Enhancement of PCR Amplification Yield and Specificity Using AmpliTaq Gold™ DNA Polymerase

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ABSTRACT

*Inadequate yields of PCR product and the generation of nonspecific PCR products can complicate genotyping studies, particularly when the DNA template is of inferior quality and/or has a low-copy number. In this study, the ability of AmpliTaq GoldÔ DNA Polymerase to enhance the specificity and yield of amplification was evaluated in a quadruplex short tandem repeat (STR) system in which a nonspecific PCR product and poor yield had been previously observed with AmpliTaqÒ DNA Polymerase usage. Because AmpliTaq Gold is inactive until heated during the PCR before thermal cycling, effects similar to those achieved with "hot-start" PCR were attained in a fast, simple and practical fashion. A significant enhancement in yield at the four STR loci and improved balance of alleles resulted with the use of AmpliTaq Gold. Furthermore, a nonspecific PCR product, the result of mispriming, was effectively eliminated. The consistency of quality results was improved, thereby promoting successful typing of suboptimal DNA samples and enhancing the accuracy of genotyping. Since PCR product yield is elevated with AmpliTaq Gold usage, and consistent performance and low background are achieved with higher amounts of AmpliTaq Gold compared with AmpliTaq, AmpliTaq Gold can be used to augment measures taken to counteract the effects of some PCR/*Taq *DNA polymerase inhibitors, such as those found in blood and some forensic specimens. Studies showed that pH affects either the activity or the activation of the polymerase. AmpliTaq Gold was found to be compatible with pH 8.3 buffers, such as GeneAmp® PCR Buffer and AmpF\STRÔ PCR Reaction Mix but not compatible with pH 9.0 buffers, such as GenePrintÔ STR 10´ Buffer (however, conditions for the usage of AmpliTaq Gold with the GenePrint CTTv system are provided). AmpliTaq Gold is useful for the development and optimization of multiplex amplification systems, particularly those in which the primers are not well designed and/or the reaction conditions are not optimal. Finally, because AmpliTaq Gold is initially inactive, preparation of reactions at ambient temperature and automation of the PCR are facilitated. Therefore throughput can be expanded significantly with the use of AmpliTaq Gold DNA Polymerase.*

INTRODUCTION

The polymerase chain reaction (PCR) is a means of amplifying specific target sequences from nanogram quantities of DNA. Each amplification system usually requires a degree of optimization to both promote high yields of PCR product and to achieve specificity of amplification. Specificity of amplification is affected by several factors. In general, mispriming is less likely to occur with stringent primer annealing temperatures and relatively low concentrations of primers, *Taq* DNA polymerase and magnesium chloride (19). However, these same conditions generally reduce the product yield. Therefore, determining an effective balance of reaction components and cycling parameters can be laborious and challenging.

AmpliTaq Gold™ DNA Polymerase is a thermostable enzyme that differs from AmpliTaq® DNA Polymerase by remaining inactive until exposed to a high temperature. It effectively simu-

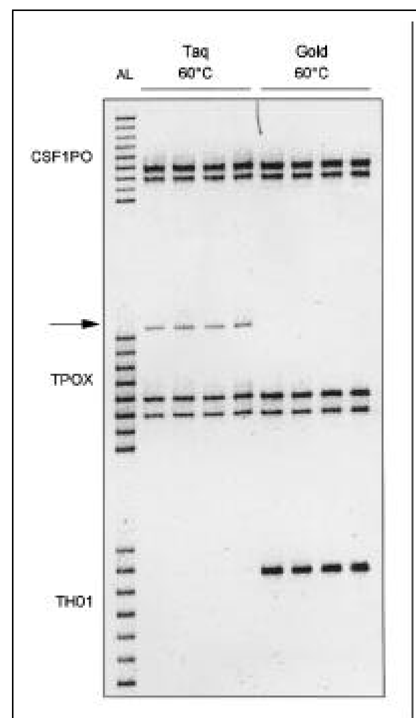

Figure 1. GenePrint Fluorescent STR System CTTv amplified with AmpliTaq and AmpliTaq Gold DNA Polymerases. K562 DNA was amplified by the manufacturer's recommended protocol (with a 60°C annealing temperature) using AmpliTaq DNA Polymerase and AmpliTaq Gold DNA Polymerase. TH01 product yield is improved considerably using AmpliTaq Gold. The nonspecific amplification product observed cathodal to the TPOX locus (arrow) is eliminated by the use of AmpliTaq Gold. Lane 1 shows the CSF, TPOX and TH01 components of GenePrint CTTv allelic ladder (AL).

