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TECHNICAL NOTE

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Development and Validation of the AmpFℓSTR® MiniFiler™ PCR Amplification Kit: A MiniSTR Multiplex for the Analysis of Degraded and/or PCR Inhibited DNA*

ABSTRACT: DNA typing of degraded DNA samples can be a challenging task when using the current commercially available multiplex short tandem repeat (STR) analysis kits. However, the ability to type degraded DNA specimens improves by redesigning current STR marker amplicons such that smaller sized polymerase chain reaction (PCR) products are generated. In an effort to increase the amount of information derived from these types of DNA samples, the AmpFℓSTR® MiniFiler™ PCR Amplification Kit has been developed. The kit contains reagents for the amplification of eight miniSTRs which are the largest sized loci in the AmpFℓSTR® Identifiler® PCR Amplification Kit (D7S820, D13S317, D16S539, D21S11, D2S1338, D18S51, CSF1PO, and FGA). Five of these STR loci (D16S539, D21S11, D2S1338, D18S51, and FGA) also are some of the largest loci in the AmpFℓSTR® SGM Plus® kit. This informative nine-locus multiplex, which includes the gender-identification locus Amelogenin, has been validated according to the FBI/National Standards and SWGDAM guidelines. Our results demonstrate significant performance improvements in models of DNA degradation, PCR inhibition, and nonprobative samples when compared to the AmpFℓSTR® Identifiler® and SGM Plus® kits. These data support that the MiniFiler™ kit will increase the likelihood of obtaining additional STR information from forensic samples in situations in which standard STR chemistries fail to produce complete profiles.

KEYWORDS: forensic science, DNA typing, mini short tandem repeat, D7S820, D13S317, D16S539, D21S11, D2S1338, D18S51, CSF1PO, FGA, humic acid, hematin

Extracting sufficient high quality DNA for conventional short tandem repeat (STR) typing from a sample of poor quality is a challenging task and at times not possible. Often, polymerase chain reaction (PCR) amplification with commercial STR multiplexes results in partial or no information due to extensive DNA fragmentation. Many laboratories often do not attempt further analysis of these limiting samples and some laboratories resort to single nucleotide polymorphism (SNP) analysis (1) or mitochondrial DNA sequencing of the hypervariable regions (2,3) so as to obtain some information. These approaches, while helpful, are inherently less discriminating than STR multiplex systems and increase analysis time and cost. Another approach increases the number of amplification cycles to the recommended PCR protocol for commercial STR multiplexes (4,5). However, low level DNA analysis is highly susceptible to stochastic effects and can result in allele drop-outs, allele drop-ins, imbalance of heterozygote peak height and area, inconsistent peak size of stutter products, and increased risk of laboratory-based contamination (6). Recently, several laboratories (7–14) have demonstrated improvements in genotyping degraded DNA samples by repositioning primers in as close as possible to the STR repeat region. These primer changes result in smaller PCR products termed "miniSTRs" that increase the potential number of template molecules available for the PCR.

The AmpFℓSTR® MiniFiler™ PCR Amplification Kit was designed to amplify as miniSTRs eight of the largest sized loci in the AmpFℓSTR® Identifiler® PCR Amplification Kit (D7S820, D13S317, D16S539, D21S11, D2S1338, D18S51, CSF1PO, FGA). Five of these loci (D16S539, D21S11, D2S1338, D18S51, and FGA) also are five of the largest loci in the AmpFℓSTR® SGM Plus® kit. Together with the gender-identification locus Amelogenin, this nine-locus multiplex enables simultaneous amplification of the loci that often fail detection during the amplification of compromised DNA samples (12). Seven of these loci (D7S820, D13S317, D16S539, D21S11, D18S51, CSF1PO, FGA) are part of the core loci required by the Combined DNA Index System (CODIS) to maintain compatibility with convicted offender database profiles generated with conventional commercial STR multiplexes. Furthermore, a population study involving 1308 samples demonstrated that the MiniFiler™ and Identifiler® kits share a very high degree of concordance (15).

This article describes the developmental validation performed according to guidelines issued by the Director of the FBI (16), and the revised guidelines issued by SWGDAM (17). Performance comparisons between the MiniFiler™, Identifiler®, and SGM Plus® kits using models of DNA degradation, PCR inhibition, and nonprobative samples are described.

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*Oral presentation at the 17th International Symposium on Human Identification, Nashville, TN 2006. Some of the data are also presented in the MiniFiler™ PCR Amplification Kit User Guide "Experiments and Results" section.

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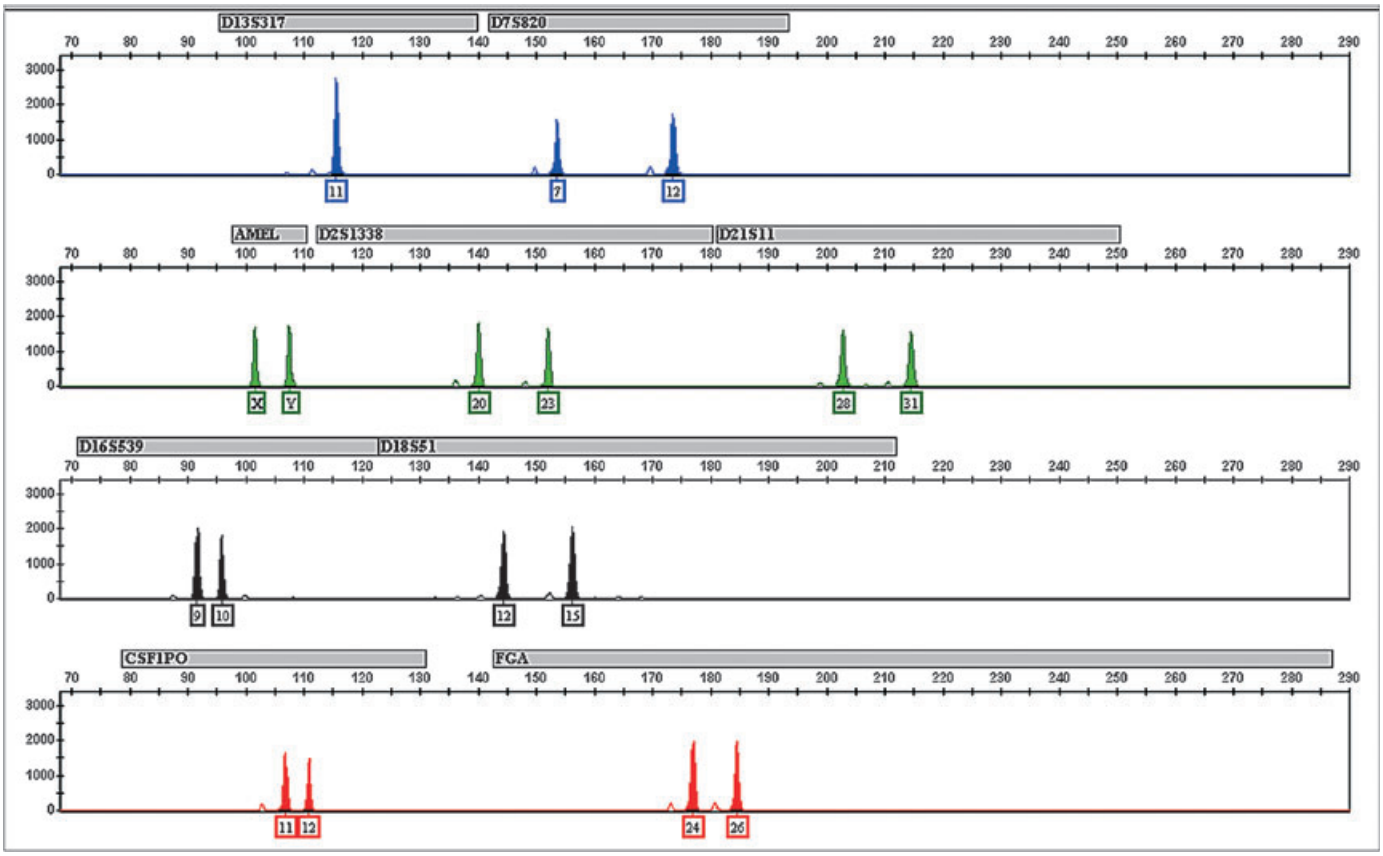

