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TECHNICAL NOTE

CRIMINALISTICS

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Multiplex Short Tandem Repeat Amplification of Low Template DNA Samples with the Addition of Proofreading Enzymes*

ABSTRACT: With <100 pg of template DNA, routine short tandem repeat (STR) analysis often fails, resulting in no or partial profiles and increased stochastic effects. To overcome this, some have investigated preamplification methods that include the addition of proofreading enzymes to the PCR cocktail. This project sought to determine whether adding proofreading polymerases directly in the STR amplification mixture would improve the reaction when little template DNA is available. Platinum Taq High Fidelity and GeneAmp High Fidelity were tested in Profiler Plus™ STR reactions alone and in combination with AmpliTaq® Gold. All reactions included the additional step of a post-PCR purification step. With both pristine low template DNA and casework samples, the addition of these polymerases resulted in comparable or no improvement in the STR amplification signal. Further, stochastic effects and artifacts were observed equally across all enzyme conditions. Based on these studies, the addition of these proofreading enzymes to a multiplex STR amplification is not recommended for low template DNA work.

KEYWORDS: forensic science, DNA typing, short tandem repeat profiling, low copy number, proofreading enzymes, capillary electrophoresis

At a crime scene, sometimes touch or trace DNA evidence, including fingerprints, saliva, hairs, and minuscule drops of blood and other bodily fluids, is the only evidence found. This type of evidence can often contain <100 pg of DNA (~15 diploid cells or less) and is referred to as low-copy DNA (LCN) evidence or low template DNA (1). Additionally, low template DNA casework samples are also often highly degraded. Because of the limited quantity and quality of DNA available, these types of samples can become difficult to analyze and interpret with traditional short tandem repeat (STR) analysis, preventing the acquisition of a full or even strong partial profile.

Several techniques have been developed in an effort to overcome the limitations of low copy number sample and/or degraded sample analysis, including mitochondrial DNA analysis (mtDNA), low copy number–polymerase chain reaction (LCN-PCR), and whole genome amplification (WGA) (1–7). Although these techniques are available, they usually do not have the same discriminating power as traditional STR analysis, most cannot be used in conjunction with the Combined DNA Index System (CODIS), and are both time-consuming and costly.

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To overcome the limitations of severe degradation or low template DNA, some researchers have investigated preamplification methods that include the addition of proofreading enzymes to the PCR cocktail (6–8). Proofreading enzymes have 3′-5′ exonuclease activity, allowing them to correct bases that are misincorporated by the traditionally used *Taq* polymerase. Typically, the addition of an enzyme that has proofreading capability results in longer fragments, although the exonuclease activity reduces the overall processivity of the reaction (9,10). Previous studies have shown that combining these proofreading enzymes with *Taq* polymerase for preamplification is the best approach for increasing fragment length and improving genome coverage, without compromising the speed of the reaction (6,7).

The performance of proofreading enzymes should be evaluated for use *directly* in the STR amplification without a preamplification step. If *Taq* is combined with a slower proofreading enzyme, such as *Pfu*, *Tgo*, or *Deep Vent*, the proofreading enzyme can likely correct mistakes that are made by *Taq* by excising the misincorporated nucleotides and replacing them with the correct bases (they possess 3′-5′ exonuclease activity), without losing the speed of the reaction. Then, not only should there be an increase in fidelity leading to a decrease in error rate, but the final product size should also increase (11). Bonnette et al. optimized a WGA method that included an addition of proofreading enzymes to the preamplification step. This study found that two proofreading enzymes used in conjunction with the WGA reaction prior to the STR multiplex amplification (Applied Biosystems' [Foster City, CA] GeneAmp DNA polymerase and Invitrogen's [Carlsbad, CA] Platinum Taq High Fidelity polymerase) significantly improved the STR results

(7). ABI GeneAmp High Fidelity DNA polymerase includes a mixture of ABI TaqGold and a proprietary proofreading enzyme, whereas Platinum Taq High Fidelity polymerase is a *Taq:Deep Vent* combination (7). The *Deep Vent* enzyme is localized from *Pyrococcus species GB-D*, has a fidelity rate six times greater than *Taq*, and has a lower error rate (2.7×10^{-6} errors per base pair) than Taq Gold (2.6×10^{-5} errors per base pair) (9–12). Less is known about GeneAmp High Fidelity polymerase, because the contents of the enzyme mixture are proprietary (13).