lates "hot-start" PCR in a fast, simple and practical fashion. The use of AmpliTaq Gold DNA Polymerase can reduce or eliminate the generation of nonspecific PCR products that can result from mispriming and primer oligomerization with the use of some primers, substandard reaction conditions and/or setup of reactions at ambient temperature. The undesired PCR products compete with targeted sequences for dNTPs and primers and, as a result, reduce the yield of specific target products. Thus, by improving specificity, AmpliTaq Gold can increase the yield of some target PCR products. Some nonspecific products can present interpretational difficulties and hinder quantitation and sequencing of PCR products. However, with the use of AmpliTaq Gold, the benefits of manual hot-start PCR can be achieved without time-consuming and onerous measures that are prohibitive of high-throughput applications.

Whereas some PCR-based systems might be improved with the use of AmpliTaq Gold, those that do not prove to be robust can benefit the most. In this study, the usefulness of AmpliTaq Gold DNA Polymerase was assessed in a multiplex short tandem repeat (STR) amplification system that exhibited a nonspecific PCR product and poor yield at two of the four loci when AmpliTaq DNA Polymerase was used.

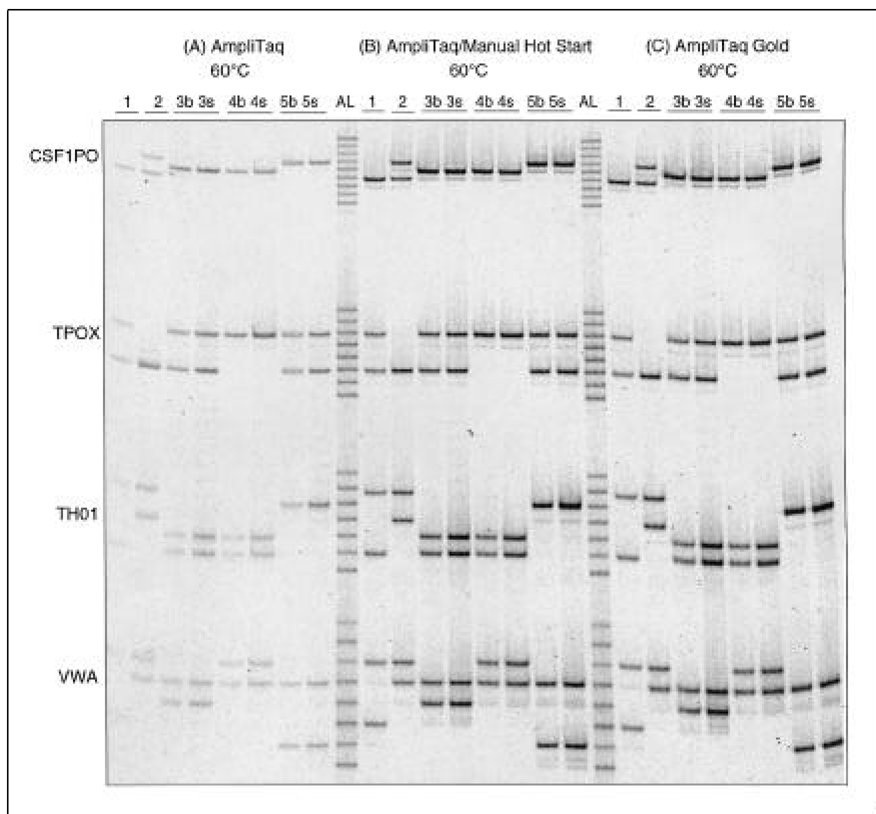

Figure 2. Comparison of the GenePrint Fluorescent STR System CTTv with the use of AmpliTaq and AmpliTaq Gold DNA Polymerases in conventional PCR and AmpliTaq in manual hot-start PCR. Human genomic DNA extracted from bloodstains and semen stains was amplified with 1 U of DNA polymerase in a 25-µL reaction as follows: (A) AmpliTaq, according to the manufacturer's recommended protocol with a 2-min 95°C heat step before thermal cycling, (B) manual hot-start PCR, which entailed withholding AmpliTaq until the final 2 min of an 11-min pre-cycling denaturation step and (C) AmpliTaq Gold in GeneAmp PCR Buffer with an 11-min, 95°C heat step before thermal cycling. The final extension step for all reactions was at 60°C for 90 min. Lanes 1, blood DNA; lanes 2, cell line 9947A DNA; lanes 3–5, matched pairs of blood (b) and semen (s) DNAs from three donors (3–5); AL represents the GenePrint CTTv allelic ladder. The results of hot-start PCR (B) and the AmpliTaq Gold reactions (C) are similar, with improved balance of the multiple loci and higher PCR product yields compared with the standard AmpliTaq reactions (A). Stutter bands and a higher background are more apparent in Panels B and C due to the overall higher PCR product yield and can be eliminated or reduced by either loading less of the PCR product onto the gel or by reducing the quantity of DNA template in the PCR.

MATERIALS AND METHODS