FIG. 1—*Representative electropherogram showing the profile of 500 pg of control DNA 007 amplified with the MiniFiler™ kit. The four panels correspond to (from top to bottom) 6-FAM™, VIC®, NED™, and PET® dye-labeled peaks. The genotype is shown with the allele number displayed underneath each peak.*

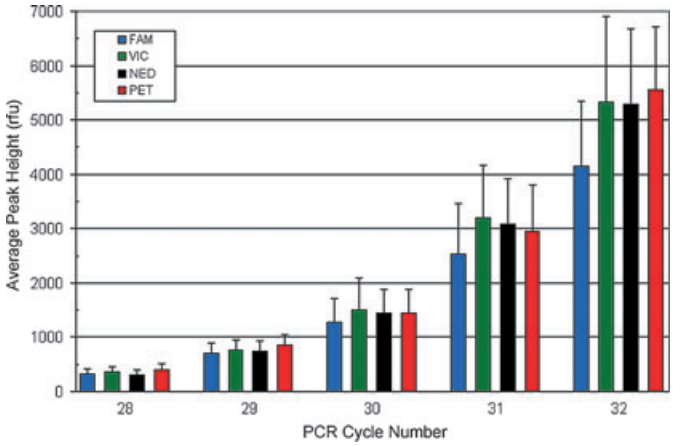

FIG. 2—*Effect of PCR cycle number on intracolor peak height average. Three genomic DNA samples (control DNA 007, 9947A, and 9948) were amplified in triplicates using as an input 500 pg of DNA. The samples were amplified on the Applied Biosystems GeneAmp® PCR System 9700 (operating in 9600 emulation mode).*

Materials and Methods

DNA Samples

Anonymous DNA samples were purchased from Seracare Life Sciences (Oceanside, CA), Raji DNA (Biochain Institute, Hayward, CA) and 9947A was purchased from Marligen Biosciences (Ijamsville, MD). The 9948 DNA sample was purchased from Coriell Cell Repositories (Camden, NJ) and the AmpFℓSTR® Control DNA 007 was obtained from Applied Biosystems (Foster City, CA). The population study encompassed DNA samples from Seracare Life Sciences as well as samples analyzed in collaboration with the National Institute of Standards and Technology (NIST) (15). The quantity of the DNA samples was determined prior to amplification using the Quantifiler® Human DNA Quantification Kit (Applied Biosystems) on the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's specifications.

Primer Set Optimization

Each of the STR loci amplified by the MiniFiler™ kit has been characterized previously by other groups and can be found in commercial STR multiplexes such as the Identifiler® and SGM Plus® Kits (18,19). The MiniFiler™ kit primers were designed and optimized to obtain small amplified products (less than currently generated using other commercially available Applied Biosystems kits) with robust signal intensity and balanced peak heights from human samples. Initially, singleplex reactions were tested using our designed primer sets to ensure locus-specific amplification. Then multiplex PCR studies were carried out through primer concentration adjustment and empirical performance testing in an effort to generate sensitive, balanced, and specific signals for the eight STR loci and the sex determining marker Amelogenin in a single PCR reaction. The MiniFiler™ kit STR loci span a range between 71 and 250 bp. To accommodate the co-amplification of eight loci

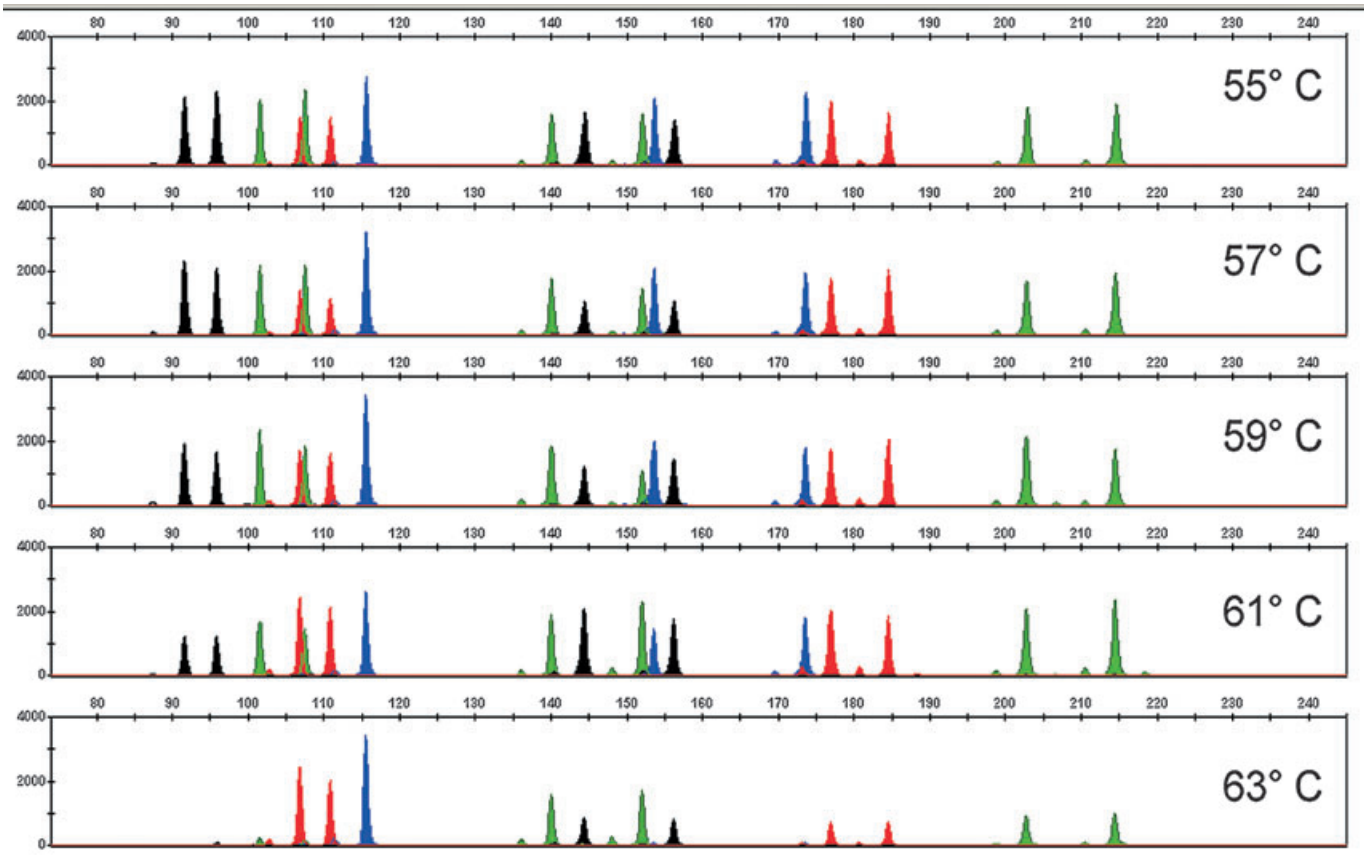

FIG. 3—*Representative electropherograms from an annealing temperature study of the control DNA 007 DNA. The DNA was amplified with the MiniFiler™ kit at the indicated temperatures. Peak heights were measured in relative fluorescent units (RFUs).*

TABLE 1—*Stutter average and range for MiniFiler™ kit loci.*

Locus	Stutter Average	Stutter Range (%)	SD
D13S317	6.3	1.9–13.7	1.7
D7S820	4.9	1.8–10.7	1.5
D2S1338	8.8	3.5–17.7	1.9
D21S11	8.1	2.2–15.7	1.5
D16S539	6.8	1.2–14.3	2.0
D18S51	7.9	2.1–17.3	2.3
CSF1PO	6.2	1.4–13.9	1.7
FGA	7.4	1–14.5	1.6

All loci have tetranucleotide repeats yielding −4 bp stutters. Stutters were determined for those samples with peak heights between 400 and 5000 RFUs. The threshold minimum stutter peak height was 20 RFUs.

within this narrow size range, we employed a five-dye fragment analysis system developed at Applied Biosystems and currently utilized in the AmpFℓSTR® SEfiler™ (20), Identifiler® (21), and Yfiler® kits (22). The five dyes (6-FAM™, NED™, PET®, VIC®, and LIZ®) expand the fluorescent detection range to 660 nm thereby enabling more loci to be multiplexed in a single PCR. The electrophoretic spacing between different loci was optimized by introducing monomeric non-nucleotide linkers between the DNA oligonucleotide sequence and the fluorescent tag during the primer synthesis process (23,24) for most loci. The resulting PCR product has a slower mobility, which correlates with the number of linkers used. This approach has been successfully applied to the Yfiler and Identifiler® kits. The corresponding allelic ladders also contain non-nucleotide linkers. Three 500 pg samples (9947A, 9948, and the control DNA 007) were amplified in triplicate at the standard primer concentration and at 10% intervals up to ±30% levels to evaluate primer performance (data not shown).

