GE Healthcare

Increased polymerase chain reaction (PCR) amplification specificity using illustra Hot Start Master Mix

Key words: *Hot Start Master Mix ● hot start PCR ● polymerase chain reaction (PCR)*

illustra™ Hot Start Master Mix is a 2× premixed formulation containing *Taq* DNA Polymerase, ultrapure deoxynucleotide triphosphates (dNTPs), Hot Start Activator protein, and an optimized polymerase chain reaction (PCR) buffer. This system offers the convenience of a master mix in which only primers, template, and water are required to be added for endpoint polymerase chain reactions.

Hot start PCR is a method developed to eliminate nonspecific amplification and limit primer-dimer formation during PCR. Both of these conditions can generate spurious bands, background smears and reduce the overall efficiency and yield of PCR. Traditional hot start methods rely on maintaining the polymerase in an inactive state through monoclonal antibodies directed against the polymerase, or by chemical modification of the enzyme itself (1, 2).

Hot Start Activator protein sequesters the primers away from the polymerase, making them unavailable for nonspecific priming during reaction setup. Unlike chemically modified hot start polymerases, which can require extensive precycling heat treatment, the DNA-binding activity of the Hot Start Activator protein employed here is readily inactivated during a much gentler precycling step. This characteristic lessens the chances for template DNA damage prior to the cycling steps, which is important when using small quantities of starting material or if amplification of long fragments is required. The net result is a more robust and reliable amplification of the target amplicons with increased yield when compared to conventional hot start master mix products. The buffer is formulated to contain $MgCl_2$, but can be easily supplemented with additional $MgCl_2$ allowing users to customize this reagent to their specific needs.

This application note highlights the hot start capability of Hot Start Master Mix, providing evidence of its polymerase-blocking ability and elimination of primer-dimer formation. In addition, this system does not interfere with common downstream applications such as restriction digestion and direct sequencing of the amplified PCR product.

Products used

Hot Start Master Mix	25-1500-01
DYEnamic™ ET Terminator Cycle Sequencing Kit	US81050
ExoSAP-IT™	US78200

Other materials used

ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems)

Custom design primers (Integrated DNA Technologies)

Taq DNA Polymerase Master Mix (USB)

Chemically inactivated Hot Start *Taq* DNA Polymerase Master Mix (Qiagen)

Monoclonal antibody–inactivated Hot Start *Taq* DNA Polymerase Master Mix (Invitrogen)

Thermocycler PTC-225 (MJ Research)

Human Genomic DNA (Promega)

Novex TBE-Urea Gel and 2× Sample Buffer (Invitrogen)

Autoclaved, deionized water

Agilent Bioanalyzer 2100 (Agilent Technologies)

DNA 1000 Lab Chip (Agilent Technologies)

Pst I restriction endonuclease and 10× buffer (New England Biolabs)

MegAlign software (DNAstar)

PCR setup

Reactions were set up according to the manufacturer's protocol in a total volume of 25 µl. Forward and reverse primer concentrations were 0.2 µM for the primer-dimer assay and 0.4 µM for the amplification of a 1018-bp fragment from the human Apo gene. Template amounts for the primer-dimer assay and the 1018-bp fragment amplification were 1 ng and 20 ng per reaction, respectively.

Thermal cycling conditions

Primer-dimer assay:

25 °C for 60 min
*95 °C for 2 min

Three-step cycling (35 cycles)
- 95 °C for 10 s
- 60 °C for 5 s
- 72 °C for 30 s for numb fragment or 2 min for p53 fragment

Final extension for 5 min at 72 °C

* Initial heating step of 95 °C for 15 min was used to activate the chemically modified polymerase.

1018-bp human Apo gene fragment:

*95 °C for 2 min

Three-step cycling (35 cycles)
- 94 °C for 30 s
- 50 °C for 30 s
- 72 °C for 1 min

Final extension for 5 min at 72 °C

* Initial heating step of 95 °C for 15 min was used to activate the chemically modified polymerase.