Genomic DNA was extracted from semen stains (4) and bloodstains (8) and quantitated by a chemiluminescent slot-blot hybridization procedure (4, 20). K652 DNA (Promega, Madison, WI, USA) and cell line 9947A DNA (Life Technologies, Gaithersburg, MD, USA) were also used. Five nanograms of DNA were amplified using the GenePrint™ Fluorescent STR System CSF1PO, TPOX, TH01, vWA (CTTv) (Promega) (15). The reactions included a final concentration of 0.16 µg/µL bovine serum albumin (BSA) (Product No. A3350; Sigma Chemical, St. Louis, MO, USA) (8,13). For reactions testing AmpliTaq Gold DNA Polymerase (PE Applied Biosystems, Foster City, CA, USA), GeneAmp® PCR Buffer with 1.5 mM $MgCl_2$ (PE Applied Biosystems) was used with the addition of 200 µM of each dNTP,

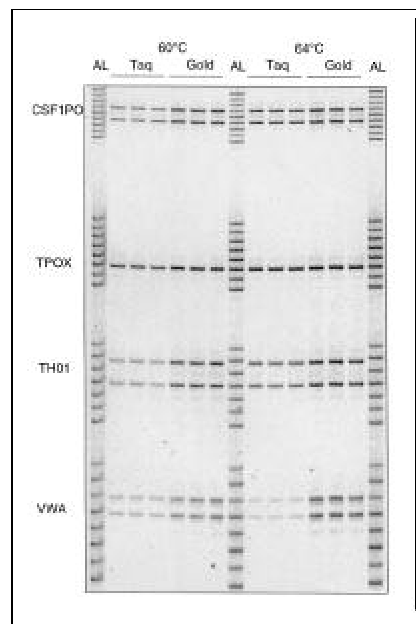

Figure 3. Annealing temperature comparison using AmpliTaq and AmpliTaq Gold DNA Polymerases. Using the GenePrint Fluorescent STR System CTTv, human cell line 9947A DNA was amplified according the manufacturer's recommendations with annealing temperatures of 60° and 64°C and using AmpliTaq and AmpliTaq Gold DNA Polymerases. Samples that were amplified with AmpliTaq Gold show an increased yield of PCR products, particularly at the TH01 and vWA loci. Improved balance of the loci is achieved using AmpliTaq Gold in the manufacturer's recommended protocol. Lane AL shows the GenePrint CTTv allelic ladder.

instead of the STR 10× Buffer that is supplied with the GenePrint kit. Amplification was carried out in the GeneAmp PCR System 9600 (PE Applied Biosystems). Before thermal cycling, the AmpliTaq Gold reactions were heated at 95°C for 11 min to activate the enzyme. Amplification was conducted according to either: *(i)* the GenePrint manufacturer's instructions, which required an annealing temperature of 60°C, thermal cycling ramp times programmed to simulate ramp times in a DNA Thermal Cycler 480 (PE Applied Biosystems), the addition of mineral oil and the placement of aluminum foil over the samples rather than the heated thermal cycler lid; or *(ii)* a modified protocol (5), in which the annealing temperature is 67°C, the duration of each cycling step is shortened to 30 s, the faster GeneAmp PCR System 9600 default ramp times are used, mineral oil is omitted and the samples are covered by the heated thermal cycler's lid. Hot-start PCR was achieved by heating reaction mixtures that lacked DNA polymerase for 8.5 min at 95°C and then adding AmpliTaq DNA Polymerase as heating continued for approximately 2.5 additional minutes. Therefore, the AmpliTaq was heated for approximately 2 min in the hot-start PCR technique, as it is in the conventional method (that is, without hot-start). A final extension of 30 min at 60°C was conducted following the cycling phase. The final extension time for some reactions that contained 1 U of AmpliTaq Gold DNA Polymerase was increased to either 60 or 90 min as noted.

