Eppendorf HotMaster—An Innovative Hot Start/Cold Stop Technology for Better PCR* Results

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ABSTRACT

Hot Start DNA polymerases have become very popular because of their convenience and their ability to reduce nonspecific amplification. Typical Hot Start enzymes utilize chemical or antibody mediated inhibition, which impart disadvantages such as a long initial denaturing step or excessive protein contamination. In contrast, Eppendorf's HotMaster uses an innovative temperature-dependent inhibitor to achieve Hot Start activity. In addition to increasing specificity and sensitivity in PCR reactions, HotMaster offers several advantages not available in other Hot Start DNA polymerases: the Mg^{++} concentration is pre-optimized and self adjusting; no heat-activation step is required, the polymerase is inhibited in a temperature-dependent manner during each annealing step of the PCR (at temperatures below ~60°C); and larger target sizes can be amplified.

INTRODUCTION

Hot Start PCR was developed as a method to minimize the deleterious effects of mispriming at lower temperatures during PCR. In a PCR reaction, even short incubations at temperatures below the optimum annealing temperature for a particular set of primers can result in mispriming, elongation and the subsequent formation of spurious bands. The Hot Start technique involves inactivating (or leaving out) one critical component of the PCR reaction until the temperature has risen above this optimal annealing temperature. Most Hot Start kits on the market today rely on chemical modification or antibodies to inhibit *Taq* polymerase; thus, their action is limited to the first cycle of the PCR reaction. In addition, they impart some disadvantages such as long pre-incubations at high temperatures or protein contamination.

Eppendorf has developed an innovative Hot Start technology. This new technology uses a ligand that binds to the *Taq* polymerase in a temperature-dependent manner and inhibits it. This ligand is thermostable, and it is active in all cycles of the PCR process. The net result is that Eppendorf's HotMaster kit not only offers Hot Start activity during the first cycle of the PCR, but also "Cold Stop" activity at temperatures below 60°C during the annealing step of each and every cycle of the PCR.

The buffer is formulated to adjust the Mg^{++} concentration automatically so that there is never a need for optimization of this critical component. It does this by weakly chelating Mg^{++} ions: when Mg^{++} is present in excess, it is bound by the chelating agent, but as it is needed by the reaction (for *Taq* or DNA), it is released. This is an innovative technology that further improves the sensitivity and specificity of HotMaster.

The series of experiments in this paper were designed to show that Eppendorf Hot Start/Cold Stop and self-adjusting Mg^{++} technology offer a superior alternative to other Hot Start *Taq* polymerases. This technology increases specificity as well as the length of the product that is amplified relative to other Hot Start polymerases. Eppendorf's HotMaster also gives increased sensitivity: we were able to reproducibly amplify a single-copy gene from as little as 10pg of human genomic DNA (about two genomic equivalents).

MATERIALS AND METHODS

All PCR reactions were performed in triplicate on the Eppendorf Mastercycler* gradient using human genomic DNA or Phage Lambda DNA (New England BioLabs, USA) as indicated. The reaction components are:

1x PCR buffer

2.5mM Mg^{++}

0.2µM of each primer

0.2µM dNTPs

1.25 units respective *Taq* polymerase

MBGW to 50µl total reaction volume

(Templates are indicated in each experiment)

Note that we have determined empirically that the optimal temperature for the elongation step using HotMaster is 65°C.

EXPERIMENT 1: SPECIFICITY

A 131 bp target in the human TNF gene was amplified using Eppendorf HotMaster *Taq* DNA polymerase, a conventional chemically modified Hot Start DNA polymerase, an antibody-mediated Hot Start DNA polymerase and standard *Taq* DNA polymerase. The following reaction conditions were used:

PRIMERS AND TEMPLATE:

Primers 131 bp TNF system

Forward Primer:	GGTTTCGAAGTGGTGGTCTTG
Reverse Primer:	CCTGCCCCAATCCCTTTATT
Template:	50ng human gDNA

HotMaster *Taq* and Standard *Taq* cycling conditions:

Initial Denaturation:	None
35 cycles:	94°C for 1 second
	55°C for 1 second
	72°C for 5 seconds
Hold:	10°C

"Chemically blocked" *Taq* cycling conditions:

Initial Denaturation:	95°C for 10 minutes
35 cycles:	94°C for 1 second
	55°C for 1 second
	72°C for 5 seconds
Hold:	4°C

"Antibody-blocked" *Taq* cycling conditions:

Initial Denaturation:	95°C for 2 minutes
35 cycles:	94°C for 1 second
	55°C for 1 second
	72°C for 5 seconds
Hold:	4°C

EXPERIMENT 2: PROCESSIVITY

A 5.0kb Phage Lambda target was amplified using Eppendorf HotMaster *Taq* DNA polymerase and Competitor A Hot Start DNA polymerase.

