

Increase Specificity, Sensitivity and Yield with HotStart Storage and Reaction Tubes

INTRODUCTION

Hot start PCR is a novel technique that can increase the yield and sensitivity of conventional PCR reactions as well as PCR reactions involving DNA that is difficult to amplify. In addition, hot start PCR can improve the specificity of the reaction by reducing false priming and primer dimerization.¹ The hot start technique entails withholding one or more key reactants (i.e. Taq DNA Polymerase) from the PCR reaction until the reaction has reached a temperature high enough to discourage false priming (generally 60° to 80°C).

Historically, mineral oil or wax beads have been used to generate a hot start PCR reaction. However, the use of mineral oil in a hot start reaction is cumbersome and prone to contamination as it requires opening each reaction tube after it has been placed in the temperature cyclers to pipet the missing key components through the oil.² Additionally, hot start PCR reactions performed with oil do not allow each reaction to begin simultaneously, thus introducing additional variables into the reaction. The use of wax beads as a physical barrier between components does enable the synchronous start up of each hot start PCR reaction, however, many researchers have found wax beads prohibitively expensive and inconvenient to work with. Typically they require delicate handling and shipping and storage at cold temperatures to prevent the beads from coagulating.

Molecular BioProducts, inc. has introduced the HotStart Storage and Reaction Tube, a thin-walled tube and pre-positioned wax bead all in one. The HotStart Storage and Reaction Tube provides a convenient and economical format to perform the hot start PCR reaction. Performing hot start PCR in the HotStart Storage and Reaction Tube can significantly increase the yield, specificity, and sensitivity of PCR reactions.

OBJECTIVE

To test the effectiveness of the hot start PCR methodology and to verify the increased yield, specificity and sensitivity obtained when using HotStart Storage and Reaction Tubes, PCR reactions were performed on DNA that could be amplified using conventional PCR conditions and DNA that could not be amplified by conventional PCR techniques.³ The following study consisted of a series of three experiments conducted in our laboratories. In experiment #1 total human genomic DNA (provided by K. L. Diggle Veterans Medical Research Foundation of San Diego) was used as a template in directly comparing the increased yield gained over conventional PCR when performing the hot start PCR protocol in the HotStart Storage & Reaction Tubes. Experiment #2 utilized the same DNA template and illustrates the increased specificity that can be achieved when performing the hot start PCR reaction in a HotStart Storage and Reaction Tube. Experiment #3 involved the use of previously unamplifiable mitochondrial D-loop DNA extracted from aged, human bones found in the New Zealand bush and coastline (provided by B. J. Scrimshaw, ESR Forensics, Ltd., New Zealand). This experiment illustrates the effectiveness of the hot start PCR reaction when performed in the HotStart Storage and Reaction Tubes with targets that are very difficult to amplify under standard conditions.

MATERIALS AND METHODS

Experiment #1

Template was total human genomic DNA purified from whole blood using the PureGene™ DNA Isolation Kit (Gentra Systems, Inc.).

Each conventional PCR reaction was performed in 20 µl total volume in a thin-walled tube as follows per reaction: 13.9 µl dH₂O, 2 µl 10x PCR buffer (100 mM Tris HCl (pH 8.8), 500 mM KCl, 15 mM MgCl₂, 0.1% gelatin), 2 µl 2.5 mM dNTP mix, 0.5 µl forward primer (20 µM stock), 0.5 µl reverse primer (20 µM stock), 0.1 µl Taq DNA polymerase (5 U/µl, Boehringer Mannheim), 1 µl total human genomic DNA template (0.1 µg/µl).

Each hot start PCR reaction was performed in 20 µl total volume in a HotStart Micro 20® tube as follows per reaction: Lower layer: 6 µl

dH₂O, 1 µl 10x PCR reaction buffer (ingredients above), 2 µl 2.5 mM dNTP mix, 0.5 µl forward primer (20 µM stock), 0.5 µl reverse primer (20 µM stock). Upper layer: 1 µl 10x PCR buffer (ingredients above), 1 µl total human genomic DNA template, 7.9 µl dH₂O, 0.1 µl Taq DNA Polymerase (5 U/µl, Boehringer Mannheim). All PCR was performed using the following conditions: 94° C one minute (1 cycle), 92° C 30 seconds, 70° C 40 seconds with ramp to 60° C one minute (30 cycles).