Samples were prepared for electrophoresis by adding 3 μL amplified DNA to 3 μL STR 2× Loading Solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol). The mixture was denatured at 95°C for 2 min and immediately placed on ice. Using a Model SA-32 Gel Electrophoresis System (Life Technologies), 5 μL of each sample were subjected to electrophoresis in 4%T/5%C denaturing polyacrylamide gels (bisacrylamide cross-linker, 7 M urea, 0.4-mm thick, 30-cm long) in 0.5× TBE (final concentration 50 mM Tris, 45 mM borate, 0.5 mM EDTA, pH 8.4). Electrophoresis was conducted at a constant power of 80 W for 5 min and then at 40 W at room temperature until the cathodal dye (xylene cyanol) migrated to approximately 6 cm from the bottom of the gel. The fluorescein-labeled PCR products were then detected in the FluorImager™ SI (Molecular Dynamics, Sunnyvale, CA, USA) using a 530-nm, band-pass filter.

RESULTS AND DISCUSSION

The GenePrint System CTTv is designed to enable the simultaneous amplification of four STR loci: CSF1PO, TPOX, TH01 and vWA (15). Previous performance testing of this system demonstrated that results obtained using the manufacturer's recommended PCR conditions were not acceptable (5). [**Note:** Some of these studies were performed before August 1996. Since then, the GenePrint manufacturer modified primer(s) to eliminate the nonspecific PCR product that appeared cathodal to the TPOX locus (James W. Schumm, Promega Corporation, personal communication). Figures 2, 3, 5 and 6 demonstrate performance of the modified kit and the absence of the non-

