Simplified hot start PCR

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The use of a thermally activated DNA polymerase PCR gives improved specificity, sensitivity and product yield without additives or extra process steps.

HIGH background and low specific product yield can occur in a polymerase chain reaction (PCR) when reaction components are mixed at room temperature[1–3]. Reaction setup below the optimal primer annealing temperature (usually 50–65 °C) permits nonspecific primer annealing and extension. Undesired, non-specific primer extension products formed this way may be amplified in the PCR, resulting in misprimed products and primer oligomers.

In 'hot start' PCR, at least one essential reagent is withheld from the PCR mixture until the system has reached a temperature that favours specific primer annealing. Hot start PCR has been implemented by mechanically separating a reagent[1–3] or by blocking the polymerase with an antibody[4,5]. In another variation, nonspecific product formed at low temperatures was destroyed by a heat-labile uracil glycosylase[6]. Hot start can greatly improve specificity, sensitivity, and yield in a PCR[1–6].

AmpliTaq Gold is a modified, inactive form of AmpliTaq DNA polymerase (both from Perkin-Elmer, Foster City, California). AmpliTaq is a thermostable DNA polymerase commonly used for PCR. Though the maximum activity of most polymerases used for PCR is at 60–70 °C, these enzymes exhibit significant activity at room temperature, whereas AmpliTaq Gold is inactive at room temperature. Heat restores the polymerase activity, allowing the addition of a hot start to an existing PCR, with no changes to the protocol except the addition of a pre-PCR heat cycle. PCR setup can be done at room temperature, as primer extension cannot occur when the DNA polymerase is inactive. The pre-PCR heat cycle can be programmed into the thermal cycler so that the transition from enzyme activation to PCR cycling occurs without going below the optimal primer annealing temperature.

Modified enzyme features

For a thermostable DNA polymerase provided in an inactive state, the enzyme unit activity given is the nascent DNA polymerase activity. To measure the nascent activity, enzyme samples are heated for 3 h at 80 °C in a buffer containing 25 mM Tris-HCl, pH 8.0 (at room temperature), 50 mM KCl, 1 mM β-mercaptoethanol, 0.5 per cent NP-40 and 0.1 per cent gelatin, and are then assayed in a DNA polymerase activity assay. Under these conditions the enzyme is activated maximally without thermally attenuating the final polymerase activity (data not shown).

To demonstrate the activation during a pre-PCR heat cycle, the DNA polymerase was incubated at 95 °C, in a commonly used PCR buffer (Fig. 1). The enzyme was activated maximally within 15 minutes. It is expected, however, that some of the potential polymerase activity was lost due to the extended incubation at 95 °C.

The new modified enzyme was substituted for unmodified DNA polymerase in a PCR amplification of a 142 bp HIV target from ten template copies in a background of 10 ng of human placental DNA (Fig. 2). This system was previously used for PCR without a hot start, using oligomer hybridization with ^{32}P-labelled probes for product detection[7]. For manual hot start, unmodified DNA polymerase was withheld from the reaction mixture, then individually added to each PCR tube during a 78 °C hold before the PCR cycling began. For modified polymerase reactions, all of the reagents were mixed at room temperature, and the only change to the PCR conditions was the addition of a nine minute, 95 °C pre-PCR heat cycle. The product was analysed by agarose gel electrophoresis with ethidium bromide staining. In the reactions done without hot start, specific product was not visible (Fig. 2, lanes 4 and 5). Manual hot start with unmodified DNA polymerase gave visible product (Fig. 2, lanes 6 and 7). However, PCR with the modified DNA polymerase

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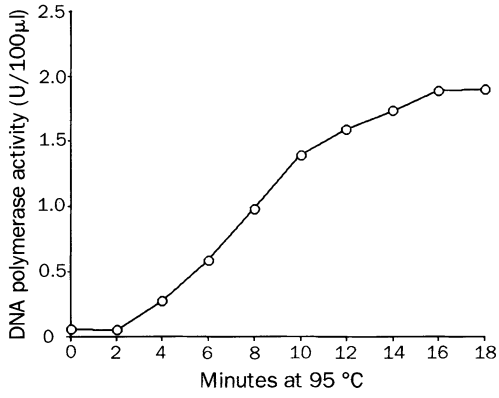

FIG. 1 Heat activation of AmpliTaq Gold. To simulate PCR conditions, AmpliTaq Gold was added at a concentration of 2.5 Units per 100 μL to a buffer containing 10 mM Tris-HCl, pH 8.3 (at room temperature), 50 mM KCl, 2.5 mM $MgCl_2$ and 200 μM each of dATP, dCTP, dGTP, and dTTP. Aliquots of 100 μL were added to PCR tubes and covered with 2 drops of mineral oil. The tubes were closed and put into a Perkin-Elmer GeneAmp DNA Thermal Cycler 480 on hold at 25 °C. The cycler was programmed to ramp to 95 °C. Two tubes were withdrawn and put on ice before starting the program, then every two minutes after reaching the target temperature. The samples were assayed for polymerase activity, as described in ref. 10.