Experiment #2

Each conventional PCR reaction was performed in 40 µl total volume in a thin-walled tube as follows per reaction: 27.8 µl dH₂O, 4 µl 10x PCR buffer (ingredients above), 3.2 µl 2.5 mM dNTP mix, 0.4 µl forward primer (50 µM stock), 0.4 µl reverse primer (50 µM stock), 0.2 µl Taq DNA polymerase (5 U/µl, Boehringer Mannheim), 2 µl total human genomic DNA template (0.1 µg/µl), 2 µl DMSO (5% final concentration).

Each hot start PCR reaction was performed in 40 µl total volume in a HotStart 50® tube as follows per reaction: Lower layer: 3.2 µl dNTP's, 2 µl 10x PCR reaction buffer (ingredients above), 0.4 µl forward primer, 0.4 µl reverse primer, 14 µl dH₂O. Upper layer: 2 µl 10x PCR buffer (ingredients above), 2 µl total human genomic DNA template, 0.2 µl Taq DNA Polymerase (5 U/µl, Boehringer Mannheim), 2 µl DMSO (5% final concentration), 13.8 µl dH₂O. All PCR was performed using the following conditions: 94° C, five minutes (1 cycle), 94° C 15 seconds, 63° C 15 seconds, 72° C 30 seconds, (40 cycles), 72° C five minutes (1 cycle).

Experiment #3

Each conventional PCR reaction was performed in 50 µl total volume in a thin walled tube as follows per reaction: 36.6 µl dH₂O, 5 µl 10x PCR buffer (ingredients above), 5 µl 2.5 mM dNTP mix, 1 µl forward primer (250 nM), 1 µl reverse primer (250 nM), 0.4 µl Taq DNA polymerase (5 U/µl, Boehringer Mannheim), 1 µl total genomic DNA template (0.1 µg/µl).

Each hot start PCR reaction was performed in 50 µl total volume in a HotStart 50 tube as follows per reaction: Lower layer: 5 µl dNTP's, 2 µl 10x PCR reaction buffer (ingredients above), 1 µl forward primer (250 nM), 1 µl reverse primer (250 nM), 16 µl dH₂O. Upper layer: 3 µl 10x PCR buffer (ingredients above), 1 µl total genomic DNA template, 0.4 µl Taq DNA Polymerase (5 U/µl, Boehringer

Mannheim), 20.6 μ l dH₂O. All PCR was performed using the following conditions: 93° C three minutes (1 cycle), 94° C 45 seconds, 56° C one minute, 72° C one minute (35 cycles), 72° C five minutes (1 cycle).

For all experiments, after the PCR reactions were completed 20 μ l of each sample was electrophoresed on a 2% agarose gel (FMC Biochemicals) and visualized with ethidium bromide (EtBr) staining.

RESULTS

Experiment #1 and #2: Increased yield and Increased specificity with HotStart Storage and Reaction Tubes.

In order to illustrate the increased yield that can be obtained using the HotStart Storage and Reaction Tubes, hot start PCR was performed on total human genomic DNA purified from whole blood. The MX6-1 and MX6-2 primers were used to detect the 155 base pair D10S173 target (see figure #1). Lanes B and C represent conventional PCR using a mineral oil overlay and lanes D and E represent hot start PCR performed in the HotStart Storage and Reaction tubes. When hot start PCR was performed in the HotStart Storage and Reaction tubes, there was a significant increase in yield of the 155 base pair target as well as a reduction in the primer dimerization which occurred in the conventional PCR reaction.

To illustrate improved PCR specificity, this same genomic DNA was used a second time in both a conventional PCR reaction and a hot start PCR reaction. The X1.31F and X1.26R primers were used to detect the 204 base pair target (see figure #2). Lanes C and D represent conventional PCR using a mineral oil overlay. Lanes E and F represent hot start PCR performed in the HotStart Storage and Reaction Tubes. When hot start PCR was performed using the HotStart Storage and Reaction Tubes, the expected 204 base pair band resulted without the unwanted 870 base pair band or smear that could be seen in the conventional PCR reaction with the mineral oil overlay.