This study sought to determine whether adding these proofreading enzymes directly into the STR reaction (without WGA) will improve STR analysis from low-level and/or severely degraded DNA samples. To determine this, *Taq* will either be replaced by a proofreading enzyme, or a proofreading enzyme will be used in conjunction with the *Taq* enzyme.

Materials and Methods

Sample Preparation and Collection

Five randomly selected donors provided reference buccal swabs (one swab per person) for use in the pristine sample study. Samples used in the mock casework study included aged bloodstains, bone, teeth, dermal ridge fingerprints, and fired cartridge cases. For the bloodstain samples, fresh whole blood samples were collected from one donor in a tube with EDTA and stored at 4°C for approximately 24 h. A bloodstain was prepared on a white cotton T-shirt. After drying, the bloodstain was divided, positioned into microcentrifuge tubes, and placed in an incubator at 80°C indefinitely. Samples were collected at 3 and 4 months. Once collected, the samples were stored at −20°C until use. One-centimeter cuttings were taken for use in this study.

Bone samples ($n = 2$) were received in powder form from the Virginia Department of Forensic Science. The Virginia Department of Forensic Science *Large Volume DNA IQ Extraction Method for Bone Samples* protocol was followed to obtain the powder as described later (14,15). Briefly, a cleaning solution of 1.2 mL TNE, 75 μL 20% Sarkosyl, and 225 μL of Type I water was prepared and preheated in a heat block at 56°C. After preheating, 15 μL of proteinase K (20 mg/mL) was added to the solution. The cleaning solution was applied to a pad of Kimwipes, and the Kimwipes were then applied to the surface of the bone to be drilled. The bone was placed in a Ziploc bag and incubated for 30 min at room temperature. Following incubation, the area that was treated was wiped with 95% ethanol and allowed to dry. The bone powder was obtained by drilling the bone with an electric drill. The drill bits (3/32″) were cleaned with 10% bleach followed by 95% ethanol before use. A weigh boat was used to collect the powder. The powder was stored at 4°C until extraction.

The teeth samples used in this study were two children's naturally expelled incisors, one recently expelled, and one removed over 5 years ago ($n = 2$). The Virginia Department of Forensic Science *Organic Extraction Method for Teeth* protocol was followed as described later (15). The outer surface of the tooth was first cleaned using a Kimwipe and 10% bleach followed by 70% ethanol. A dremel tool with a heavy duty cut-off wheel was used to remove the crown portion of the tooth. The tool and bits were cleaned with 10% bleach and 70% ethanol before use. After the crown was removed, the tooth was placed into a sterile Ziploc plastic bag. This plastic bag was then placed into several other sterile Ziploc plastic bags. A hammer (covered with sterile plastic bags) was used to pulverize the tooth, taking care not to puncture the plastic bags. The pulverized tooth was transferred to a sterile 1.5-mL microcentrifuge tube and stored at 4°C until extraction.

To obtain the dermal ridge fingerprint samples, three individuals were given a 50-mL conical tube to hold. The individual grasped the conical tube with a full fist, taking care to place the thumb in a designated area and held it for 10 sec. Each conical tube was swabbed in the designated thumbprint area using the double-swab method (16). The swabs were stored at −20°C until extraction.

Four fired olive steel cartridge cases (7.62 × 39 mm) (Wolf® Performance Ammunition, Placentia, CA) were obtained from the Virginia Department of Forensic Science. The rifle was cleaned with water and isopropanol before firing. To better simulate a realistic situation, the cartridge cases were not cleaned prior to shooting, but came from a new box. One cartridge case was loaded by each of four individuals. After each firing, the case was retrieved with a swab and placed into a sterile plastic bag. Each case was swabbed using the double-swab method (16) and stored at 4°C until extraction.