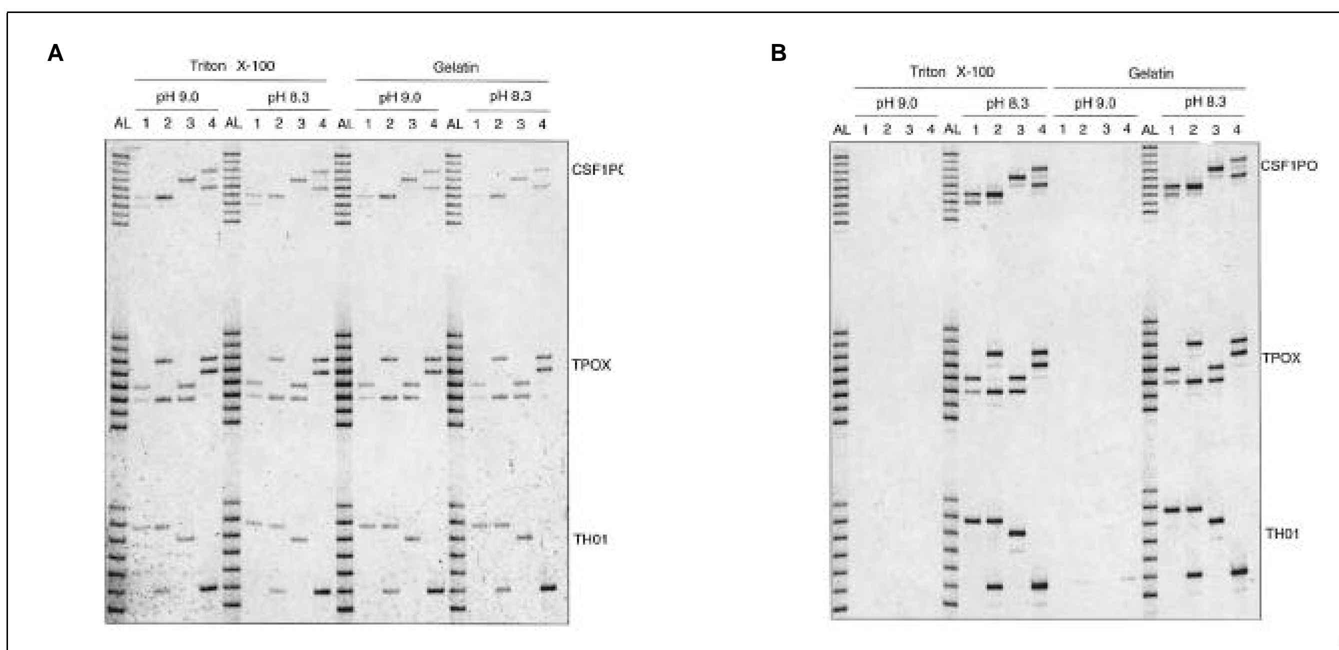

Figure 4. The effects of pH and PCR buffer components on AmpliTaq Gold amplification. Four buffers were prepared to test the performance of AmpliTaq and AmpliTaq Gold DNA Polymerases: *(i)* pH 9.0 buffer with Triton X-100 (10× STR Buffer formulation; Promega); *(ii)* pH 8.3 buffer with Triton X-100; *(iii)* pH 9.0 buffer with 0.01% (wt/vol) gelatin and *(iv)* pH 8.3 buffer with 0.01% (wt/vol) gelatin (GeneAmp PCR Buffer formulation). A modified PCR protocol with a 67°C annealing temperature (5) was used to amplify four human genomic DNAs (lanes 1–4) extracted from bloodstains, using the GenePrint Fluorescent STR System CTTv. Each buffer was shown to work with AmpliTaq (Panel 4A). However, AmpliTaq Gold (Panel 4B) was not compatible with the pH 9.0 buffers but was used successfully in the pH 8.3 buffers. Lane AL shows the GenePrint CTTv allelic ladder.

specific PCR product with the use of AmpliTaq and AmpliTaq Gold DNA Polymerases.] First, using AmpliTaq DNA Polymerase according to the manufacturer's recommended protocol, an additional PCR fragment of undefined origin was observed cathodal to TPOX allele 13 (Figure 1) in some DNA samples (e.g. K562, human genomic DNAs extracted from adjudicated forensic case specimens and reference samples). This fragment was shown to be the result of nonspecific primer binding since it increased in intensity as the annealing temperature was lowered to 57°C and disappeared as the annealing temperature was raised to 64° or 67°C (5). Second, using the manufacturer's recommended protocol with AmpliTaq DNA Polymerase, the product yields of the TH01 and vWA loci were low relative to CSF1PO and TPOX products (Figures 1 and 2). A possible explanation for the low TH01 yield is the formation of a hairpin structure in the primer and/or primer-binding site that interferes with primer binding and/or complete denaturation of the template DNA (5). By increasing the annealing temperature to 67°C, the artifact band was eliminated, and the yield of TH01 products was increased; however, vWA amplification could not be achieved at the higher annealing temperature (5). Alternatively, with a 60°C annealing temperature, the use of AmpliTaq Gold DNA Polymerase effectively eliminated the nonspecific product (Figure 1), substantially increased TH01 and vWA yields (Figures 1 and 2) and augmented the