PRIMERS AND TEMPLATE:

Forward Primer:	GGCAAGCATAAGCACACAGA
Reverse Primer:	CAGCATAAGCGGCTACATGA
Template:	10ng Lambda DNA

HotMaster *Taq* cycling conditions:

Initial Denaturation:	94°C for 2 minutes
35 cycles:	94°C for 45 seconds
	61°C for 30 seconds
	65°C for 5 minutes
Final extension:	65°C for 5 minutes
Hold:	10°C

Competitor A cycling conditions:

Initial Denaturation:	95°C for 10 minutes
35 cycles:	94°C for 45 seconds
	61°C for 30 seconds
	72°C for 5 minutes
Final extension:	72°C for 5 minutes
Hold:	10°C

EXPERIMENT 3: SENSITIVITY

A 201 bp target in the human SRY gene (a single-copy gene on the Y chromosome) was amplified using Eppendorf HotMaster *Taq* DNA polymerase.

PRIMERS AND TEMPLATE:

Forward Primer:	CTCCGGAGAAGCTCTTCCTT
Reverse Primer:	CAGCTGCTTGCTGATCTCTG
Template:	0pg-100ng human male DNA

HotMaster *Taq* cycling conditions:

Initial Denaturation:	94°C for 2 minutes
35 cycles:	94°C for 20 seconds
	62°C for 20 seconds
	65°C for 30 seconds
Hold:	10°C

Cont.

Eppendorf HotMaster—An Innovative Hot Start/Cold Stop Technology for Better PCR* Results (cont.)

RESULTS AND DISCUSSION

Specificity

In order to determine the performance of the HotMaster *Taq* polymerase versus other Hot Start systems, we chose a set of primers that normally gives multiple nonspecific bands. When these primers were used to amplify a 131 bp fragment of the human TNF target, HotMaster *Taq* (*Fig. 1, lanes 4-6*) clearly outperformed the chemically modified Hot Start enzyme (*Fig. 1, lanes 7-8*) and the antibody-inhibited Hot Start enzyme (*Fig. 1, lanes 10-11*). Though the chemically modified Hot Start enzyme gave expected bands, the yield of the reaction is significantly lower than that of Eppendorf HotMaster. The low yield is probably caused by the partial loss of enzymatic activity during the long initial denaturation step at high temperature (95°C for 10 minutes). The yield for the "antibody-blocked" Hot Start enzyme is comparable to that of the regular *Taq* polymerase. However, the nonspecific bands were produced presumably because the antibody had been denatured in the early cycles and was no longer able to provide inhibition of the polymerase at sub-optimal annealing temperatures. As expected, HotMaster produces a greater yield and more specific amplification than standard *Taq* (*Fig. 1, lanes 1-3*).