DNA Isolation and Purification

DNA was extracted from both the pristine and mock case samples using the Qiagen QIAamp® DNA Mini kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. Samples were eluted into 100 μL of buffer AE after a 5-min room temperature incubation. All extracted DNA samples were stored at −20°C until needed.

DNA Quantitation and STR Amplification

Following extraction, the samples were quantified using the Applied Biosystems Quantifiler® Human DNA Quantification Kit and the ABI 7500 Real-Time PCR System according to the manufacturer's instructions (Applied Biosystems). For the pristine sample study, LCN DNA input values were generated by serially diluting each of the five reference samples to obtain DNA input amounts of 0.0625, 0.03125, 0.0156, and 0.0078 ng, each in a volume of 5 μL ($n = 20$ for each enzyme condition).

Profiler Plus™ Multiplex STR Amplification

Reduced-volume reactions were used for AmpF*l*STR® Profiler Plus™ STR amplification; however, there were no changes in the reaction component concentrations. For each sample, 10 μL of PCR master mix was added to the DNA sample for amplification. The master mix consisted of the following components for each sample: 5.7 μL of AmpF*l*STR® PCR Reaction Mix, 2.0 μL of AmpF*l*STR® Profiler Plus™ primers, 2.1 μL of ddH_2O, and 0.20 μL of 5 U/μL of AmpliTaq® Gold DNA polymerase (Applied Biosystems). In some reactions, AmpliTaq® Gold DNA polymerase was replaced or combined with either GeneAmp High Fidelity DNA polymerase or Platinum Taq High Fidelity polymerase (proofreading enzyme[s]) as described later. When GeneAmp High Fidelity DNA polymerase was used, the ddH_2O was replaced with 2.10 μL of GeneAmp High Fidelity 10× buffer without $MgCl_2$ (Applied Biosystems). When Platinum Taq High Fidelity polymerase was used, the ddH_2O was replaced with 1.5 μL of Platinum Taq High Fidelity 10× PCR buffer and 0.6 μL of $MgSO_4$ (Invitrogen). No further pH or magnesium changes were made. Positive and negative controls (0.5 ng 9947A DNA and 5 μL of ddH_2O, respectively) were also amplified. All amplifications were performed in a PerkinElmer 9600 GeneAmp PCR system (PerkinElmer, Waltham, MA) using the following

parameters: 95°C for 11 min; 28 cycles of 94°C for 1 min; 59°C for 1 min; 72°C for 1 min; 60°C for 90 min; and hold at 4°C.

Samples used in the pristine sample study were amplified using the Profiler Plus™ Multiplex STR amplification kit as described above with the following six enzyme conditions ($n = 20$ for each): 1 U of AmpliTaq® Gold DNA polymerase (conditions A and G) (Applied Biosystems), 2 U of Taq Gold (condition B), 1 U of Platinum Taq High Fidelity polymerase (condition C) (Invitrogen), 1 U of Taq Gold and 1 U of Platinum Taq High Fidelity (condition D), 1 U of GeneAmp High Fidelity DNA polymerase (condition E) (Applied Biosystems), and 1 U of Taq Gold and 1 U of GeneAmp High Fidelity DNA polymerase (condition F). All enzyme conditions and modifications are listed in Table 1.

Typically, one nanogram of DNA (in 5 μL) was targeted for STR amplification, when available. However, many of the casework samples used in this study yielded low or undetectable (0 ng/μL) quantities of DNA, as expected. Samples that yielded more than one nanogram of DNA were expected to be highly degraded. As a result, all available mock casework samples were used in this study, regardless of the DNA yield. Those samples that had concentrations less than the target (1 ng) were concentrated to 20 μL prior to STR amplification using Microcon® YM-100 (Millipore, Billerica, MA) centrifugal filter devices per the manufacturer's protocol; from these samples, 5 μL (~0–100 pg or ¼ of the sample) was used for STR amplification. Samples with high concentrations were diluted to 0.2 ng/μL and 5 μL (1 ng) used for STR amplification. For the mock casework samples, only four enzyme conditions were amplified using the Profiler Plus™ STR amplification kit (conditions A, B, C, and D as described earlier).

