec; and a final extension at 72°C for 5 min. in an Agilent Aria Mx Real Time PCR Cycler.

Long Term Inhibition of Taq DNA polymerase:

The results in Figure 1 suggested that ThermaStop™ and hot-start antibodies inhibit DNA polymerases by different mechanisms. This finding suggested that the two reagents together could prevent enzymatic activity for extended periods of time. To test this hypothesis, a basic master mix was assembled containing all the components for the 10 plex reaction described above including 1.25 unit/25µl of an antibody-based hot-start Taq DNA polymerase (MyTaq DNA polymerase, Bioline USA, Taunton, MA). One aliquot of the basic master mix also received lambda and mouse DNA, while a second aliquot received only buffer. Finally, each set of reactions was amplified in the absence or presence of ThermaStop™. Sets of reactions were incubated for either 48 hours at 22°C, or for 60 days at 4°C and then tested for their ability to generate amplification products. Figure 5 shows that reactions without ThermaStop™ were poorly amplified after prior incubation for 48 hours at 22°C (*i.e.*, the hot start antibody alone was not sufficient to prevent primer-dimer formation

over the course two days at room temperature). In contrast, inclusion of ThermoStop™ along with the antibody resulted in far less primer-dimer amplification and far more correct product amplification once the reaction was started, Figure 5. The combination of ThermoStop™ plus antibody was also very effective at preventing amplification of non-specific products during storage of the complete master mix for 60 days at 4°C prior to amplification, Figure 6.

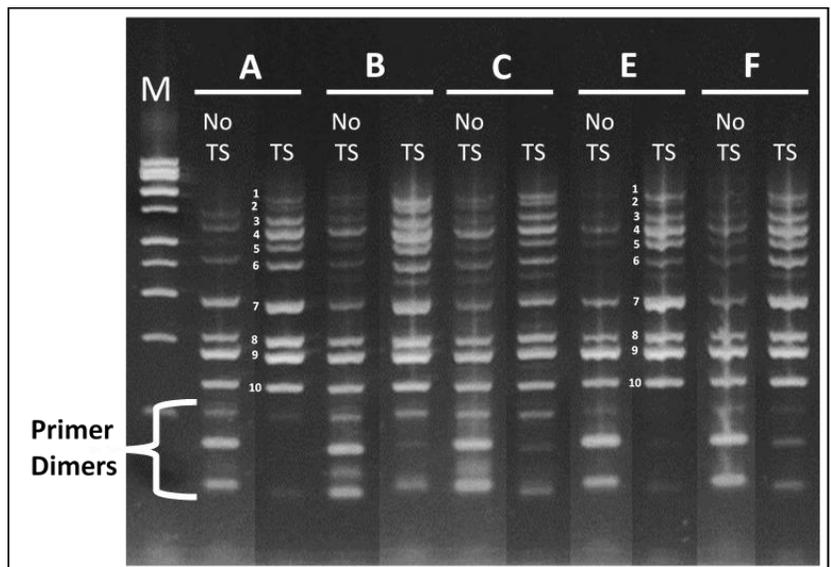
Figure 6 Legend: ThermoStop™ Combined with Hot-Start Antibody Enhanced Long Term Stability of Complete PCR Master Mixes for 60 Days at 4°C. 25µl reactions contained 1X PCR buffer, 300nM of each primer, 0.4mM dNTPs, 3mM Mg++, 0.24X SYBR Green, either 3.75 units ThermoStop™ (lanes labeled “ThermoStop™”) or an equivalent volume of 10mM Tris-Cl, pH. 8.3 (lanes labeled “No ThermoStop™”), 1.25 units of MyTaq™ HS polymerase (Bioline USA, Taunton, MA), and either 500 genomes of lambda DNA plus 500 genomes of mouse DNA (lanes labeled “+ Target”) or an equivalent volume of 10mM Tris-Cl, PH 8.3 (lanes labeled “NTC”). All samples were incubated for 60 days at 4°C prior to amplification. Cycling conditions were one cycle at 95°C for 3mins; 40 cycles at 95°C, 10 sec, 60°C, 15 sec, and 72°C, 30 sec; and a final extension at 72°C for 5 min. in an Agilent Aria Mx Real Time PCR Cycler.