PCR Components

The buffer components and concentrations are proprietary and include a DNA polymerase, salts, dNTPs, carrier protein, and 0.05% sodium azide. Each of these components was tested individually at a series of titrations around the standard condition used in the AmpFℓSTR® MiniFiler™ Master Mix. The components were tested in increments of ±10% up to ±30% from the standard concentration to test for reliability and robustness of the amplification components. Each concentration was tested with three samples (9947A, 9948, and control DNA 007) and amplified in triplicate using 500 pg of template DNA (data not shown).

Thermal Cycling Parameters

Thermal cycling parameters were evaluated to establish the optimal performance window of amplification for the MiniFiler™ kit. Cycling parameters around the standard set of conditions were tested. For each study, 500 pg of DNA from three samples (9947A, 9948, and control DNA 007) were prepared in triplicate and stored at 4°C while awaiting amplification in the same thermal cycler for each of the parameters tested.

The following thermal cycler parameters were examined (the recommended parameters are shown in bold):

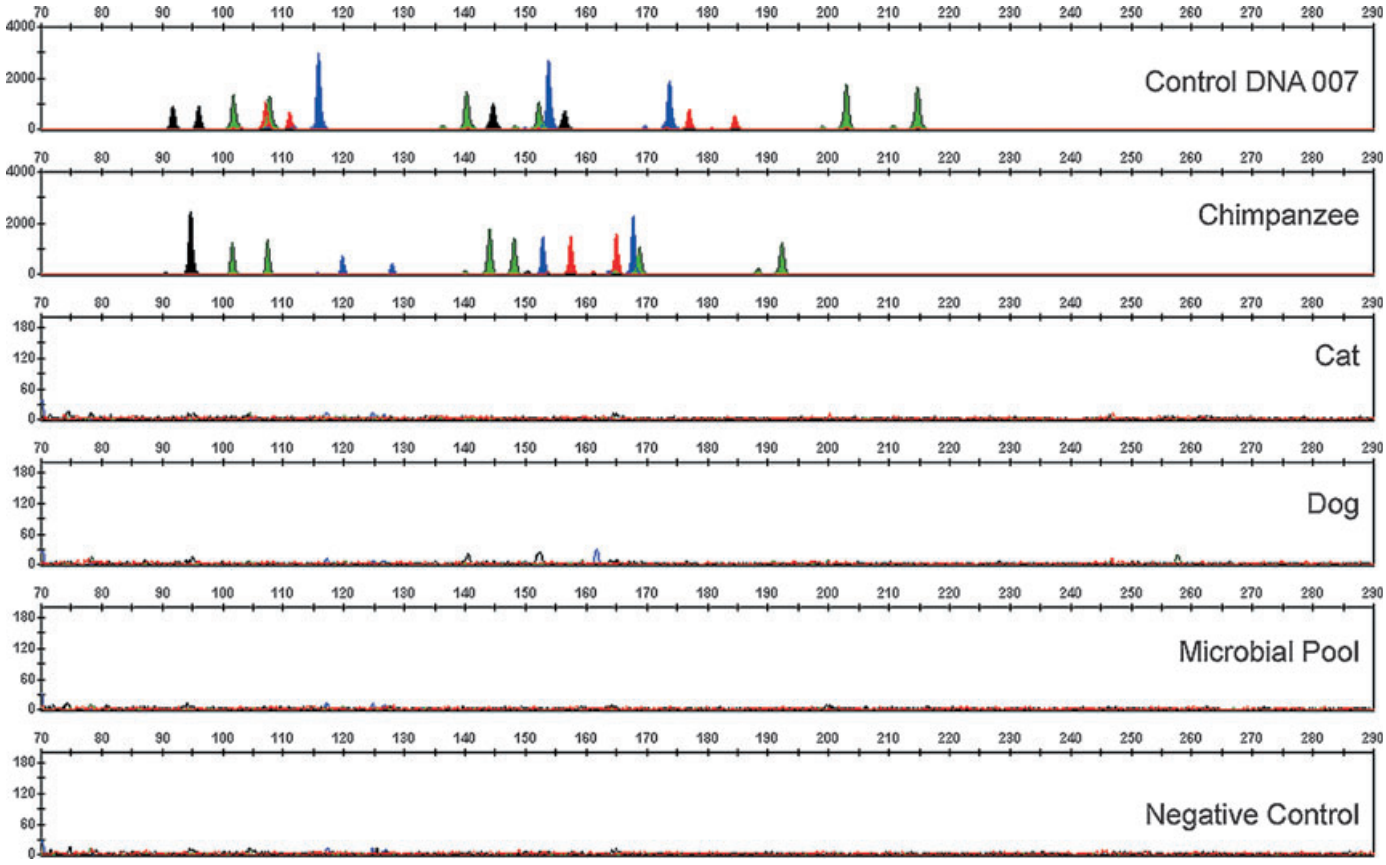

FIG. 4—*Representative electropherograms from a species specificity study. From top to bottom: 1 ng of the control DNA 007 (human), 1 ng of chimpanzee DNA, 10 ng of cat and dog DNA, a microbial pool (*c. *100,000 copies each of* Candida albicans, Escherichia coli, *and* Lactobacillus rhamnosus *amplified together), and a negative control. Different RFU scales (*y*-axis) are shown to demonstrate specificity.*

1. Cycle number: 28, 29, **30**, 31, and 32 cycles.
2. Denaturation temperature: 92.5°C, **94.0°C,** and 95.5°C.
3. Annealing temperature: 55°C, 57°C, **59**°C, 61°C, and 63°C.
4. Final extension time: 15, 30, **45**, 60, and 90 min.

Accuracy, Precision, and Stutter Studies

Forty-two DNA samples from Seracare Life Sciences and Coriell Cell Repositories were used to measure the deviation of each sample allele size from the corresponding allelic ladder allele size. The DNA samples (0.5 ng input) were amplified with the MiniFiler™ kit using the standard conditions and subjected to electrophoresis on the Applied Biosystems 3130*xl* Genetic Analyzer. Allelic ladder sizing precision was calculated from multiple injections of ladder run on the Applied Biosystems 3130*xl* Genetic Analyzer. Percent stutter was calculated on 1264 alleles from the population study data set (15,25) by dividing the height of the stutter peak by the height of the true allele peak.

PCR Amplification

Unless noted otherwise, the protocols in the AmpFℓSTR® MiniFiler™ kit user guide were followed (25). The PCR amplification was performed in a reaction volume of 25 μL containing 10 μL of AmpFℓSTR® MiniFiler™ Master Mix, 5 μL of AmpFℓSTR® MiniFiler™ Primer Set, and a maximum volume of 10 μL of target DNA. Samples were amplified in MicroAmp® reaction tubes (Applied Biosystems) in the GeneAmp® PCR System 9700 with a gold-plated silver or silver block (Applied Biosystems). The standard thermal cycling conditions in the 9600 emulation mode consisted of enzyme activation at 95°C for 11 min, followed by 30 cycles of denaturation at 94°C for 20 sec, annealing at 59°C for 2 min, and extension at 72°C for 1 min. A final extension was performed at 60°C for 45 min with a 4°C temperature hold, if the PCR product was to remain in the thermal cycler. Thermal cycling conditions for the Identifiler® and SGM Plus® kit PCR reactions were performed for 28 cycles of amplification as described in their user's manuals (18,19).