Postamplification Purification and Concentration

Amplified samples from conditions A–F were purified and concentrated to 10 μL using Qiagen's MinElute® purification kit (Qiagen Inc.) after STR amplification. This was performed following the manufacturer's recommendation with modifications described by Smith and Ballantyne (17). Samples were washed three times and, at the final step, eluted into 10 μL of 100% Hi-Di™ formamide (Applied Biosystems).

Capillary Electrophoresis

Following STR multiplex amplification, products were size-separated via capillary electrophoresis using the ABI 3100*Avant* Genetic Analyzer (Applied Biosystems). The samples were prepared for capillary electrophoresis analysis by adding 1.2 μL of amplified samples from conditions A–F or 1 μL of Profiler Plus™ allelic ladder to 0.5 μL of GeneScan™ 500 ROX™ size standard (Applied Biosystems) and 12.0 μL of Hi-Di™ Formamide (Applied Biosystems) in a MicroAmp™ optical 96-well reaction plate (Applied Biosystems). The plate was denatured at 95°C for 5 min, snap-cooled on ice for 5 min, and then loaded into the ABI 3100*Avant* Genetic Analyzer. Electrophoresis was performed with default STR conditions, using ABI 3100 POP-4™ polymer (Applied Biosystems) and a 36-cm capillary. However, the injection time was increased to 20 sec for conditions A–F. The STR fragment data for all samples were sized and typed using ABI GeneMapper® ID version 3.2 software (Applied Biosystems) along with the laboratory's validated analytical threshold of 75 relative fluorescent units (RFU).

TABLE 1—*Postamplification modifications and enzymes used for each condition.*

Condition	Enzyme Used	MinElute	Injection Time (s)
A	TaqGold (1 U)	Yes	20
B	TaqGold (2 U)	Yes	20
C	Platinum Taq High Fidelity (1 U)	Yes	20
D	Platinum Taq High Fidelity (1 U) and TaqGold (1 U)	Yes	20
E	GeneAmp High Fidelity (1 U)	Yes	20
F	GeneAmp High Fidelity (1 U) and TaqGold (1 U)	Yes	20
G*	TaqGold (1 U)	No	10

*Standard STR amplification with no low template modifications.

Data Analysis

For each enzyme condition evaluated, several parameters were used to measure success of STR results. First, percent STR allele success was determined by dividing the number of correct alleles present by the number of expected alleles and multiplying by 100. To determine whether there was a significant difference between the enzyme conditions, a Student *t*-test ($\alpha = 0.05$) was calculated. Next, heterozygote peak balance was calculated by dividing the height of the minor peak (in RFU) by the height of the major peak (in RFU) and multiplying by 100. For a single sample, heterozygote peak balance was calculated for any heterozygous locus where both alleles were present above threshold. All expected alleles that were above threshold were used for peak height and inter-locus peak height calculations. To determine whether there was a significant difference between the enzyme condition results in heterozygote peak balance and peak height, a one-way ANOVA test ($\alpha = 0.05$) was calculated. A two-way ANOVA test ($\alpha = 0.05$) was calculated to determine whether there was a significant difference in peak height across all loci between the different enzyme conditions and between the DNA inputs tested. Microsoft Excel was used for all statistical calculations.