Polymerase-blocking activity of Hot Start Master Mix

The ability to maintain a DNA polymerase in an inactive state underpins all hot start PCR systems. This goal is normally achieved via antibody-mediated inhibition or chemical modification of the polymerase. Hot Start Master Mix uses a novel method for hot start PCR in which the oligonucleotides are sequestered from the polymerase: this reduces the potential for nonspecific priming. Thus, the activity of the polymerase is blocked by the removal of one of its substrates.

Polymerase blocking ability was demonstrated by using an assay that compared the polymerase activity of Hot Start Master Mix to that of a master mix in which the Hot Start Activator protein was heat-inactivated by incubation at 95 °C for 2 min. The reaction mixture contained 1× master mix in a final volume of 25 µl, and 2 pmol of overlapping, extendable oligonucleotides with a 9-bp overlap. One of the oligonucleotides was labeled with HEX fluorescent dye while the other was not. After incubation at 25 °C for 4 h, the reaction mixtures were resolved on a 15% polyacrylamide TBE-Urea denaturing gel. The presence of an active polymerase would lead to an extension of the labeled 23-bp oligonucleotide to 50 bp but an inactive or blocked polymerase could not extend the labeled oligonucleotide.

Figure 1 shows results for the polymerase blocking assays in which reactions were set up in triplicate using a non–hot start master mix (lanes 1–3), Hot Start Master Mix (lanes 4–6), and commercially available master mixes whose polymerase was either inactivated by a monoclonal antibody or by chemical modification (lanes 7–9 and 10–12, respectively). Reactions were divided equally and incubated at 25 °C for 4 h (Panel A) or were heat-treated to reverse the inhibitory mechanism prior to 25 °C incubation (Fig 1, Panel B). Heat treatment consisted of incubation at 95 °C for 2 min (lanes 1–9) or 95 °C for 15 min (lanes 10–12).

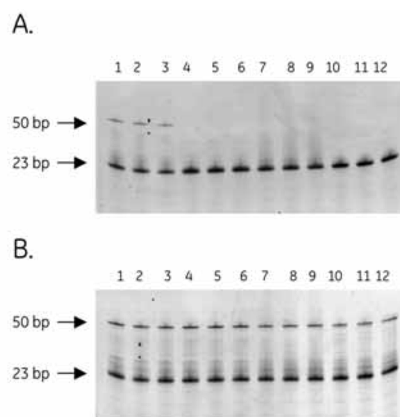

Fig 1. Comparison of Hot Start Master Mix to other commercially available hot start systems in a polymerase blocking assay. Reactions in lanes 1–3 used PCR master mix, lanes 4–6 used Hot Start Master Mix, lanes 7–9 used antibody-inhibited polymerase master mix and lanes 10–12 used a chemically inhibited polymerase master mix. Reactions in Panel A were incubated for 4 h at 25 °C. Reactions in Panel B were heat-treated at 95 °C for 2 min (lanes 4–9) or at 95 °C for 15 min (lanes 10–12) to stop the respective inhibitory mechanisms of the master mixes. All reactions were resolved on a 15% polyacrylamide TBE-Urea denaturing gel and quantitated using ImageQuant software.

Panel A shows a significant accumulation of the extended, 50-bp product in the reactions where non–hot start master mix was used (lanes 1–3). In the reactions where a hot start method was employed, there was no discernible 50-bp fragment formed (lanes 4–12). This is in contrast to reactions where the inhibitory mechanism was reversed by heat inactivation. A marked increase in the amount of 50-bp product formed in all the hot start-capable reactions suggest that the polymerase had been activated (Panel B, lanes 4–12).

The amount of 50-bp fragment was quantitated for each reaction using ImageQuant™ software. A ratio between the amounts of product formed in the non-heat treated samples to those that were heat-treated was established. This was then multiplied by 100% to yield the relative polymerase activity for a given reaction.

$$\% \text{ Activity} = \frac{\% \text{ 50-bp fragment formed in nonheated samples}}{\% \text{ 50-bp fragment formed in heated samples}} \times 100\%$$

Subtracting the activity from 100% yields the relative amount of polymerase activity blocked when a hot start method is used. The results show that Hot Start Master Mix blocks polymerase activity to 99.7% while the monoclonal antibody–inhibited and chemically modified polymerases blocked activity to 97% and 99.4%, respectively. This demonstrates that Hot Start Master Mix blocks polymerase activity by primer sequestration to the same degree as commercially available hot start master mixes.