Experiment #3: HotStart Storage and Reaction Tubes with Hard-to-Amplify DNA.

In order to prove the effectiveness of hot start PCR performed in the HotStart Storage and Reaction Tubes with DNA that is hard-to-copy, we attempted to amplify mitochondrial D-loop DNA extracted from aged bones using primers for hypervariable region one of the mitochondrial D-loop [Anderson S. et al, Nature 290, 457-465, 1981]. Some bones were estimated to be over 150 years old. According to previous research, this DNA could not be amplified previously using a conventional PCR reaction with a mineral oil overlay. Using the HotStart Storage and Reaction Tubes, hot start PCR was performed on this DNA. Results from this experiment are shown in figure #3. It can be seen that signif-

icant amplification occurred when hot start PCR was performed using the HotStart Storage and Reaction Tubes whereas no amplification resulted utilizing conventional PCR conditions. In this experiment, enough DNA was amplified using the hot start PCR reaction in the HotStart Storage and Reaction Tube to purify the DNA from the gel for further analysis.

CONCLUSIONS

Hot start PCR performed in HotStart Storage and Reaction Tubes can increase the yield and specificity of PCR reactions by reducing false priming and primer dimerization.

When compared to conventional PCR, hot start PCR performed in HotStart Storage and Reaction Tubes yielded superior results and increased sensitivity on DNA that is hard-to-amplify.

Hot start PCR performed in the HotStart Storage and Reaction Tube can significantly increase the sensitivity in PCR reactions involving the detection of multiple target sequences with complex, genomic templates.

Molecular BioProducts' HotStart Storage and Reaction Tubes provide an effective format for performing the hot start PCR reaction. The tubes are a practical, economical alternative to mineral oil overlays or individual wax beads.

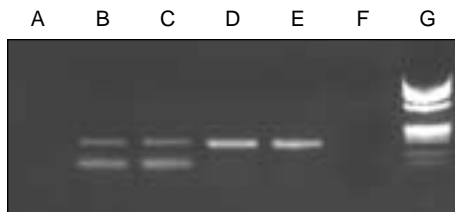


Figure 1. Lane A negative control with mineral oil overlay, Lanes B & C conventional PCR performed with mineral oil overlay, Lanes D & E hot start PCR performed in HotStart Storage and Reaction Tubes, Lane F negative control in HotStart tube, Lane G ϕ X174/Hae III Marker. Both methods amplified the 155 b.p. target, however primer dimerization occurred in lanes B & C.

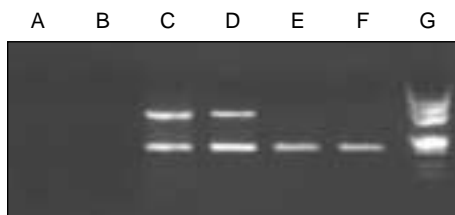


Figure 2. Lane A negative control with mineral oil overlay, Lane B negative control in HotStart Storage and Reaction Tube, Lanes C & D conventional PCR performed with mineral oil overlay, Lanes E & F hot start performed in HotStart tubes, Lane G ϕ X174/Hae III Marker. The 204 b.p. target was amplified with the HotStart tubes without the unwanted 870 b.p. sequence seen in lanes C & D.

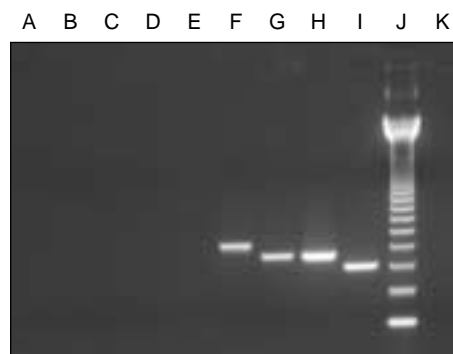


Figure 3. Lanes A,B,C,D & E conventional PCR performed with a mineral oil overlay, Lanes F,G,H,I & K hot start PCR performed in HotStart Storage and Reaction Tubes, Lane J 1kb ladder. Lanes A & F H16401-L15926 primers, Lanes B & G H16325-L15926 primers, Lanes C & H H16401-L15977 primers, Lanes D & I H16325-L15977 primers. Only with hot start PCR performed in HotStart Storage and Reaction Tubes was amplification possible.

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