Results

Pristine Samples

STR analysis was performed with all six enzyme conditions to evaluate STR allele success and data quality. All positive controls worked as expected and all negative controls were clean, displaying no signs of contamination. Overall, the low-copy input samples amplified using GeneAmp High Fidelity (conditions E and F, $p < 0.0001$), 2 U of Taq Gold (condition B, $p = 0.0003$), or 1 U of Taq Gold + 1 U of Platinum Taq High Fidelity in combination (condition D, $p = 0.057$) yielded a reduction in the number of STR alleles detected when compared to the control condition (condition A), and thus, no further analysis was performed for these data sets. However, conditions A (Taq Gold control) and C (Platinum Taq High Fidelity) yielded comparable results ($p = 0.9081$) (Table 2). Additionally, the use of Platinum Taq High Fidelity (condition C) sometimes provided a more informative profile at very low input values (~7.8 pg) (data not shown).

The average heterozygous peak balance for the samples using the control condition A was 64.9% while the average heterozygous peak balance using condition C was 61.3% (Fig. 1, $p = 0.2937$). Between conditions A and C, there was no significant difference in peak height across input values (Fig. 2, $p = 0.0601$). Furthermore, there was no significant difference in peak heights between conditions A and C when examined within each locus (inter-locus) and

no differences in peak heights across loci of varying sizes for either enzyme condition ($p = 0.4691$, data not shown).

Mock Case Samples

Quantitation values varied for the mock case samples, ranging from "undetermined" (0 ng/μL) to 3.61 ng/μL (data not shown). Of the sample types, the teeth yielded more than any other group with an average total yield of 182.48 ng, followed by the heat-degraded bloodstains with 82.6 ng average total DNA yield. However, the majority of sample types tested yielded very low to undetected DNA quantities, including the fired cartridge cases with an average total yield of 0.1 ng. Input DNA amounts used for multiplex STR amplification are indicated in Table 3. The real-time PCR internal positive control for all samples was normal with no signs of inhibition. The positive and negative controls gave the expected results.

To further test the ability of Platinum Taq High Fidelity to improve STR analysis from compromised forensic samples, STR analysis was performed using enzyme conditions A, B, C, and D. All positive controls worked as expected; the negative controls had one drop-in allele that was contributed to contamination traced back to a contaminated water aliquot. However, this spurious allele did not affect accurate calling or data analysis for the study samples. Interestingly, within each type of mock case sample evaluated, a condition other than A had the highest allele success. However, it was inconsistent which condition performed best among the sample types, and there were no significant differences between the four enzyme conditions at each sample type (Table 3). Overall, all four enzyme conditions yielded similar results with the control condition (A) having the lowest overall allele success at 47.9% and

TABLE 2—*Average STR allele success for all low-copy pristine samples.*

	Average % STR Allele Success					
	Control	Proofreading Enzymes				
STR Input (ng)	A	B	C	D	E	F
0.0625	83.2	56.0	78.5	59.2	6.4	4.8
0.03125	96.5	51.5	79.5	54.7	0	6.9
0.0156	58.8	37.7	70.3	49.3	0	0
0.0078	22.8	2.4	36.6	25.9	0	2.4
Average	65.3	36.9*	66.2	47.2	1.6*	3.5*

*$p < 0.05$.

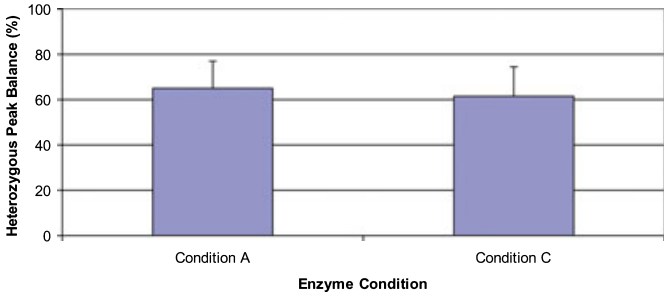

FIG. 1—*Average heterozygous peak balance (%) between condition A and C for all low-copy pristine samples. The peak ratio for both enzyme conditions was consistently >60% (*n = *15 for Taq Gold and* n = *18 for Platinum Taq High Fidelity,* p = *0.2937). Error bars represent standard deviation.*