Sample Electrophoresis and Data Analysis

Amplification products were separated and detected on the Applied Biosystems 3130 and 3130*xl,* ABI PRISM® 3100 and 3100–*Avant*, and ABI PRISM® 310 Genetic Analyzers using the specified G5 variable binning module as described in the user's guide (25). For example, sample preparations and electrophoresis on the Applied Biosystems 3130*xl* analyzer occurred as follows: 1 μL of the amplified product or allelic ladder and 0.3 μL of GeneScan™-500 LIZ® size standard were added to 8.7 μL of deionized Hi-Di™ Formamide (Applied Biosystems), denatured at 95°C for 3 min, and then chilled on ice for 3 min. Samples were injected for 10 sec at 3 kV and electrophoresed at 15 kV for 1500 sec in Performance Optimized Polymer (POP-4™polymer) with a run temperature of 60°C as indicated in the GeneScan36vb_POP4DyeSetG5Module. The data were collected using the Applied Biosystems 3130*xl* Data Collection Software application v3.0. Electrophoresis results were analyzed using GeneMapper® *ID*

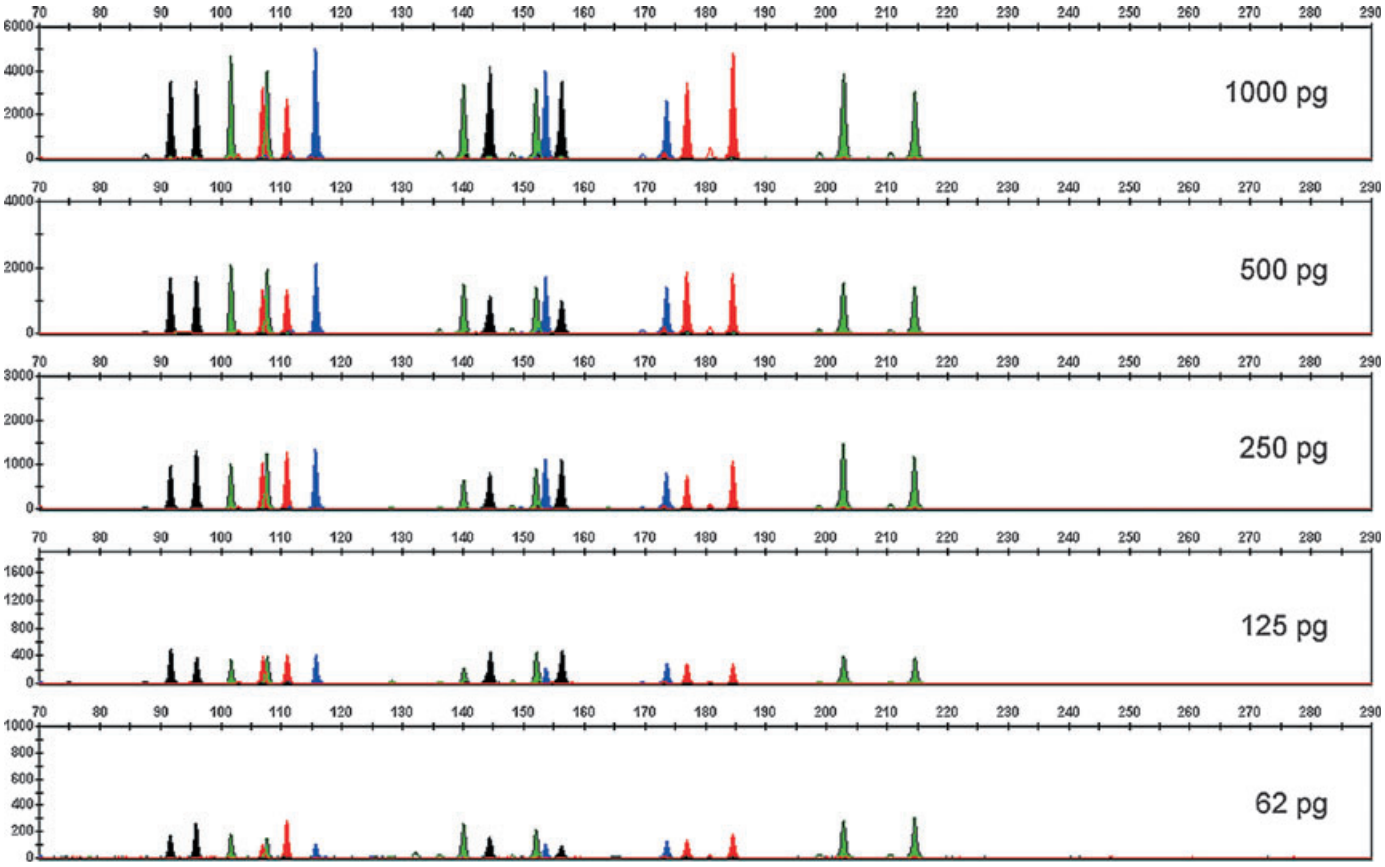

FIG. 5—*Effect of varying inputs of template DNA on peak height. The results depicted are representative of the amplification of the control DNA 007 at the indicated amounts. The data were analyzed with a peak amplitude threshold of 50 RFUs.*

Software v3.2. Allele peaks were interpreted when greater than or equal to 50 relative fluorescence units (RFUs).

Species Specificity

DNA samples from primates (1 ng each from gorilla, chimpanzee, orangutan, and macaque), nonprimates (10 ng each from mouse, dog, pig, cat, horse, chicken, and cow), and micro-organisms (*c*. 100,000 copies each from *Candida albicans, Staphylococcus aureus, Escherichia coli, Neisseria gonorrhoeae, Bacillus subtilis, and Lactobacillus rhamnosus*) were subjected to PCR amplification using the MiniFiler™ kit in triplicates. The microbial DNAs were pooled together prior to amplification.

Sensitivity Study

Two DNA samples (9948 and control DNA 007) were serially diluted to amounts of 1 ng, 500, 250, 125, 62, and 31 pg and amplified in replicates of four with the MiniFiler™ kit.

Models of DNA Degradation and PCR Inhibition

Performance of the kit with inhibited and degraded samples was examined using laboratory models. Degraded DNA was produced first by sonicating Raji DNA followed by a 20-min incubation with increasing amounts (0–6 U) of DNase I (Ambion Inc., Austin, TX). The resulting DNA was examined by agarose gel analysis to assess the level of DNA degradation at each dose. An inhibition study was performed with hematin (Sigma, St. Louis, MO) diluted to 1 mM in 0.1 N NaOH and added to the PCR to obtain final concentrations ranging from 0 to 80 μM. Another inhibition study also was performed by diluting humic acid (Sigma) to 250 ng/μL in TE buffer (10 mM Tris pH 8.0, 0.1 mM EDTA) and adding it to the PCR to obtain final concentrations ranging from 0 to 50 ng/μL.

Nonprobative Samples

DNA from the formaldehyde-fixed paraffin-embedded (FFPE) tissue specimen was extracted from two 2-μm sections from tissue block using the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion). This procedure included an initial deparaffinization with xylene, followed by an ethanol rinse and thorough drying. The resulting deparaffinized sample was digested with proteinase K and incubated for 48 h. The DNA solution was applied to a glass-fiber filter, washed, treated with RNase, and eluted into a microcentrifuge tube using reagents provided by the manufacturer.

The bone sample from a skeletal remain was obtained and analyzed by the Armed Forces DNA Identification Laboratory. The bone was processed and the DNA extracted according to their established procedures.

Mixture Studies

Mixtures of two DNA samples were examined at various ratios (1:1, 1:3, 1:7, 1:10, and 1:15) while holding the total amount of input DNA constant at 1 ng. For example, a 1:1 mixture contains 0.5 ng of each individual. Mixture ratios of 1:3, 1:7, 1:10, and 1:15