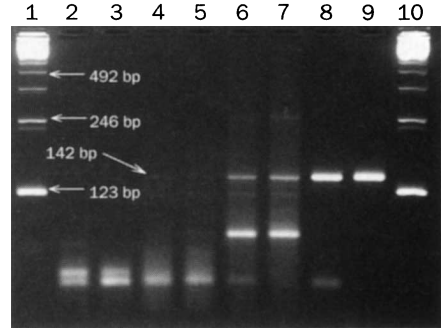

FIG. 2 100 μl PCR amplifications of a 142bp product of the HIV-1 genome (Perkin-Elmer) were run in duplicate using manual or no manual hot start methods. This procedure used 2.5 Units of AmpliTaq DNA Polymerase or AmpliTaq Gold, with 1× PCR Buffer II, 2.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, 400 μM dUTP, 50 pmoles of primers SK145 and SK431 (ref. 7), 1 unit of UNG, and 10 input copies of HIV-1 Positive Control DNA in 10 ng of HIV-1 Negative Control DNA in MicroAmp reaction tubes and a GeneAmp PCR System 9600. Following 43 cycles of amplification (1 initial preincubation cycle at 95 °C for 9 min; plus 43 cycles at 95 °C for 30 sec and 60 °C for 1 min, plus a final cycle at 60 °C for 10 min), the samples were analysed on an ethidium bromide stained 3 per cent Nusieve GTG and 1 per cent SeaKem GTG agarose gel (FMC BioProducts, Rockland, Maine) for 2 hours per 25 cm at 200 volts. Lanes 2 to 3: 0 input copies, AmpliTaq DNA polymerase, no preincubation, no manual hot start; lanes 4 to 5: 10 input copies, AmpliTaq DNA polymerase, no preincubation, no manual hot start; Lanes 6 to 7: 10 input copies, AmpliTaq DNA polymerase, no preincubation, manual hot start; Lanes 8 to 9: 10 input copies, AmpliTaq Gold, 9 min preincubation, no manual hot start; Lanes 1 and 10: 123bp DNA ladder, 1 μg.

gave a stronger product band and reduced nonspecific products (Fig. 2, lanes 8 and 9). The increased specific product yield with AmpliTaq Gold is consistent with increased functional enzyme activity ('timed release') in later cycles, when enzyme activity usually becomes limiting.

Applications

The increased amplification specificity of modified DNA polymerase is illustrated by improvements seen in the co-amplification of the genes encoding the small subunit ribosomal RNA (16S rRNA) and the β-subunit of RNA polymerase (rpoB) from *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). Amplification of the 16S rRNA gene allows the detection and identification of the pathogen[8]. Mutations in the *rpo*B gene have been linked to resistance to rifampin[9], one of the primary drugs used in the treatment of TB. Co-amplification of both target genes is desirable because the presence *M. tuberculosis* and its rifampin resistance/sensitivity genotype can be determined from products of a single amplification reaction. The relative performance of the unmodified and the modified DNA polymerase on the co-amplification assay was examined. DNA isolated from *M. tuberculosis* was amplified with unmodified or modified polymerase in a bicine-buffered reaction in a thermal cycler. As shown in Fig. 3, significant amounts of nonspecific amplification products were formed when co-amplification was carried out with unmodified DNA polymerase, especially in the presence of heterologous DNA. In contrast, when amplification was carried out with the modified enzyme, nonspecific products were greatly reduced and the amplification efficiency of the specific targets improved. This was apparent in the rpoB product, which showed a barely visable band in the unmodified-enzyme amplification, but was a more prominent band in the modified-enzyme amplification. Furthermore, the yields of the two products were much more balanced with the modified enzyme.