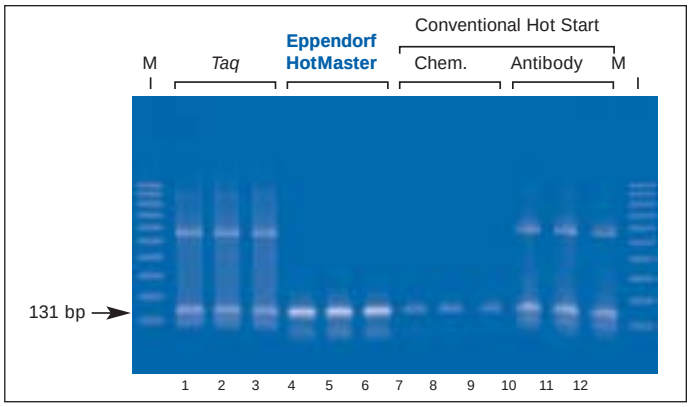


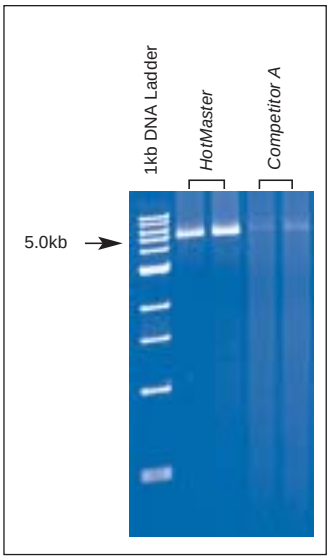
Figure 1:
Amplification of a 131 bp fragment of the human TNF gene using HotMaster and competing Hot Start technologies.

Lanes 1-3: Standard *Taq*
Lanes 4-6: Eppendorf HotMaster *Taq*
Lanes 7-9: Chemically blocked *Taq*
Lanes 10-12: Antibody-blocked *Taq*
M: 100 bp ladder (NEB)

Processivity:

HotMaster *Taq* can be used to amplify fragments up to 5.0kb long. HotMaster easily amplifies a 5.0kb fragment from Lambda Phage. In contrast, a chemically modified Hot Start enzyme barely produces any amplification of this same fragment (*Fig. 2*).

Figure 2:
Amplification of a 5.0kb Lambda DNA target using Eppendorf HotMaster *Taq* and Competitor A Hot Start *Taq*. 1µL of the PCR reactions was loaded onto a 1% agarose gel. Replicates are shown for each enzyme.



Sensitivity

To test the limits of the amount of template that can be amplified in a highly complex background, a fragment of the male-specific human single-copy gene SRY was amplified using HotMaster *Taq*. Based on the knowledge that 6pg of human gDNA equals one copy of the genome, serial dilutions of male DNA were spiked into a constant background of 100ng female DNA so that the PCR reactions contained 0, <10, 10, 100, 1000 or 10,000 copies of the target gene. The data show (*Fig. 3*) that HotMaster *Taq* DNA Polymerase is able to amplify even extremely low (<10) target DNA molecules in a high background of non-specific DNA.

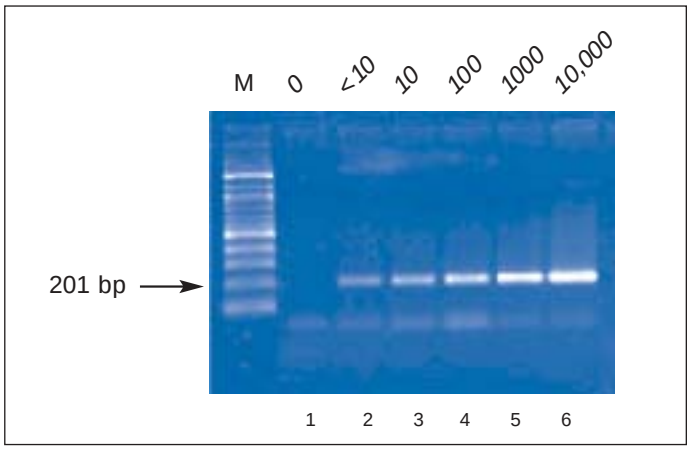


Figure 3:
Amplification of a 201 bp SRY gene target using HotMaster *Taq* with varying amounts of template. 10µL of the PCR reactions were loaded onto a 2.5% agarose gel.

CONCLUSIONS

Eppendorf HotMaster *Taq* DNA Polymerase offers several advantages over other Hot Start DNA polymerases. First and foremost, HotMaster *Taq* both reduces non-specific PCR products and increases specific yield to a greater extent than the major Hot Start DNA polymerases on the market. This is primarily due to the significant advantages of this kit such as "Hot Start/Cold Stop" technology. In addition, no heat activation is required with HotMaster, and target sizes of up to 5.0kb can be efficiently and reproducibly amplified. Furthermore, the Mg^{++} concentration is self-adjusting, which makes it optimal for virtually all targets. Lastly, because of its unique technology, HotMaster will not contaminate PCR reactions with unwanted proteins as do the Hot Start DNA polymerases that employ antibodies to achieve inhibition. Testing has also shown that HotMaster *Taq* can be used in real-time PCR applications (data not shown).

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