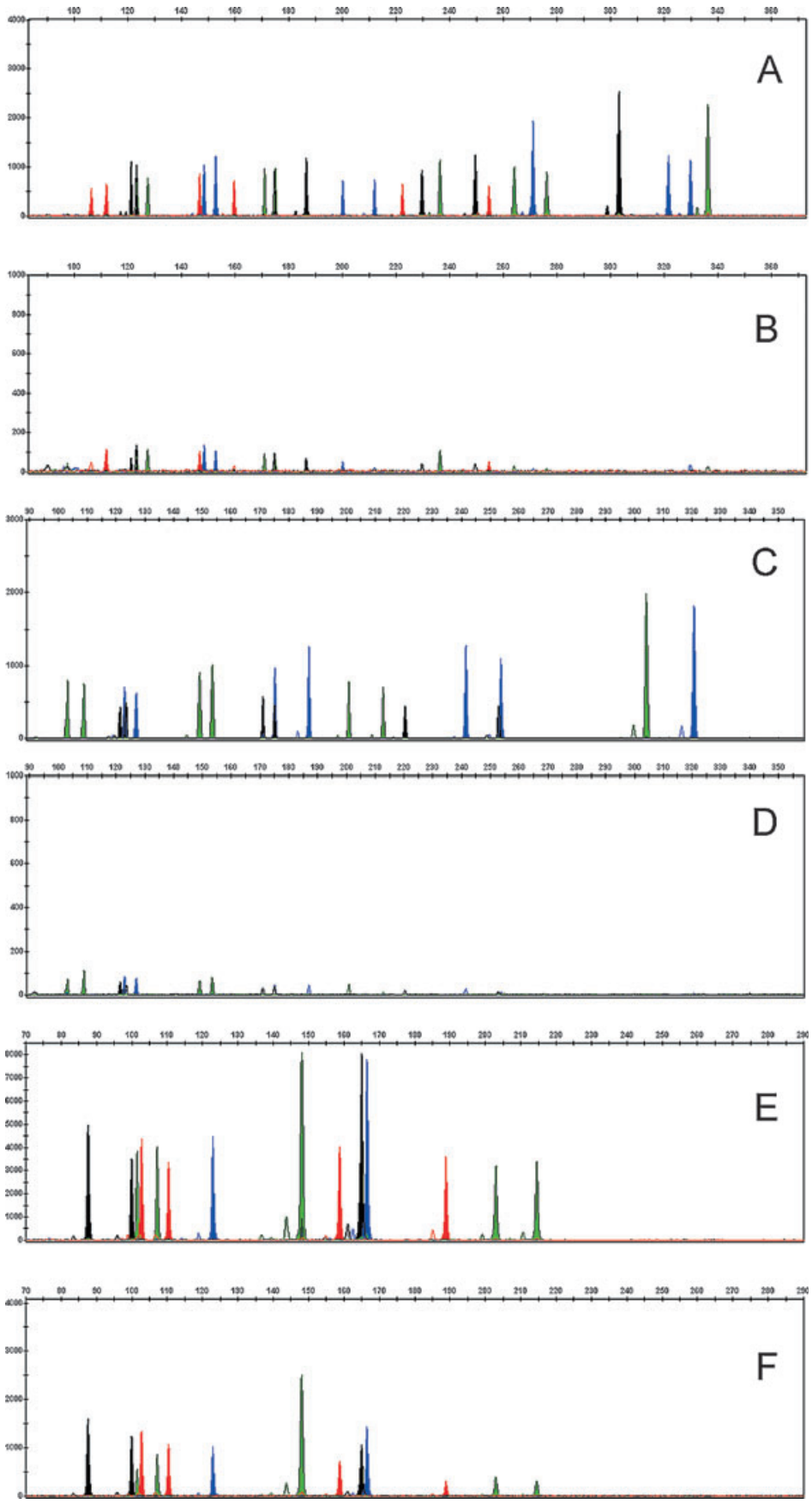

FIG. 6—*Representative electropherograms from a model of DNA degradation. Panels A, C, and E are 1 ng of untreated Raji DNA and Panels B, D, and F correspond to 2 ng samples that were sonicated and treated with 5 U of DNase I for 20 min (Materials and Methods). Panel A and B correspond to DNA amplified with the Identifiler® kit. Panels C and D correspond to DNA amplified with the SGM Plus® kit. Panels E and F correspond to DNA amplified with the MiniFiler™ kit.*

TABLE 2—*Amplification efficiency of Raji DNA incubated with increasing doses of DNaseI for 20 min.*

DNaseI	MiniFiler™ Kit (n = 3)	Identifiler® Kit (n = 3)	SGM Plus® Kit (n = 4)
0	1.0	1.0 (1.0)	1.0 (1.0)
4 U	1.0	0.36 ± 0.19 (0.65 ± 0.08)	0.43 ± 0.17 (0.71 ± 0.09)
5 U	1.0	0.26 ± 0.04 (0.52 ± 0.05)	0.20 ± 0.0 (0.44 ± 0.08)
6 U	0.98 ± 0.04	0 (0.024 ± 0.021)	0.05 ± 0.06 (0.025 ± 0.029)

A full profile in MiniFiler™ consists of 14 amplification products (14/14 = 1.0). Identifiler® and SGM Plus® kits were scored for the loci shared with the MiniFiler™ kit and for their total number of amplification products as shown in brackets (28 and 20 amplification products respectively for Identifiler® and SGM Plus® kits). The values are expressed as mean ± SD.

TABLE 3—*Combined probability of Identity (P_I) values for the Identifiler® and MiniFiler™ kit loci.*

	African American	U.S. Caucasian	U.S. Hispanic	Native American
Identifiler® kit loci (without MiniFiler™ kit loci)*	2.01×10^{-08}	6.10×10^{-08}	7.32×10^{-08}	1.75×10^{-07}
MiniFiler™ kit loci	6.52×10^{-11}	8.21×10^{-11}	1.05×10^{-10}	2.08×10^{-10}
Identifiler® kit loci	1.31×10^{-18}	5.01×10^{-18}	7.65×10^{-18}	3.62×10^{-17}

*The Identifiler® kit loci included in the P_I calculation are D8S1179, D3S1358, TH01, vWA, D5S818, and TPOX.

contain 0.25, 0.125, 0.091, and 0.0625 ng of the minor component, respectively.

Statistical Analysis

Peak height ratios were calculated by dividing the lower peak height by the higher peak height within a heterozygote at a locus. Intracolor peak balance was calculated by dividing the lowest peak height value by the highest peak height value within a color (homozygote peak heights are divided by two and heterozygote peak heights are averaged for each marker).

The probability of identity (P_I) for each locus was calculated by summing the square of the genotype frequencies (26). The combined P_I was determined by multiplying the individual P_Is for each locus tested. The population data set and relevant statistics were documented in the Identifiler® kit user's manual (18).

Results

Thermal Cycling Parameters

A representative MiniFiler™ kit STR profile using as template 500 pg of the control DNA 007 is shown in Fig. 1. The optimal thermal cycling parameters were determined to be in the middle of a window that balances specificity and sensitivity. PCR cycle numbers did not have a significant effect on peak height balance in the range studied (28–32 cycles) (Fig. 2). Each increase in cycle number generally led to an approximately corresponding twofold increase in overall peak height. The average peak height at 28 cycles for a heterozyogus peak was 346 ± 94 RFUs and the average peak height at the standard cycle number of 30 was 1420 ± 470 RFUs. Several peaks were offscale at 32 cycles (average heterozygous peak height 5080 ± 1329 RFUs), while no offscale peaks were detected at lower cycle numbers. The optimal annealing temperature was based on a balance among specific amplification of DNA, sensitivity, and reproducible intracolor peak balance. No locus dropout was observed with annealing temperatures between 55°C and 61°C. However, a significant decrease in peak heights in the D7S820 locus and Amelogenin products as well as occasional allele dropouts in D16S539 were noted at 63°C (Fig. 3). These experiments indicated that a 2°C window around the set point of 59°C yields specific PCR products with the desired sensitivity for the DNA samples tested. At the standard annealing temperature (59°C), intracolor peak balance greater than 40% was reproducibly observed for 500 pg of input DNA (data not shown).

Although, the primers used in the MiniFiler™ kit were designed to promote nonspecific terminal nucleotide addition by the DNA polymerase (27), a 60°C, 45-min final extension step was added to the protocol. The final extension step ensures the completion of +A addition to the 3′ end of all double-stranded PCR products. This is especially important in samples containing PCR inhibitors. It is possible that some types of PCR inhibition not encountered in this study may require an even longer final extension step.

Accuracy, Precision, and Stutter Studies

Determining sizing accuracy and precision includes measurement error and assessing performance for accurate and reliable genotyping. Forty-two DNA samples were used to measure the deviation of each sample allele size from the corresponding allelic ladder allele size. All sample alleles tested were within ±0.5 bp of a corresponding allele in the allelic ladder (25). Allelic ladder sizing precision was calculated from multiple injections of ladder run. The standard deviation (SD) of the mean was calculated and shown to be within 0.15 bp or less (25).