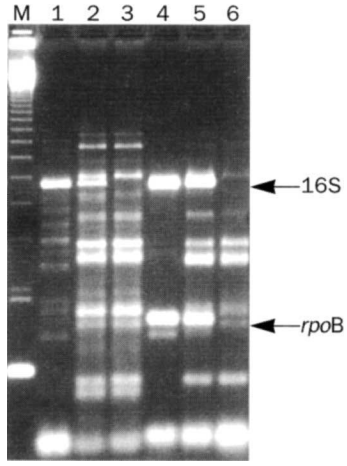

FIG. 3 PCR products from co-amplification of 16S and rpoB gene targets from ten copies of *Mycobacterium tuberculosis* DNA. Lanes 1–3: products generated with AmpliTaq DNA polymerase (Perkin-Elmer). Lanes 4–6: products generated with AmpliTaq Gold (Perkin-Elmer). Target DNA was amplified alone (lanes 1 and 4), and in the presence of DNA from 10^5 human leukocytes (lanes 2 and 5). Products from the amplification of leukocyte DNA alone are in lanes 3 and 6. Lane M: 1 μg of 123 base pair DNA marker (Gibco BRL, Gaithersburg, Maryland). Aliquots of 10 μl volume were electrophoresed through two per cent NuSieve GTG and one per cent SeaKem Agarose (both, FMC BioProducts) in Tris-borate/EDTA buffer, and stained with ethidium bromide. Locations of the specific amplification products are denoted by the arrows.

The ability to amplify multiple targets in a single PCR has great utility. Examples include the identification of several pathogens in a biological sample, mutational analysis of multiple gene exons, and simultaneous interrogation of multiple loci for human genetic linkage or oncogenic studies. Benefits of multiplexing include experiental simplification, decreased effort, greater cost effectiveness and reduced time. However, increasing the number of primer pairs in a PCR exacerbates the poor specificity and low product yield that result from primer interactions and mis-priming. Extensive primer testing and reaction optimization are required to minimize these problems when using an unmodified DNA polymerase. With a modified enzyme amplifications can be done simultaneously with little or no primer sequence optimization. A comparison of multiplex amplifications using unmodified DNA polymerase and the modified form is shown in Fig. 4. The reactions with the modified enzyme gave reduced primer artefact and background, leading to increased yield and specificity relative to the unmodified DNA polymerase reactions. In addition, one of the PCR products (100 bp) was not amplified with unmodified polymerase, but was clearly detected in the modified polymerase reactions. The polymorphic markers shown in Fig. 4 have been co-amplified with 20 exons from the cystic fibrosis gene using the modified enzyme to give a total of 34 products clearly resolved by agarose gel (data not shown, G. A. Zangenberg).

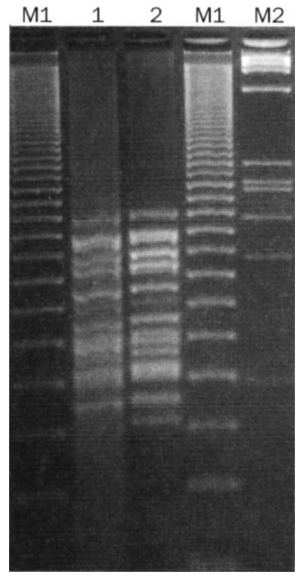

FIG. 4 Amplicon generated at 18 different DNA loci using 2 ng of genomic DNA, separated on a 3.5 per cent MetaPhor (FMC BioProducts) agarose gel. M1 = Superladder-low, 20 bp ladder (GenSura, Del Mar, California), M2 = (X 174 DNA-Hae III Digest (New England Biolabs, Beverly, Massachusetts). Lane 1 shows multiplex PCR results using AmpliTaq DNA polymerase, lane 2 shows the same reaction but using AmpliTaq Gold with dramatically decreased primer-dimer (size range of 40 to 60bp). PCR product sizes range from 100 bp to 242 bp.

Extending the technology

AmpliTaq Gold can be substituted directly for conventional thermostable DNA polymerases in most PCR systems using a standard PCR buffer (GeneAmp 10× PCR buffer or 10× PCR buffer II and $MgCl_2$ solution, Perkin-Elmer), when a pre-PCR heat cycle is used. However, optimizing the reaction conditions, such as pH, KCl concentration or $MgCl_2$ concentration, may further improve PCR results. Because of the enhanced specificity of a modified DNA polymerase PCR, increasing both the enzyme concentration and cycle number may increase specific product yield without increasing background. The pre-PCR heat cycle can be eliminated, allowing the enzyme to activate during cycling. If a pre-PCR heat cycle is not used, ten or more extra cycles may be necessary to give equivalent product yield (data not shown). The simplicity of the activation makes the enzyme well suited for applications like automated PCR and *in situ* PCR, as well as in high-throughput situations where pre-mixing and aliquotting of reagents is required.

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