Inhibition of primer-dimer formation by Hot Start Master Mix

PCR amplification using primers with homology to one another is often unavoidable due to the sequence constraints of the region to be amplified. This situation often results in the formation of primer-dimers in which the primers anneal and are extended by active polymerase in the presence of dNTPs during reaction setup. This renders the primers unusable for subsequent cycling reactions because of the loss of specificity for the intended target, resulting in a decrease in the yield of specific PCR product.

To demonstrate the ability of Hot Start Master Mix to inhibit primer-dimer formation, forward and reverse primers that were specific for the amplification of either a 303-bp fragment from the human numb locus or a 1114-bp fragment from the human p53 locus were designed. The pair of primers—forward and reverse—was designed with a 3-bp overlap at the 3′ ends. During reaction setup and a precycling incubation at 25 °C, this overlap allowed the oligonucleotides to form base pairs resulting in a duplex DNA with an exposed 3′-hydroxyl group. The latter is a recognizable substrate for DNA polymerases. Active polymerase, in the presence of dNTPs, recognizes this exposed end and extends the primer sequences using complementary forward and reverse primers as templates. The newly extended sequence lacks homology to the original intended target sequence and cannot act as a primer for amplification.

Equal amounts of forward and reverse primers (2 pmol) and 1 ng human genomic DNA template were used in reactions with non–hot start master mix (Fig 2A and 2B, lanes 1–4), Hot Start Master Mix (Fig 2A and 2B, lanes 5–8) and master mixes that use either monoclonal antibody–inactivated or chemically inactivated polymerase (Fig 2A and 2B, lanes 9–12 and 13–16, respectively). No-template control reactions for each master mix were in lanes 4, 8, 12, and 16. All reactions were incubated at 25 °C for 60 min prior to cycling. Equal volumes of each reaction were resolved on a 1.5% agarose-TAE gel stained with ethidium bromide and the average overall yield for a 25-µl reaction of the specific, amplified product for each master mix was determined with an Agilent Bioanalyzer 2100.

Figure 2A shows the results of the primer-dimer inhibition assay for the 303-bp numb locus DNA fragment. In the absence of hot start there was a weak amplification of the specific 303-bp fragment (Fig 2A, lanes 1–3) with an average yield of only 15.7 ng for a 25-µl reaction as quantitated using an Agilent Bioanalyzer 2100. However, when Hot Start Master Mix was used, the amount of 303-bp product increased dramatically (compare lanes 5–7 to 1–3 of Fig 2A) with an average yield of 168 ng for a 25-µl reaction. This represents a greater than 10-fold increase in overall product yield for Hot Start Master Mix over a non–hot start master mix reagent reaction. Although there was an increase in the specific amplified fragment, the results were less dramatic for the amplification of the numb locus fragment using either the antibody-inhibited or chemically inhibited polymerase master mixes (Fig 2A, lanes 8–11 and 13–15). The average overall yield for these 25-µl reactions were 31 ng and 32 ng for the antibody and chemically inhibited polymerase master mixes, respectively. Compared to non–hot start reactions, these master mixes showed an increase in yield of only about two-fold, which is five times lower than the increase observed with Hot Start Master Mix.