Evaluating And Rapidly Optimizing Pre-Mixed Enzyme Kits:

Some manufacturers of molecular toolkits sell proprietary sets of pre-mixes containing both Type A and Type B DNA polymerases with a range of additives. End users use these kits to determine which pre-mix is best suited to their particular PCR reaction. The 10-plex reaction described above was used to test one such kit from Lucigen comprised of a set of 12 pre-mixes (FailSafe PCR Systems, Lucigen, Middleton, WI). The primers

Figure 7 Legend: ThermoStop™ Enhances PCR Multiplexing Under a Variety of Reaction Buffer Conditions and PCR Additives. 25µl reactions contained 1X FailSafe PCR System’s ready mixes (Lucigen, Middleton, WI), either 3 units ThermoStop™ (lanes labeled “TS”) or an equivalent volume of 10mM Tris-Cl, pH. 8.3 (lanes labeled “No TS”), 500nM each all 10 primer pairs listed in Table I, and 30,000 copies of mouse genomic DNA plus 50,000 copies of lambda genomic DNA. Lanes A-F show the results from the five best ready mixes. Lane M, Fisher Bioreagent™ Routine DNA ladder (Fisher Scientific, Hampton, NH). Reactions were incubated at 25°C for 30 minutes prior to cycling for one cycle at 95°C, 3mins; 40 cycles at 95°C, 10 sec, 60°C, 15 sec, and 72°C, 30 sec; and a final extension at 72°C for 5 min in an Agilent Aria Mx Real Time PCR Cycler.



and targets for the 10-plex reaction were added to each ready-mix in the absence or presence of ThermaStop™ without knowing the compositions of the different mixes. Figure 7 shows the results from the five best pre-mixes without and with ThermaStop™. In each case ThermaStop™ suppressed amplification of primer-dimers and increased the yield of the 10 intended amplification products. These results demonstrate that ThermaStop™ works in a variety of reaction conditions and PCR additives.

Improving the Specificity of High-Fidelity and Type B DNA Polymerases:

Type B DNA polymerases are structurally different from Type A enzymes and exhibit greater processivity. High-fidelity Type B DNA polymerases retain their 3'-to-5' editing function and are therefore the enzymes of choice for PCR amplification prior to next generation sequencing. The following experiments were carried out to investigate the impact of ThermaStop™ addition on non-hot start and hot start Type B enzymes.

The primers and targets of the 10-plex reaction were amplified using the non-hot-start versions of both Kapa HiFi DNA polymerase (Kapa Biosystems, Inc, Wilmington, MA) and Roche HiFi DNA polymerase (Roche Sequencing, Pleasanton, CA), Figure 8. Both enzymes generated primer-dimers in the absence of ThermaStop™, even in their own recommended buffers. In contrast, addition of ThermaStop™ at 3 units of reagent to 1 unit of each of these enzymes in the manufacturer recommended buffers dramatically inhibited primer-dimer formation and significantly enhanced amplification of all 10 products, Figure 8. The hot-start