Stutter products are a result of strand slippage (28) during PCR amplification. The most common stutter is one unit in length smaller than the true allele resulting in a product that could be, for example, four bases smaller for the tetranucleotide repeat markers in the MiniFiler™ kit. Percent stutter was calculated on 1264 alleles from the population study data set (15,25) (Table 1). All loci showed the trend of increasing stutter percentages with increasing allele size. On average, the stutter product formation for MiniFiler™ is slightly higher than for the Identifiler® kit (18). This could be due to the higher number of amplification cycles for the MiniFiler™ kit (30 vs. 28 cycles) increasing the chance of slippage events. Alternatively, the MiniFiler™ kit has a higher concentration of $MgCl_2$ in the amplification reaction potentially contributing to more slippage events by the polymerase (29,30).

Species Specificity

A variety of animal and microbial species were tested to assess the human specificity of the assay. Of the primate DNA samples, not surprisingly chimpanzee (Fig. 4) and to a lesser extent gorilla (data not shown) yielded partial profiles. Chimpanzee STRs homologous

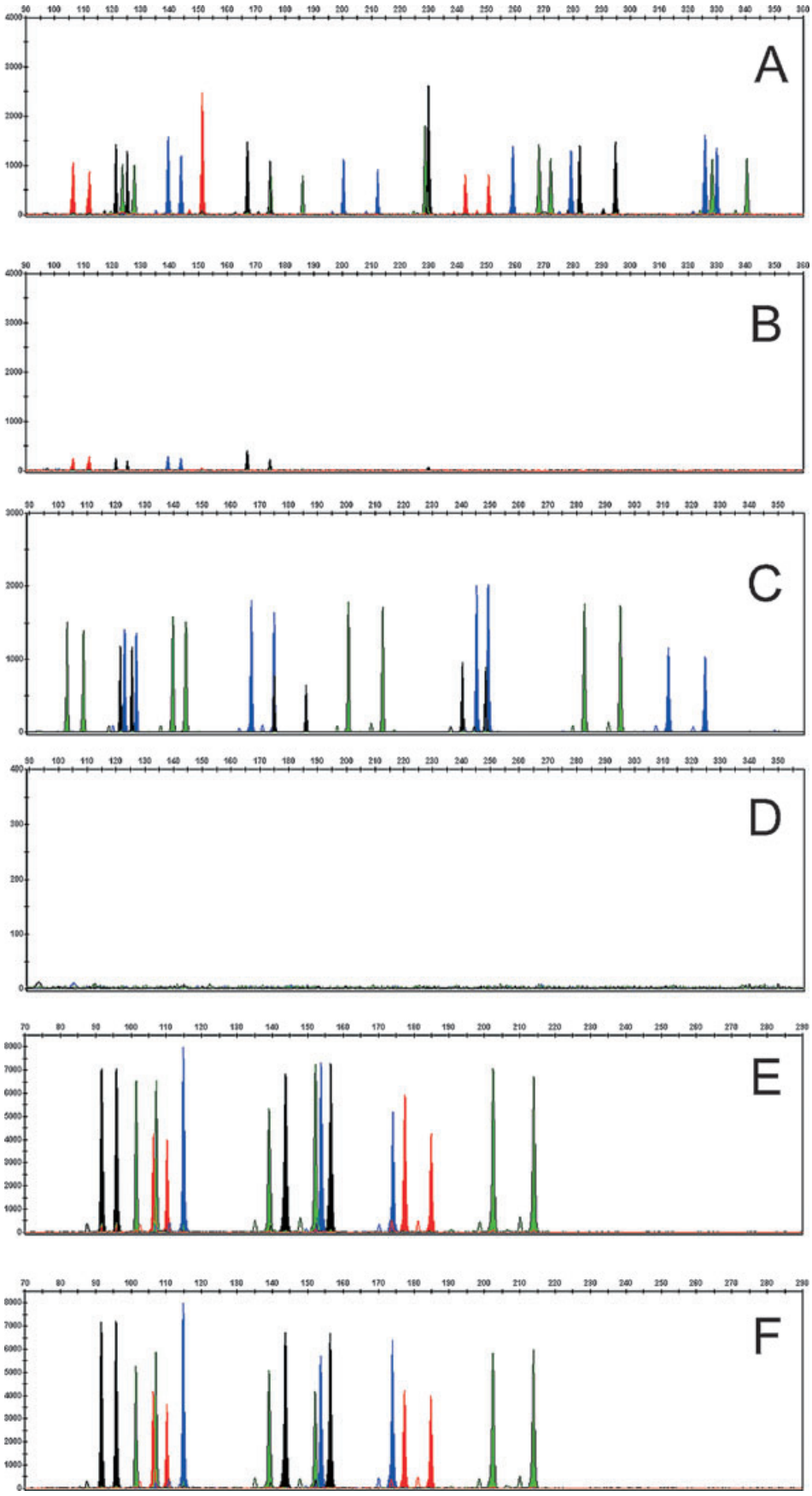

FIG. 7—*Representative electropherograms from a model of hematin inhibition. Panels A, C, and E are 1 ng of untreated control DNA 007, and Panels B, D, and F correspond to 1 ng samples that were amplified in the presence of 80 μM hematin. Panels A and B correspond to DNA amplified with the Identifiler® kit. Panels C and D correspond to DNA amplified with the SGM Plus® kit. Panels E and F correspond to DNA amplified with the MiniFiler™ kit.*

TABLE 4—*Amplification efficiency of control DNA 007 in the presence of increasing amounts of hematin.*

Hematin (μM)	MiniFiler™ Kit (n = 3)	Identifiler® Kit (n = 3)	SGM Plus® Kit (n = 5)
0	1.0	1.0 (1.0)	1.0 (1.0)
20	1.0	1.0 (1.0)	1.0 (1.0)
40	1.0	0.84 ± 0.27 (0.91 ± 0.16)	1.0 (1.0)
60	1.0	0.08 ± 0.07 (0.16 ± 0.16)	0.13 ± 0.03 (0.12 ± 0.07)
80	1.0	0 (0)	0 (0)

A full MiniFiler™ profile consists of 17 amplification products (17/17 = 1.0). Identifiler® and SGM Plus® kits were scored for the loci shared with the MiniFiler kit and for their total number of amplification products as shown in brackets (29 and 22 amplification products respectively for Identifiler® and SGM Plus® kits). The values are expressed as mean ± SD.

to human STRs have been previously characterized (31). The amplification of genomic DNA from nonprimate species and pooled DNA from a number of microorganisms did not yield reproducible PCR products in triplicate amplifications. A representative electropherogram containing selected species is shown in Fig. 4.

Sensitivity

Sensitivity studies were performed using serial dilutions of two DNA samples including the control DNA 007 contained within the kit (Fig. 5). The optimal quantity of template DNA for the MiniFiler™ kit PCR ranged from 0.5 to 0.75 ng. Full profiles were obtained with amounts as low as 0.125 ng. Low input DNA amounts (<100 pg) yielded more variable results occasionally resulting in partial profiles. Depending on the sensitivity of the instrument, some minor dye artifacts may appear in the electropherogram (FAM70, FAM117, FAM127, VIC80, VIC117, and NED166) (25). These artifacts can be distinguished from actual allele peaks by using appropriate negative controls and should therefore not compromise the accurate typing of samples. PCR artifacts may occur when the amount of input DNA exceeds the recommended amount (500–750 pg). These artifacts were characterized as secondary stutter products in D13S317 and D21S11 and their mobility varies with that of the main amplification product (25). Electrophoretic artifacts may arise if the ambient temperature falls below 20°C. Under these conditions, D16S539 and D18S51 may yield broad and occasionally split peak profiles (data not shown).

Model of DNA Degradation

Environmental insults on forensic samples may result in DNA degradation or damage at random locations. As with any multilocus system, the possibility exists that not every locus will amplify if the DNA sample has been severely degraded with the largest sized loci being the most susceptible. The ability of the MiniFiler™ kit primers to amplify degraded DNA was investigated and compared to the Identifiler® and SGM Plus® kits (Fig. 6, Table 2). High molecular weight genomic DNA sonicated and incubated with DNase I for several time periods was tested (Materials and Methods). Two nanograms of degraded DNA (and 1 ng nondegraded DNA) was amplified. As expected, with increasing DNase I digestion, there was a reduction in PCR product yield at all loci. At each of the DNase I doses shown in Table 2, the MiniFiler™ kit outperformed both the SGM Plus® and Identifiler® kits. The information content in Identifiler® and SGM Plus® comes primarily from the smaller loci.