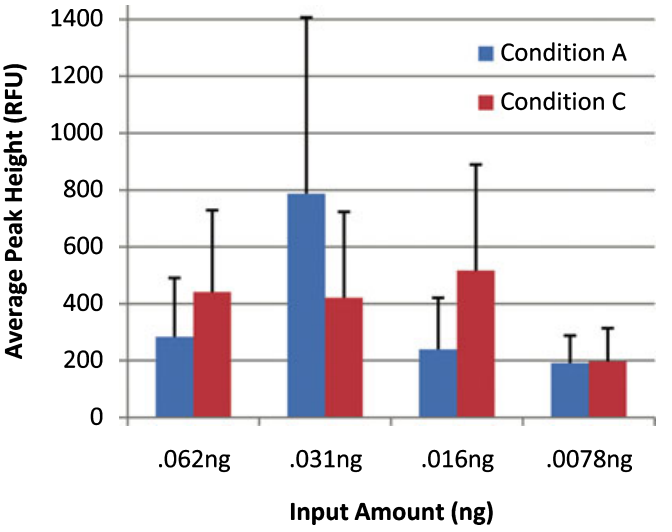

FIG. 2—*Average peak height for conditions A and C in the pristine sample study. Samples for each input value were tested (*n = *18 for Taq Gold STR and* n = *20 for Platinum Taq High Fidelity,* p = *0.0601). Error bars represent standard deviation.*

TABLE 3—*Percent STR allele success and average DNA yield by sample type for mock case sample study.*

	STR DNA Input for Each Sample Tested (ng)	% Allele Success by Sample Type			
		Condition A	Condition B	Condition C	Condition D
Teeth $n = 2$	1, 1	84.90	94.12	93.72	**97.06**†
Bones $n = 2$	0.082, 0	25.00	**43.75**	28.13	21.88
Fired cartridge cases $n = 4$	0.097, 0, 0, 0	1.47	6.27	9.21	**12.15**
Prints $n = 3$	0.160, 0.044, 0	68.10	68.63	**76.47**	72.55
Heat degraded blood $n = 2$	1, 1	**96.67**	90.00	93.33	**96.67**
	Overall average	47.9	52.8	53.6*	53.6

*$p = 0.03$.
†Numbers in bold indicate the condition with the highest allele success per sample.

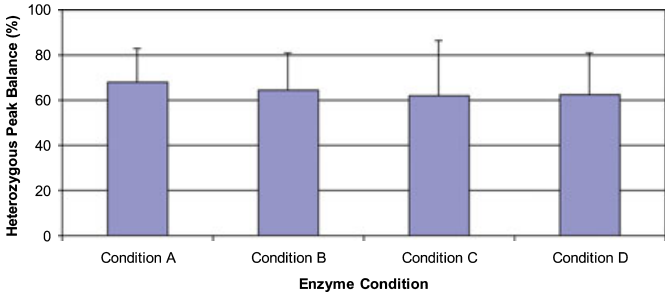

FIG. 3—*Heterozygous peak balance (%) between all four enzyme conditions for mock case sample study. The peak ratio for all enzymes tested was consistently >60% (*n = *7–8 for all enzyme conditions,* p = *0.3007). Error bars represent standard deviation.*

conditions C and condition D having the highest allele success at 53.6% (Table 3).

For loci with both peaks of a heterozygous pair present, there was no significant difference observed between all four enzyme conditions with regard to heterozygous peak balance (Fig. 3, $p = 0.3007$). Further, there were no differences found in average peak heights or in inter-locus peak heights among all four enzyme conditions tested ($p = 0.9704$ and $p = 0.6715$, respectively; data not shown). Overall, adding a proofreading enzyme did not affect the STR data quality observed (Fig. 4).