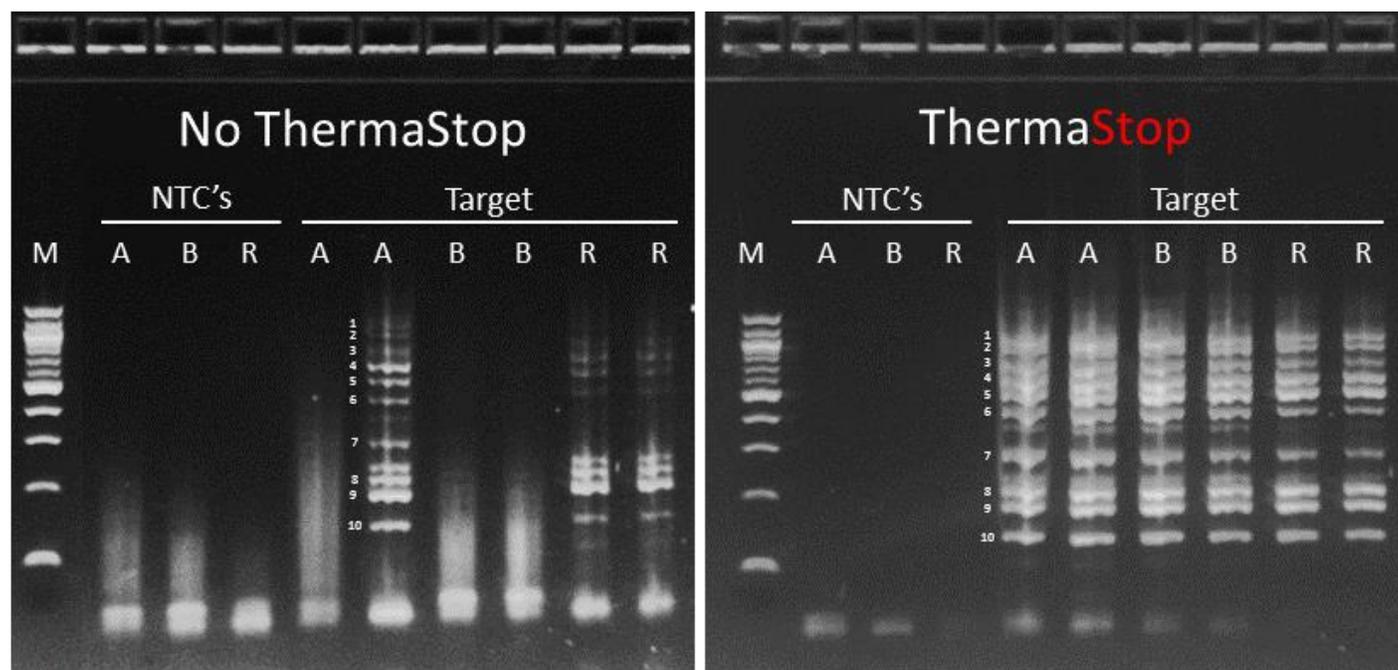


Figure 8 Legend: ThermaStop™ Improves PCR Amplification from High-Fidelity and Type B DNA Polymerases. 25µl reactions contained 1X manufacturer-recommended PCR buffer for each DNA polymerase, 3mM MgCl₂, 400 nM dNTPs, 200nM of each primer (Table I), either 7.5 units ThermaStop™ (lanes labeled “ThermaStop™”) or an equivalent volume of 10mM Tris-Cl, pH. 8.3 (lanes labeled “No ThermaStop™”), 2.5 units non-hot start Kapa DNA Polymerase (Kapa Biosystems, Wilmington, MA) or 2.5 Units non-hot start HiFi DNA polymerase (Roche Sequencing, Pleasanton, CA), and either 500 genomes of lambda DNA plus 500 genomes of mouse DNA (lanes labeled “Target”) or an equivalent volume of 10mM Tris-Cl, PH 8.3 (lanes labeled “NTC”). Lanes A or B – Kapa HiFi polymerase with buffer A or B respectively; R – Roche HiFi polymerase. Lane M Quick-Load® Purple 1 kb DNA Ladder (New Englan BioLabs, Ipswich, MA). Cycling conditions were one cycle at 95°C, 2min; 35 cycles at 95°C, 15 sec; 60°C, 30 sec; 72°C, 2 min; and a final extension at 72°C for 5 min in an Agilent Aria Mx Real-Time PCR Cycler

Kapa HiFi enzyme amplified some of the 10 amplicons, but not as efficiently as the non-hot start version of this enzyme with ThermaStop™, (results not shown).

Similar tests using non-hot-start HiFi Q5® DNA polymerase from New England BioLabs (Ipswich, MA) showed that in the absence of ThermaStop™ this enzyme amplified all 10 products but also generated high levels of

primer-dimers and additional non-specific amplicons when 500 genomes of lambda and mouse DNA were used to initiate amplification. Moreover, the amplification efficiency of this enzyme decreased dramatically when the number of lambda and mouse genomes was less than 500 copies. These artifacts decreased or disappeared when ThermaStop™ was added at 0.5 units per 0.5 units of enzyme. Under these conditions, all 10 products were amplified down to the near-digital level, Figure 9. The absence of a few bands in some samples initiated with <5 genomes was most likely due to the absence of the corresponding target sequences at this low number of starting templates due to Poisson distribution effects. In conclusion, ThermaStop™ alone is a robust hot-start for the non-hot-start and HiFi versions of these Type B DNA polymerases.