The probability of Identity values were calculated for the Identifiler® kit loci with and without the MiniFiler™ kit loci so as to estimate the discrimination power of a partial STR profile missing the largest loci in Identifiler® when compared to a full profile with the MiniFiler™ kit. Table 3 demonstrates that a full profile with the MiniFiler™ kit is more discriminating than a partial STR profile consisting of the Identifiler® loci without the MiniFiler™ loci.

Models of PCR Inhibition

DNA samples from crime scenes may contain inhibitors that can affect amplification of DNA samples. For example, heme compounds have been identified as PCR inhibitors in DNA samples extracted from bloodstains (32). The effect of hematin on the amplification efficiency of the MiniFiler™ kit was examined by varying concentrations of hematin (0–80 μM) in the PCR. Overall inhibition of the Identifiler® and SGM Plus® PCR reactions was observed as the concentration of hematin increased to 60 μM (Fig. 7, Table 4). Complete PCR inhibition for Identifiler® and SGM Plus® kits was seen at 80 μM. Complete profiles were reproducibly obtained with the MiniFiler™ kit at the tested concentrations.

Forensic evidence collected from soil may result in the coextraction of other soil components, such as humic acids (HA) which negatively interfere with DNA analytical processes (33). The influence of increasing amounts of HA (0–50 ng/μL) on the amplification of DNA with the MiniFiler™, Identifiler®, and SGM Plus® kits was examined. Complete inhibition of the Identifiler® and SGM Plus® kits was observed as the concentration of HA reached 30 ng/μL (Fig. 8, Table 5). Full profiles were reproducibly achieved by the MiniFiler™ kit even at 50 ng/μL. We observed a 50% peak height reduction in the MiniFiler™ kit loci D7S820, D16S539, and Amelogenin loci at the highest concentration tested (50 ng/μL HA).

This overcoming of inhibition is not directly related to reduction in amplicon size. The small sized loci in the Identifiler® and SGM Plus® kits failed to amplify in the presence of high concentrations of inhibitor where the Minifiler™ loci were successfully amplified. Yet, these loci are similar in size. The introduction of the proprietary PCR buffer overcomes some of the inhibitory effects and thus contributes to the overall robustness of the Minifiler™ kit.

Nonprobative Sample Analysis

The ability to isolate and identify DNA from archived tissue samples provides a powerful tool in retrospective identity studies of clinical tissue samples. The standard preservation technique of FFPE tissues results in extensive cross-linking between the nucleo-histone matrices. During long-term storage, chemical modification can cause nucleic acid fragmentation causing a potential problem in obtaining DNA typing results. Although not typeable with conventional STR typing kits, the MiniFiler™ kit enabled successful analysis of DNA extracted from several samples including a breast tumor tissue sample dating back to 1997 and extracted 9 years later (2006) (Fig. 9). A bone sample from a skeletal remain dating back to World War II

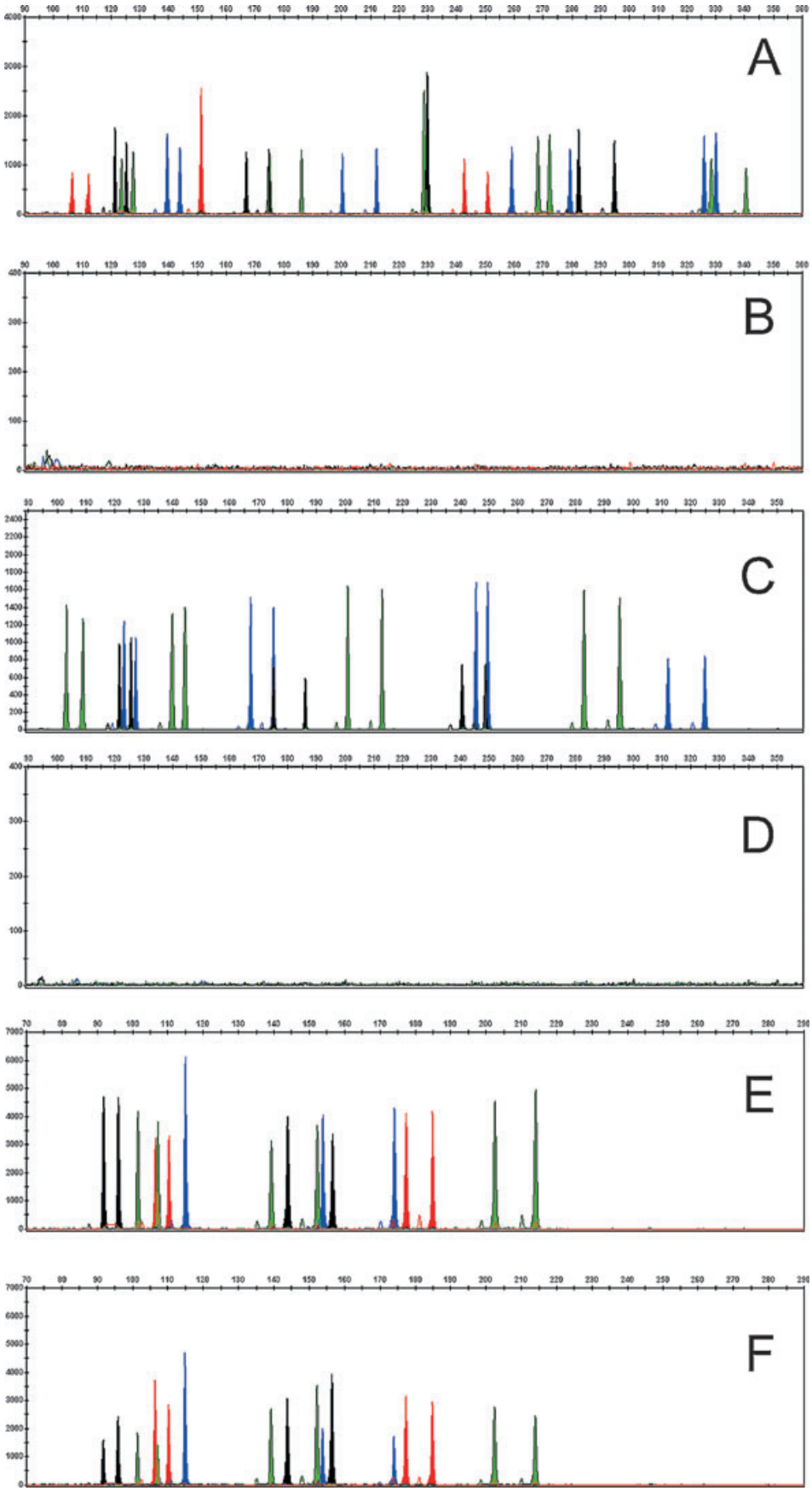

FIG. 8—*Representative electropherograms from a model of humic acid inhibition. Panels A, C, and E are 1 ng of untreated control DNA 007 and Panels B, D, and F correspond to 1 ng samples that were amplified in the presence of 30 ng/μL humic acid. Panels A and B correspond to DNA amplified with the Identifiler® kit. Panels C and D correspond to DNA amplified with the SGM Plus® kit. Panels E and F correspond to DNA amplified with the MiniFiler™ kit.*

TABLE 5—*Amplification efficiency of control DNA 007 in the presence of increasing amounts of humic acid.*

Humic acid (ng/μL)	MiniFiler™ Kit (n = 3)	Identifiler® Kit (n = 3)	SGM Plus® Kit (n = 5)
0	1.0	1.0 (1.0)	1.0 (1.0)
10	1.0	0.96 ± 0.08 (0.98 ± 0.05)	1.0 (1.0)
30	1.0	0 (0)	0 (0)
50	1.0	0 (0)	0 (0)

A full MiniFiler™ profile consists of 17 amplification products (17/17 = 1.0). Identifiler® and SGM Plus® kits were scored for the loci shared with the MiniFiler™ kit and for their total number of amplification products as shown in brackets (29 and 22 amplification products respectively for Identifiler® and SGM Plus® kits). The values are expressed as mean ± SD.

was amplified with the Identifiler® kit and resulted in no genetic profile. In contrast, analysis of the sample with the MiniFiler™ kit yielded typeable results at all loci (Fig. 10).