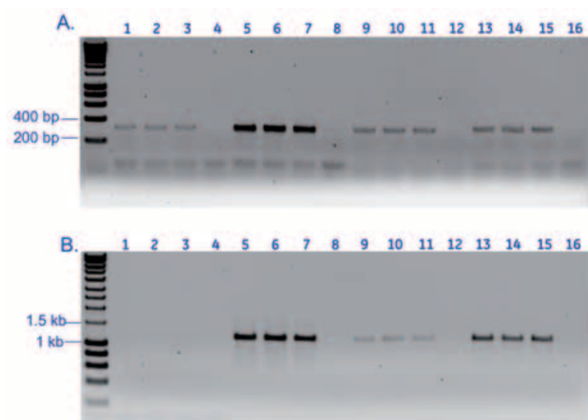

Fig 2. Primer-dimer inhibition. In all the reactions, we used 1 ng of human genomic DNA as template and 0.2 µM each of forward and reverse primers specific for the human numb locus (A) or human p53 locus (B), which amplified fragments of 303-bp and 1114-bp, respectively. For Panels A and B, lanes 1–4 used non–hot start PCR master mix reactions, lanes 5–8 used Hot Start Master Mix, lanes 9–12 used antibody-inhibited polymerase master mix, and lanes 13–16 used chemically inhibited polymerase master mix. Lanes 4, 8, 12, and 16 represent no-template control reactions. Products were resolved on a 1.5%-agarose TAE gel stained with ethidium bromide.

Figure 2B depicts the results of the primer-dimer inhibition assay for the 1114-bp p53 locus DNA fragment. As with the numb locus primers, amplification of the 1114-bp p53 fragment was weak and barely visible on an agarose gel (Fig 2B, lanes 1–3). Quantitation using the Agilent Bioanalyzer 2100 was unable to detect a significant peak indicating a complete failure of amplification. In sharp contrast, reactions using the Hot Start Master Mix (Fig 2B, lanes 5–7) produced an average yield of 102 ng. Comparatively, this represents a greater than 13-fold more product than the antibody-inhibited polymerase master

mix, whose overall average yield was only 7.4 ng. The chemically inhibited polymerase master mix performed slightly better than the antibody-inhibited polymerase, with an overall average yield of 48 ng, but this is still two-fold less than reactions using Hot Start Master Mix.

These results demonstrate that Hot Start Master Mix effectively eliminates primer-dimer formation to a degree that is equal to or better than that for other hot start methods that employ either monoclonal antibody–mediated inhibition or chemical inhibition of the DNA polymerase. This leads to an increased yield in the amplification of the desired product.

Compatibility with downstream applications

To demonstrate that products amplified with Hot Start Master Mix are compatible with downstream applications, sequencing and restriction digestion reactions were carried out with fragments amplified using Hot Start Master Mix, monoclonal antibody–inhibited polymerase and chemically inhibited polymerase master mixes.

A 1018-bp fragment of the human Apo gene was amplified in duplicate 25-µl reactions using Hot Start Master Mix, antibody-inhibited polymerase, or chemically inhibited polymerase master mix. Following amplification and quantitation, 100 ng of each reaction product was digested with *Pst* I restriction endonuclease. A unique *Pst* I restriction site within this fragment at nucleotide position 400 ensures that a complete digestion will yield two fragments of 400 and 618 bp, respectively.

Negative control reactions were set up alongside reactions containing the restriction enzyme with 50 ng of product. All reactions were incubated at 37 °C for 2 h and resolved on a 1% TAE agarose gel stained with ethidium bromide. The results are shown in Figure 3 where lanes 1–6 represent negative control reactions and lanes 7–12 are reactions with *Pst* I. Products in lanes 1, 2, 7, and 8 were amplified with Hot Start Master Mix. Products in lanes 3, 4, 9, and 10 were amplified with an antibody-inhibited polymerase master mix. Products amplified with a chemically inhibited polymerase master mix are shown in lanes 5, 6, 11, and 12. All the PCR products that were digested with *Pst* I yielded two fragments with one at ~ 400 bp and the other at ~ 600 bp. These results demonstrate that PCR products amplified with Hot Start Master Mix are amenable to restriction analysis and show no difference compared to other hot start master mixes.

1 2 3 4 5 6 7 8 9 10 11 12
1000 bp
600 bp
400 bp
200 bp

Fig 3. Restriction digest of 50 ng of product amplified with Hot Start Master Mix (lanes 1, 2, 7, and 8), antibody-inhibited polymerase master mix (lanes 3, 4, 9, and 10) and chemically inhibited polymerase master mix (lanes 5, 6, 11, and 12) were incubated at 37 °C for 2 h in 1× restriction buffer minus *Pst* I (lanes 1-6) or plus *Pst* I (lanes 7-12). All the reactions were resolved on a 1% TAE agarose gel stained with ethidium bromide.