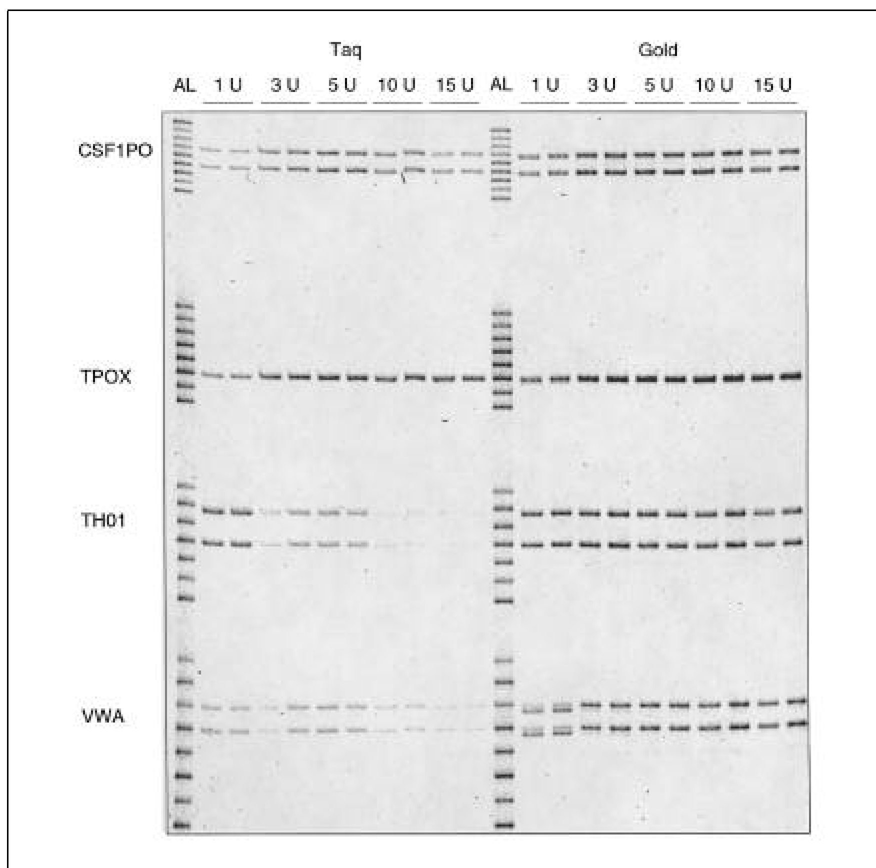

Figure 5. The effects of increasing the amount of AmpliTaq and AmpliTaq Gold DNA Polymerases used to amplify the GenePrint STR System CTTv. Cell line 9947A DNA was amplified with an annealing temperature of 60°C according the manufacturer's recommended protocol using AmpliTaq DNA Polymerase (with GenePrint 10× STR Buffer) and AmpliTaq Gold DNA Polymerase (with GeneAmp PCR Buffer) in the following amounts: 1, 3, 5, 10 and 15 U. As the amount of AmpliTaq is raised from 1–5 U, samples show increasing yield at the CSF1PO locus but decreasing yield at the TH01 locus. Using AmpliTaq Gold, the product yield at all loci is improved compared with using AmpliTaq and is constant from 1–15 U. Due to high enzyme concentrations, up to 15 U of AmpliTaq Gold can be used with no apparent inhibition of the PCR. Lane AL shows the GenePrint CTTv allelic ladder.

overall product yields at the CSF1PO and TPOX loci (Figure 2).

To determine whether the improvement in yield, particularly at the TH01 locus, could be attributed primarily to either the 11 min pre-cycling heat step or the application of AmpliTaq Gold, a manual hot-start PCR with AmpliTaq was conducted (Figure 2). The hot-start PCR results (Panel B) (which entailed withholding AmpliTaq until approximately the final 2 min of an 11-min pre-cycling heat step) were compared with those from both standard reactions (without hot start) with AmpliTaq (denatured for 2 min before cycling) (Panel A) or with AmpliTaq Gold (denatured for 11 min before cycling) (Panel C). A significant enhancement in yield was observed at all loci with the hot-start PCR application, and the balance of product yield of the four loci was improved. The reactions using AmpliTaq Gold were comparable to the hot-start reactions. Therefore, the benefits of manual hot-start PCR can be achieved with AmpliTaq Gold without additional cumbersome steps that are prohibitive of high-throughput applications.

The use of mineral oil to overlay reactions amplified in the GeneAmp PCR System 9600 (as recommended by Promega for the GenePrint CTTv system) appeared to be beneficial to some but not to all AmpliTaq reactions. However with the use of AmpliTaq Gold, higher PCR product yield, improved reliability of amplification and better balance of the product yield of the four loci were consistently achieved both with and without mineral oil. The elimination of mineral oil facilitates PCR product sampling and is desirable for high-throughput applications, including automation of the PCR.

The actual melting temperature (T_m) of one (or both) of the vWA primers in the GenePrint CTTv multiplex is likely to be around 64°C, since with the use of AmpliTaq DNA Polymerase, amplification success at 64°C is inconsistent and sometimes negligible. Highest yield of vWA product was, in fact, obtained at 64°C, which is close to the apparent T_m, with the use of AmpliTaq Gold (Figure 3). The effective T_m of the vWA primer(s) thus appears to be affected by the modified polymerase, making AmpliTaq Gold particularly useful in the development and optimization of this multiplex system.