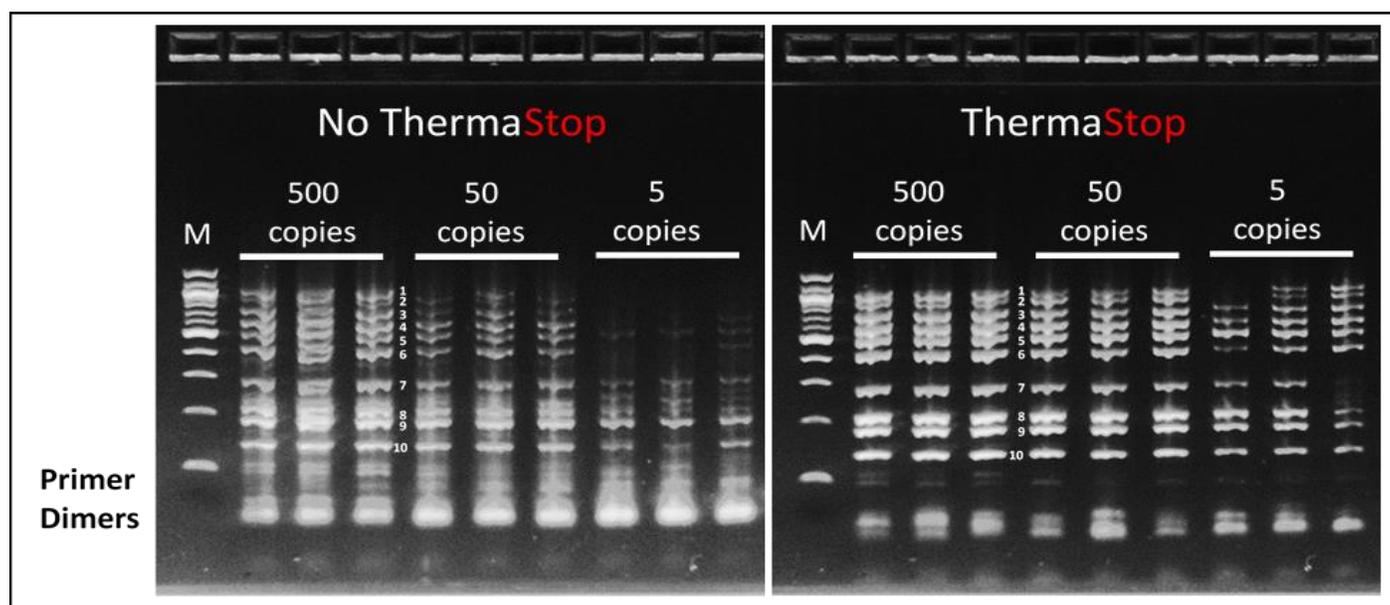


Figure 9 Legend: ThermaStop™ Improves PCR Multiplex Amplification with Non-Hot Start HIFI Q5 DNA Polymerase (New England Biolabs, Ipswich, MA). 25µl reactions contained 1X Q5 DNA Polymerase PCR buffer (New England BioLabs, Ipswich, MA), 3mM MgCl₂, 400nM dNTPs, 500nM of each primer (Table I), either 0.5 units ThermaStop™ (lanes labeled “ThermaStop™”) or an equivalent volume of 10mM Tris-Cl, pH. 8.3 (lanes labeled “No ThermaStop™”), 0.5 units non-hot start Q5 DNA Polymerase, and 500 to 5 genomic copies each of lambda DNA and mouse DNA. Lane M Quick-Load® Purple 1 kb DNA Ladder. Cycling conditions were one cycle at 95°C, 3min; 40 cycles at 98°C, 15 sec; 62°C, 30 sec; 72°C, 90 sec; and a final extension at 72°C for 5 min in an Agilent Aria Mx Real-Time PCR Cycler.

Preventing Low Temperature Errors during Rapid Amplification:

Several companies have recently introduced rapid and very rapidly cycling devices which reduce the total amplification time to less than an hour or even a few minutes, *e.g.*, NEXTGENPCR from Molecular Biology Systems (Goes, The Netherlands). Because of the very rapid temperature oscillations, these devices require high levels of DNA polymerases and primers for successful amplification. Under these conditions annealing temperature and the extension temperature of the traditional thermal cycle as replaced by a temperature zone in which primer annealing and extension overlap. Rapid cycling, however, will increase rather than decrease the risk of

mis-priming when the temperature is dropped below optimum temperature for primer specificity during every thermal cycle. Not surprisingly, conventional hot-start antibodies and chemistries that are irreversibly denatured during the first thermal cycle cannot prevent mis-priming of primers strands and product strands during rapid cycle below the primer melting temperature. ThermaStop™, which is not irreversibly denatured, interacts directly with the DNA polymerase to prevent mis-priming as the temperature drops in every thermal cycle.