Several data quality issues were noted during data analysis of the STR results for the mock case samples across all enzyme conditions tested including the control condition (A). The most common effects noted were A^-/A^+, stutter, and baseline artifacts (no allelic drop-in was observed). However, many of the undesirable effects were likely caused by the high level of DNA detected in some samples (i.e., teeth) and would likely be remedied with lower injection times. It is important to note that these issues were observed throughout all four enzyme conditions tested with the mock case samples; thus, the presence of stochastic effects and/or

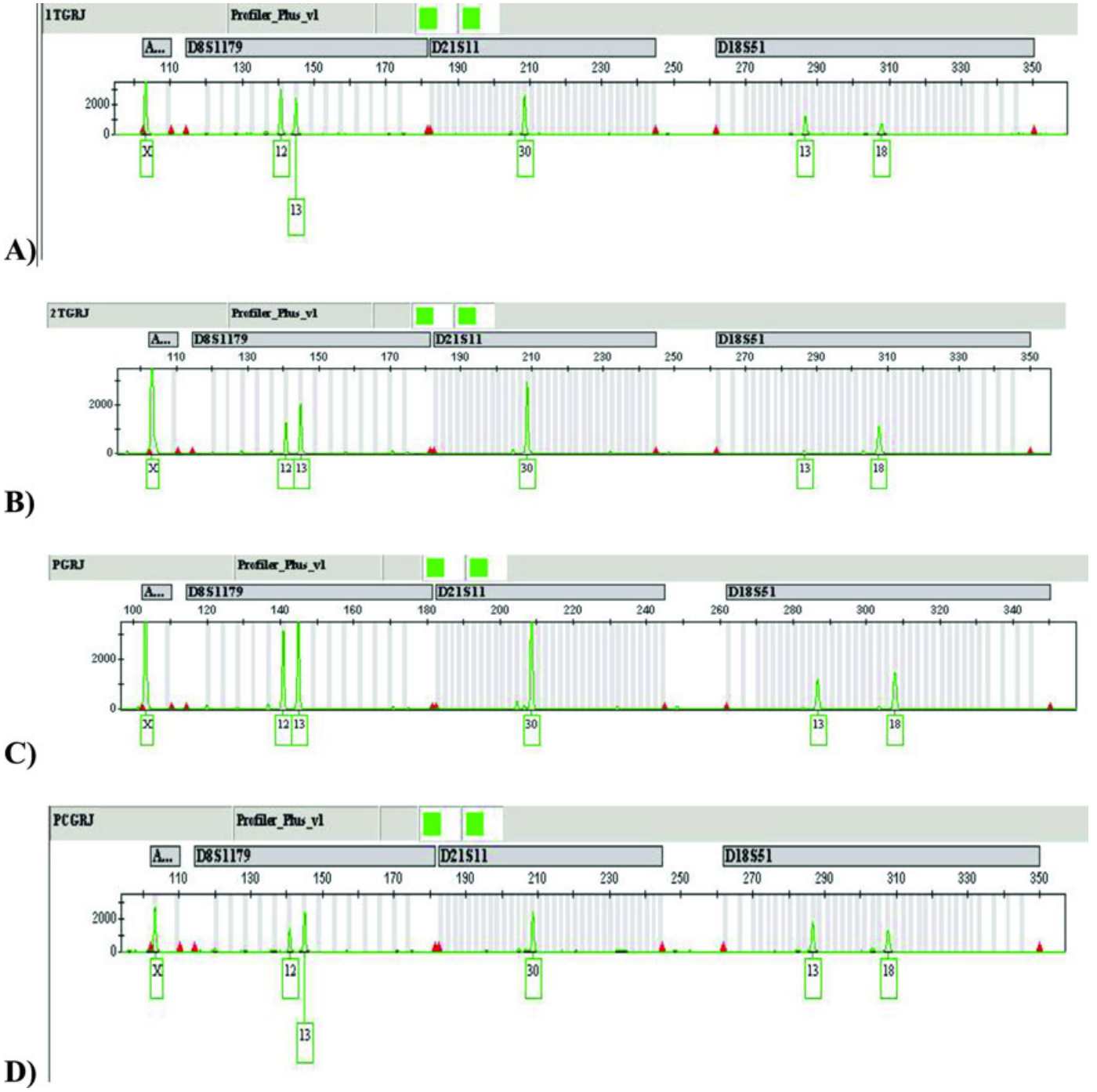

FIG. 4—*An electropherogram of a dermal ridge print from the same source (0.15 ng input) (A) using condition A; (B) using condition B; (C) using condition C; (D) using condition D. Green channel only shown.*

other artifacts is not impacted by the addition of proofreading enzymes to the STR cocktail.