For amplification systems using GeneAmp PCR Buffer or AmpF*l* STR PCR Reaction Mix (PE Applied Biosystems), AmpliTaq Gold DNA Polymerase can be substituted directly for AmpliTaq DNA polymerase. However, the STR 10× Buffer supplied with the GenePrint STR Systems is not compatible with AmpliTaq Gold. To determine which buffer component(s) limits AmpliTaq Gold performance, buffers were prepared according the manufacturers' formulations and as described in Figure 4. Each of the prepared PCR buffers enabled AmpliTaq activity (Figure 4A), but only the pH 8.3 buffers (one with gelatin and the other with Triton® X-100) were compatible with AmpliTaq Gold (Figure 4B). Thus, AmpliTaq Gold performs well in the pH 8.3 buffers and either does not perform well in the pH 9.0 buffers tested or requires a certain pH environment to be activated.

High yields of specific PCR products can be generated by empirically determining appropriate concentrations of reaction components and cycling parameters. To enhance PCR performance and reliability, primer sequences may require modification. Also, the concentration of primers used for the simultaneous amplification of multiple target sites might need manipulation to achieve a balance of the alleles. It is desirable for primers selected for multiplexing to have similar melting profiles. If the T_ms are dissimilar, the cycling parameters might have to be changed to accommodate the primer set with the lowest annealing temperature requirements, which could result in undesirable effects. For example, the addition of primers for the vWA locus to the GenePrint CTT Multiplex System necessitated lowering the annealing temperature from 64°C (which is recommended for the GenePrint CTT triplex) to 60°C. Nonspecific products and reduction in TH01 yield resulted from this reduction in stringency (5). While the recommended approach to developing multiplex systems should entail primer balancing and experiments to foster reliable product performance, the use of AmpliTaq Gold instead could improve specificity and yield during the PCR. In this study, balance of PCR products at the four STR loci and high yields of PCR products were achieved with the use of AmpliTaq Gold and a 64°C annealing temperature. Reasonable results were also obtained with the use of AmpliTaq Gold and a 60°C annealing temperature (Figure 3).

To overcome the effects of PCR/*Taq* DNA polymerase inhibitors (such as indigo dyes from certain substrates and heme) that might co-extract with the DNA from some forensic specimens (1, 7,9–11,13,14,17,18) the following may be done: *(i)* BSA is typically included in the amplification reaction (8,12), *(ii)* the amount of *Taq* DNA polymerase in the PCR is sometimes increased (2) and/or *(iii)* the reaction volume may be increased to dilute the inhibitors. The use of AmpliTaq Gold can augment the measures used to counteract the effects of some PCR/*Taq* DNA polymerase inhibitors. In some instances, high concentrations of *Taq* DNA polymerase may result in a higher background in the sample lane and/or reduced yield of the desired fragment(s) (2). Alterna-

tively, higher concentrations of AmpliTaq Gold could improve specificity and would be particularly beneficial with low-copy number DNA templates. For the GenePrint CTTv multiplex system, while the background was not affected by increasing amounts of AmpliTaq, the yield of amplified products was affected when the amount of AmpliTaq used in the PCR was varied (Figure 5). The CSF1PO locus showed an increase in product yield with 5 U of AmpliTaq compared with 1 and 3 U; whereas, the highest product yield for the TH01 locus was with 1 U of AmpliTaq. The yield at the TPOX locus was slightly lower with 1 U of AmpliTaq but was relatively constant from 3–15 U; whereas, vWA product yield, while lower than the other loci, was greatest at 5 U. Conversely, amplification product yield with the use of AmpliTaq Gold was relatively consistent across enzyme amounts ranging from 1–15 U. However, with the use of 1 U of AmpliTaq Gold, most notably, the vWA locus in some DNA samples exhibited a lower propensity for non-templated 3′ nucleotide addition (Figure 5) (6,16). Although the “minus-A/plus-A” occurrences did not hinder the ability to accurately type the alleles, it may be desirable to promote the adenine addition (Figures 2 and 6) by doing one of the following: *(i)* lengthening the final extension time at 60°C if a low amount of AmpliTaq Gold is used, *(ii)* using more than 1 U of AmpliTaq Gold or *(iii)* modifying the VWA primer sequences (3). No undesirable effects were