The experiment described in Figure 10 was designed to determine the levels of mis-priming using conventional antibody hot-start and ThermaStop™ in a moderately rapid cycling reaction carried out on the MIC PCR Cycler, a portable air-cooled device manufactured by Bio Molecular Systems (Upper Coomera, Queensland, Australia). The primer pair used for this demonstration was known to mis-prime in the absence of a hot-start antibody at an annealing temperature of 60°C in a standard thermal cycler (8, 9). Under optimal conditions in a conventional thermocycler in the presence of ThermaStop™, these primers generate an 82 bp amplicon from the human cystic fibrosis transmembrane conductance regulator (CFTR) gene. To push the limits of low stringency the annealing temperature was lowered to 40°C, the number of amplification cycles

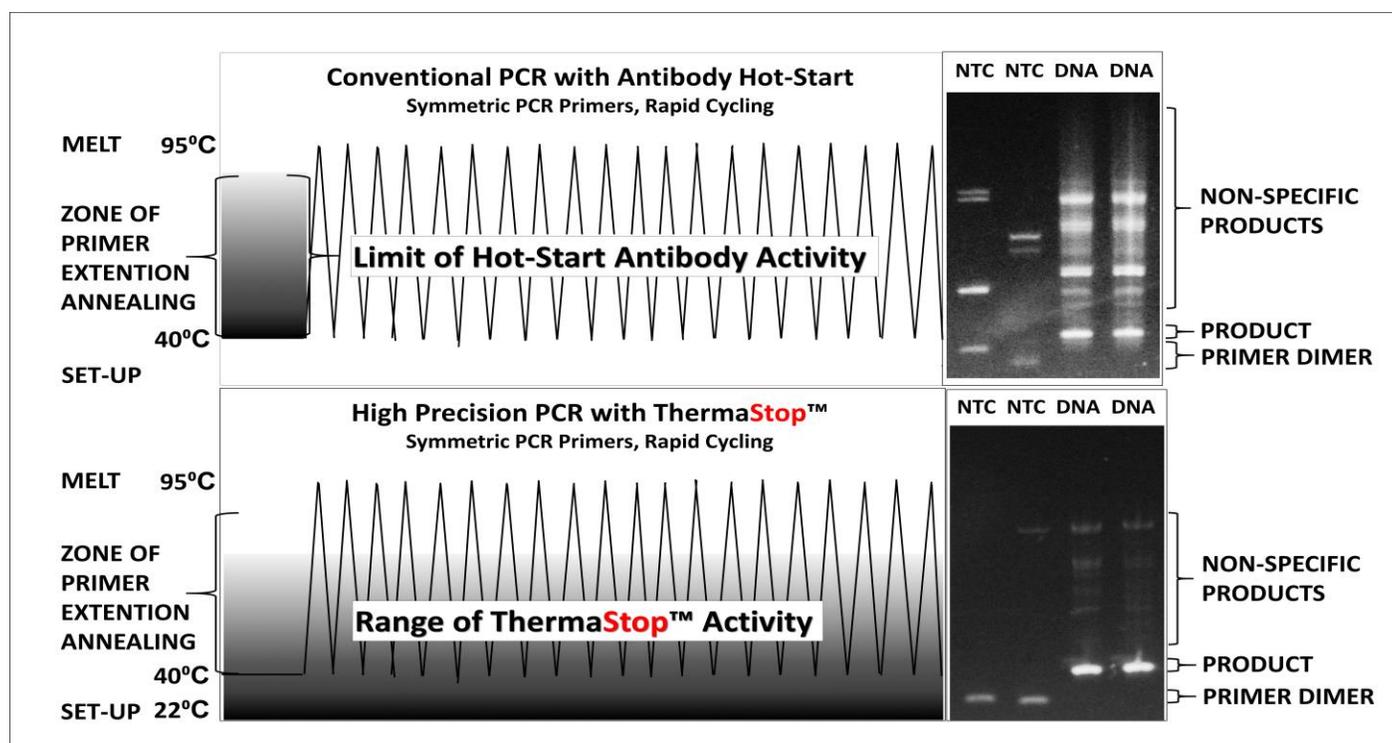


Figure 10 Legend: ThermaStop™ Prevents Low Temperature Errors During Rapid Amplification. 12.5µl reactions contained 1X PCR buffer (Thermo Fisher Scientific, Waltham, MA), 3 mM Mg⁺⁺, 0.25mM dNTPs, 0.24X SYBR Green, 300nM each cystic fibrosis forward and reverse primers (8, 9), either 1.25 units Platinum Taq DNA polymerase antibody or 1.25 units ThermaStop™, 1.25 units MyTaq™ DNA polymerase, and 50 copies human DNA. The calculated melting temperature of the two primers at 300 nM each were 67.7°C and 66.2°C, respectively. In each instance, the DNA polymerase was pre-incubated for 5 minutes at room temperature with an equal volume of Platinum Taq DNA polymerase antibody prior to addition to the PCR mastermix. In the case of ThermaStop™, the reagent was added directly to the PCR master mix and vortexed prior to addition of the DNA polymerase. Cycling conditions were one cycle at 94°C, 2 min.; 60 cycles at 94°C, 0 sec., 40°C, 0 sec., and 72°C for 0 sec (for fluorescence signal acquisition). Nine microliter samples were analyzed by gel electrophoresis as described in Figure 1.

was doubled, and the reaction was initiated with only 300 pg of human genomic DNA (50 copies). Extensive mis-priming occurred in both the no-template-control reactions and in the reactions containing genomic DNA when 1.25 units of Bioline MyTaq™ DNA polymerase with antibody hot-start (Bioline USA, Inc., Taunton, MA) was used under these conditions, Figure 10. In contrast, addition of 1.25 units of ThermaStop™ without hot-start antibody resulted in very little mis-priming in both the no-template-control reactions and reactions containing human DNA. And, as in all previous examples, there was a higher yield of the intended product when mis-priming was suppressed by ThermaStop™.

DISCUSSION

This paper confirms and extends the observation by Stevens *et al.* [2] that many commercial hot-start Taq antibodies fail to fully inhibit the activity of thermostable DNA polymerases prior to first heating. As a result, primer-dimers occur at the low temperatures at which reactions are set-up. The resulting errant molecules are subsequent amplified and oligomerized during thermal cycling. These artifacts are observed in both monoplex and multiplex no-template control reactions, as well as in reactions containing target DNA, particularly reactions started with small numbers of genomes. Importantly, these artifacts reduce both the sensitivity and the quantitative accuracy of the reactions in which they occur. This conclusion should be disturbing to all PCR practitioners who want to determine the sequences present or absent of each target in their samples.