To further illustrate downstream application compatibility, the 1018-bp products were sequenced using a nested primer that anneals within the 1018-bp Apo sequence. PCR products were treated with ExoSAP-IT to remove unincorporated dNTPs and primers from the amplification step. Sequencing was carried out using the DYEnamic ET Terminator Cycle Sequencing Kit for ABI instruments and run on an ABI PRISM 3100. A portion of the electropherogram spanning the same sequence for one of each template is shown in Figure 4. There is negligible difference in peak heights and sequence quality in all cases demonstrating that products amplified with Hot Start Master Mix do not interfere with PCR cleanup and sequencing reactions.

Hot Start Master Mix
mAb
Chemical

Fig 4. Representative electropherograms for sequenced PCR fragments. A portion of the electropherogram spanning the same sequence for each template shows consistent, high-quality sequencing results from each fragment.

Sequence results were aligned with MegAlign software using the Jotun-Hein sequence alignment method. A 500-bp stretch of the known Apo sequence starting at 50 bp 3' to the end of the sequencing primer was included in the alignment to determine the percent identity of each sequence to the known sequence. Figure 5 shows the alignment of the known Apo sequence to the sequences obtained using the PCR-amplified fragments as sequencing template. Phred20 scores for each sequenced fragment were determined. Hot Start Master Mix exhibited a Phred20 score of 385 bp while fragments amplified by antibody-inhibited and chemically inhibited master mix showed Phred20 scores of 350 bp and 353 bp, respectively. These results demonstrate that PCR products amplified with Hot Start Master Mix are better than other commercially available hot start master mixes in compatibility with downstream applications and have no adverse affect on these processes.

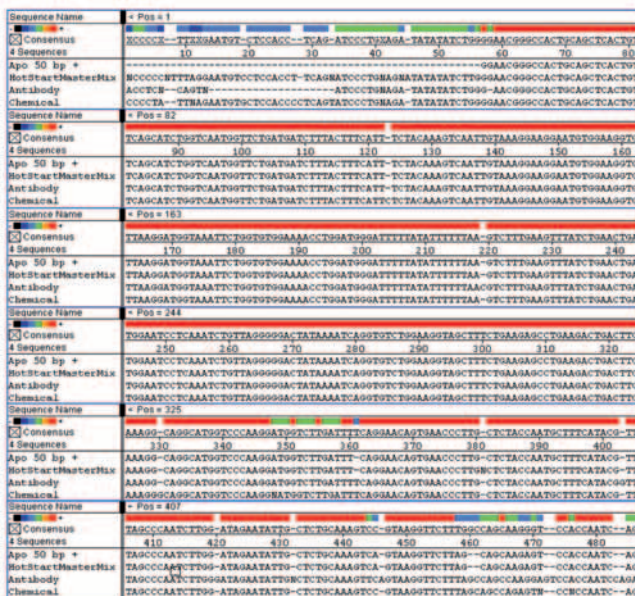

Fig 5. Alignment of sequences from templates amplified with different hot start PCR master mixes. The sequences were aligned with MegAlign software using the Jotun-Hein method. Apo 50 bp + is the known Apo sequence to which all sequences were compared. Red, green, blue, and no shading represent 100%, 75%, 50%, and 25% identity, respectively, across all sequences.

Conclusions

The high degree of polymerase blocking during reaction set up, as a consequence of using Hot Start Master Mix, means that the specificity is at least as high as that achieved using other commercially available hot start master mix systems.

Hot Start Master Mix more effectively inhibits the formation of primer-dimers during PCR than other commercially available products. This results in an increased yield, when compared to conventional hot start master mix products, for reactions where there is a high tendency for the formation of primer-dimers.

Hot Start Master Mix can be used with confidence in downstream applications. The Hot Start Activator protein does not interfere with downstream processes such as sequencing or restriction digests.

References

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