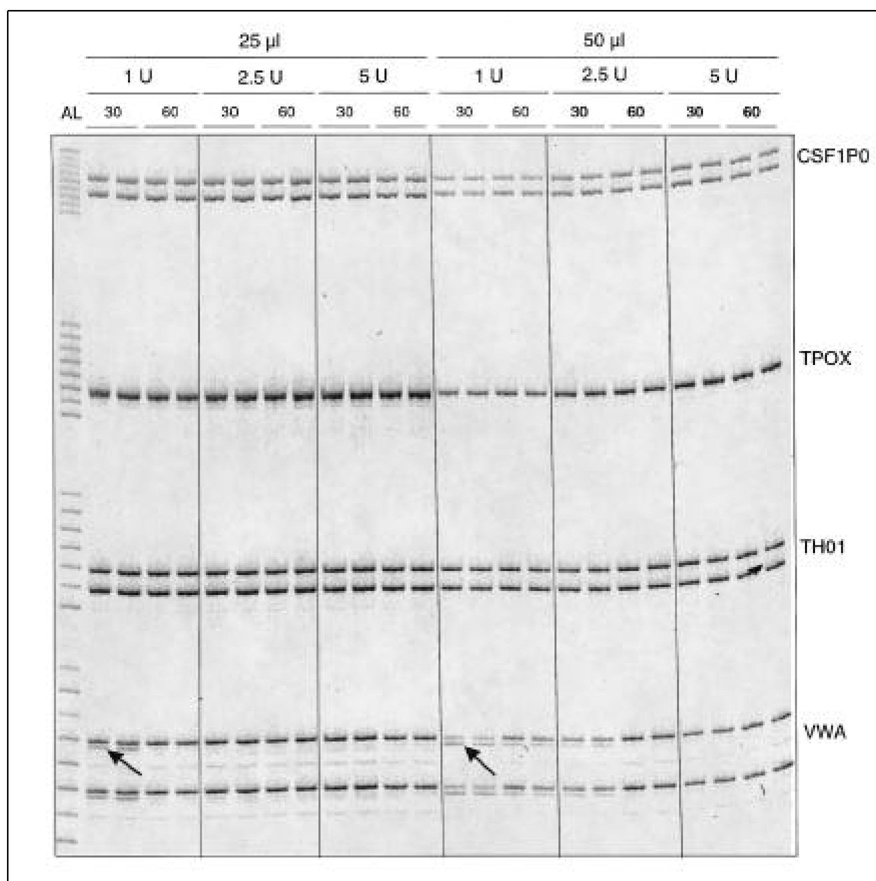

Figure 6. The effects of various concentrations of AmpliTaq Gold DNA Polymerase on GenePrint CTTv amplification. 1, 2.5 and 5 U of AmpliTaq Gold were used to amplify 5 ng of DNA extracted from blood in 25 and 50 µL total reaction volumes. Since 3 µL of PCR product were electrophoresed in each lane, the overall product yield is weaker, as expected, in the reactions with 5 ng template/50 µL compared with the reactions having 5 ng template/25 µL. With the use of 1 U of AmpliTaq Gold, the vWA locus exhibited less template-independent 3′ dATP addition (arrow indicates the “minus A” band). However, the A-addition can be promoted by lengthening the final extension time at 60°C from 30 to 60 min, as shown in lanes 30 and 60, respectively, or by increasing the amount of AmpliTaq Gold. Lane AL shows GenePrint CTTv allelic ladder.

observed at the higher concentrations of AmpliTaq Gold. Thus, AmpliTaq Gold allows for the usage of an increased amount of the polymerase that could be advantageous for the analysis of some forensic specimens (2).

For the development of effective PCR-based multiplex systems, performance testing of various reaction components in the PCR is generally necessary, particularly when AmpliTaq DNA Polymerase is used. Certain additional electrophoretic bands could adversely affect interpretation of DNA profiles and the determination of the potential contributor(s) of a sample. The use of AmpliTaq Gold might improve specificity of the PCR, and the yield of PCR products and might also enhance the balance of loci in some multiplex systems, particularly those in which the primers are not well designed and/or the reaction conditions are not optimal. Specificity of amplification of STR loci is important for forensic applications.

An ancillary benefit of the use of AmpliTaq Gold is that the preparation of reactions at room temperature is facilitated. For high-throughput applications, such as multiplex STR typing for convicted offenders databases and other genotyping studies, AmpliTaq Gold DNA Polymerase will enable automation of the PCR in addition to simplifying optimization of the multiplex reactions, thus promoting successful typing of suboptimal DNA samples and enhancing the overall consistency of quality results.

ACKNOWLEDGMENTS

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