As a solution, this paper introduces ThermaStop™, a chemically modified oligonucleotide that forms a secondary structure whenever the reaction temperature is below 60°C. In this configuration ThermaStop™ binds to and inhibits both Type A and Type B DNA polymerases prior to and during amplification anytime the reaction temperature is below 60°C. Thus ThermaStop™ acts as both a robust hot-start and a robust cold-stop in all PCR buffers tested. The chemical modifications and universal activity against a large variety of Type A and Type B DNA polymerases distinguish ThermaStop™ from aptamers against DNA polymerases. The benefits of adding ThermaStop™ during rapid thermal cycling when the annealing temperature is below the primer annealing temperature are particularly striking. ThermaStop™ also acts synergistically with various antibody hot-starts making it possible to assemble and store complete master mixes for days or months without activation, depending on the storage temperature. These attributes of ThermaStop™ are very useful for preparation of volumes of liquid master mix used in high through-put automated analyses of large numbers of PCR samples. In all cases, when mis-priming is suppressed by ThermaStop™ there is a higher yield of the correct product(s) and increased sensitivity.

ThermaStop™ enhances PCR-based tests by reducing their cost (less expensive hot-start) and improving their quantitative accuracy. This is particularly critical for PCR cancer diagnosis tests. Because of the heterogeneous nature of tumors, PCR cancer tests are increasingly being carried out on very small amounts of biopsied material or even on single cells. And, because of the multigenic nature of cancer, these assays typically begin with targeted amplification of multiple sequences that may contain alleles indicative of malignancy or drug resistance. Next generation sequencing and digital PCR are rapidly becoming the tools of choice for analysis of the large sets of single nucleotide polymorphisms that shed light on the state and the evolution of a particular individual's malignancy, but both of these technologies are currently compromised

by the quality of the PCR amplification products used during library construction or single-molecule analysis. The results shown in this paper, particularly those using high fidelity enzymes like Q5® and other Type B DNA polymerases typically used for next generation sequencing and digital PCR, demonstrate that suppression of mis-priming at the very first step in the multi-step chain of molecular diagnosis should readily and simply improve the sensitivity and the accuracy of the amplicons used for diagnosis.

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