Discussion

The goal of this study was to determine whether adding a proofreading enzyme (with a post-PCR purification step) would enhance the overall STR allele success and data quality from LCN samples. If successful, this would provide a simple, inexpensive, less time-consuming alternative to the currently available LCN techniques. To explore this further, two sample sets were analyzed: pristine samples diluted to LCN amounts (<100 pg) and nonprobative/mock case samples similar to those frequently encountered in the forensic laboratory. While the addition of a post-PCR purification step and the increase in injection time did improve the number of STR alleles recovered as expected (vs. traditional STR amplification methods), adding a proofreading enzyme did not further improve STR results in these samples.

The two proofreading enzymes chosen for this experiment were the two that performed best when added to the WGA master mix in a previous study by Pavlova et al. (Platinum Taq High Fidelity and GeneAmp High Fidelity polymerase) (7,18). Unfortunately, neither Platinum Taq High Fidelity (conditions C and D) nor GeneAmp High Fidelity (conditions E and F) performed as well in this study. This may be attributed to two major factors. First, in the previous study, the enzyme selection was based on adding proofreading enzymes to the whole genome preamplification, not directly to a manufactured STR multiplex amplification reaction mix. Further, because the STR multiplex amplification kit provides a single PCR mix, which combines multiple components at proprietary ratios and concentrations, it is possible that either the presence/absence of a particular component or the concentration of a component (i.e., $MgCl_2$) could have negatively affected the proofreading polymerase(s).

While no proofreading enzyme condition tested outperformed the control condition, it should be noted that the control condition A (1 U Taq Gold) and condition C (1 U Platinum Taq High Fidelity) were comparable overall at all STR data measures. Although there was not a significant difference between the two enzyme conditions, condition C yielded an average of 13.9% more alleles than condition A at very low input quantities (7.8 pg) from pristine LCN samples. Further, while a slight improvement was found with condition C in the mock case sample study, the relatively minor increase in STR alleles detected (5.7%) is not enough to warrant a change to the standard validated STR amplification methods.

As has been previously described (17), there were several data quality issues observed among all mock case samples. Because these issues were not observed with the pristine low template DNA samples, it is possible that interfering components within the mock case DNA samples (gunpowder on the fired cartridge samples, bone and teeth components, or heme from the bloodstains) were not completely removed during extraction. This has also been observed in other studies that used a concentration step (such as MinElute) prior to CE analysis (17). Additionally, these issues were most prevalent in samples that yielded large quantities of DNA, which are not the type of samples typically targeted for LCN applications. Nonetheless, the stochastic effects and artifacts observed were seen equally across all four polymerase conditions tested using the mock case samples (including *Taq*), ruling out the possibility that they resulted from the enzyme utilized in the reaction.

Finally, low template DNA samples from this study that had been post-PCR purified and amplified using either Taq Gold (condition A) or Platinum Taq High Fidelity (condition C) were compared with data from a similar set of pristine samples that had been previously amplified using traditional STR amplification and analysis procedures (without any modifications, condition G). While the newer, modified methods increased the number of STR alleles detected (65.3% and 66.2% for conditions A and C, respectively, versus 14.6% for the traditional method, data not shown), it is not possible to make an argument for the use of a proofreading enzyme. Based on this data, it is evident that most of the increases in STR success are likely caused by the increased injection time and the addition of the post-PCR purification step. Thus, the addition of proofreading enzymes to the reaction cocktail for multiplex STR amplification is not generally needed. However, if a sample yields a very low quantity of DNA, Platinum Taq High Fidelity enzyme may be employed to obtain a more complete STR profile.

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