Mixture Studies

Evidence samples that contain body fluids and/or tissues originating from more than one individual are commonly encountered in forensic casework. Table 6 depicts the genotype of the minor component of two individuals mixed at several mixture ratios. Most of the alleles at each locus for the two individuals are different and thus do not overlap; however, some alleles of the minor contributors do reside at stutter positions of alleles from the major contributor. The minor component at 1:1, 1:3, and 1:7 mixture ratios was readily typeable. At a 1:10 ratio, all three profiles yielded complete profiles. However at 1:15 ratios, alleles at the D7S820 and D18S51 loci for one of the profiles were not detected because they were below the stutter filter threshold. In summary, ratios greater than 1:10 (where the minor component is *c*. 91 pg) may result in partial profiles for the minor component.

Population and Concordance Studies

Concordance data for four population groups (U.S. Caucasian, U.S. Hispanic, Asians, and African Americans) have been published (15) and documented in the MiniFiler™ kit user guide (25). In summary, 1308 samples were evaluated with both the MiniFiler™ and Identifiler® STR kits: 449 African American, 445 Caucasian, 207 Hispanic, and 207 Asian individuals. Full concordance between the Identifiler® and MiniFiler™ kits was observed in 99.7% of the STR allele calls compared (15). Twenty-five of the 27 discordant results were due to allele dropout. Two discordant results in the Identifiler® kit amplicon outside of the Minifiler™ kit primer binding region were due to a base insertion in one sample and a five base deletion in the other. The population set was also analyzed for peak height ratios as shown on Table 7. The mean peak height ratios indicate that the two alleles of a heterozygous individual are generally very well balanced. However, occasional low peak heights ratios were observed as outlying data points as shown in the column labeled as "Minimum" on Table 7.

Discussion

The MiniFiler™ PCR amplification kit simultaneously amplifies eight miniSTR loci and the sex determining marker amelogenin, making it a highly discriminating STR detection system for human identification. The combination of five-dye chemistry (6-FAM™, NED™, PET®, VIC®, and LIZ® dyes) and the inclusion of nonnucleotide linkers made it technologically possible to simultaneously amplify all loci in a single PCR. Because the primers for the MiniFiler™ loci were redesigned from the conventional STR kits to yield smaller amplicons, these loci may be typeable with the MiniFiler™ kit when previously not possible. Additionally, as shown in Table 3, the MiniFiler™ kit is more discriminating than a partial profile with the Identifiler® kit (minus the MiniFiler™ loci). Thus, a forensic analyst has alternatives in deciding which multiplex system to use, especially when confronted with a compromised DNA sample that yields sufficient DNA only for a single PCR. If there is sufficient DNA for multiple PCRs but the DNA is sufficiently degraded, then the combination of the results that may be derived from a partial Identifiler® or SGM Plus® kit profile and a MiniFiler™ kit profile may notably increase the discrimination power of the STR profile.

The validation of the MiniFiler™ kit encompassed the verification of the best reaction conditions and reagent concentrations for the amplification of pristine as well as compromised DNA. The performance criteria included overall peak heights, peak height ratios, intracolor peak balance, and lack of cross-reactive peaks in the presence of nonhuman DNA. The thermal cycling parameters of the MiniFiler™ kit differ from other autosomal AmpFℓSTR® kits in three ways: (1) the number of amplification cycles (30 cycles), (2) the denaturation time (20 sec), and (3) annealing time (2 min). These changes enhanced sensitivity for detection of small amounts of DNA in the presence of inhibitors of the PCR. The sensitivity studies demonstrated that an input amount of 500 pg of DNA does not produce off-scale peaks while yielding enough signal strength for reproducible detection of 125 pg or less of template DNA (Fig. 5). The DNA mixture study (Table 6) demonstrates that the MiniFiler™ kit is capable of producing robust profiles for the minor contributor even at ratios of 1:10.

In summary, the results from the models of PCR degradation and inhibition and nonprobative samples demonstrate that the MiniFiler™ kit can be extremely useful for amplifying DNA under conditions where other commercial autosomal STR kits yield partial or no profiles. As such, this kit is a useful and robust complement to conventional STR kits and will be especially applicable to challenging situations involving the identification of human remains.

Finally, Applied Biosystems performed the developmental validation studies described in this paper. It is recommended that each laboratory conduct its own internal validation according to FBI/National Standards and SWGDAM guidelines (16,17).

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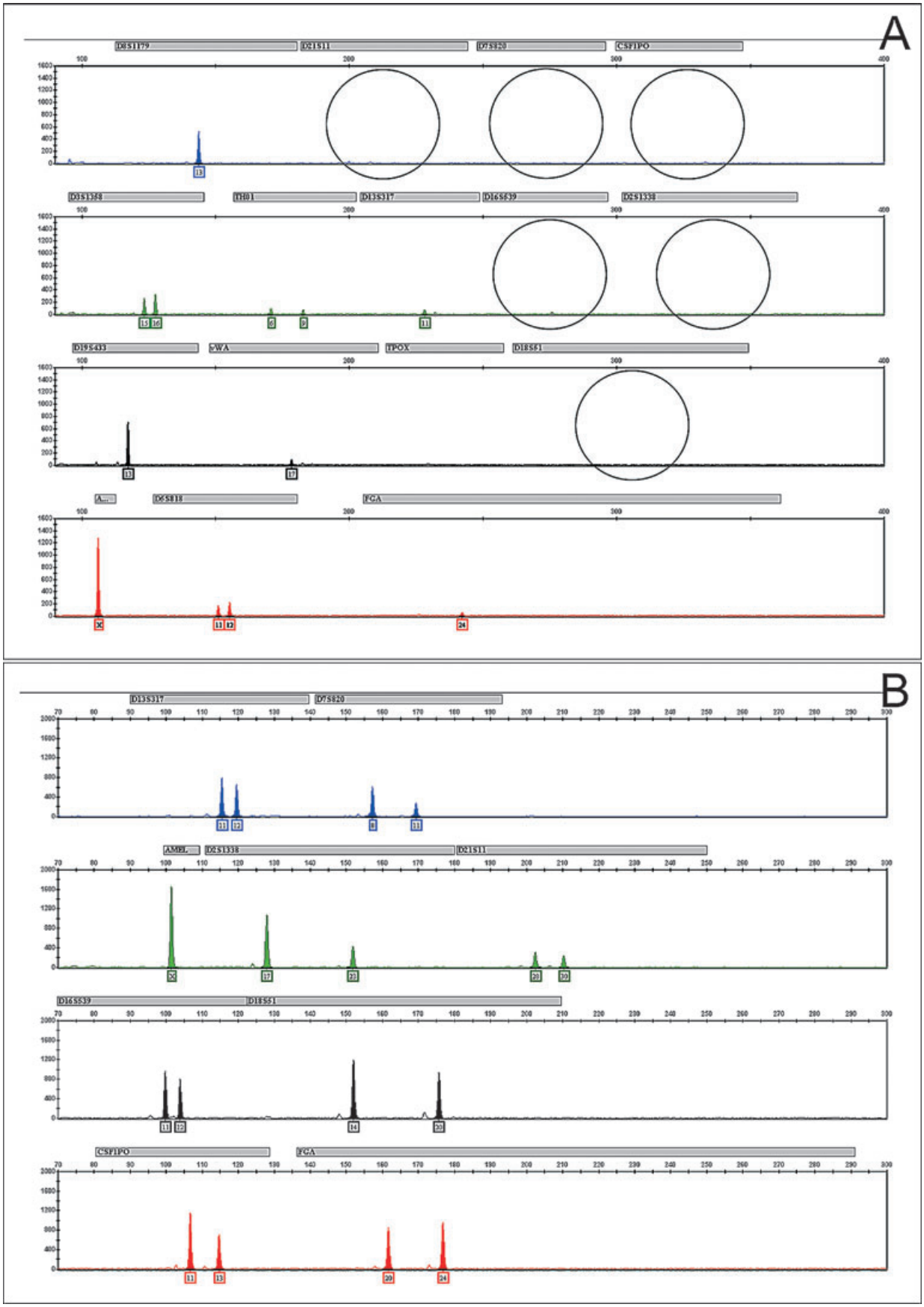

FIG. 9—*Results from the amplification of 1 ng of degraded DNA isolated from a formalin fixed paraffin-embedded breast tumor tissue sample from 1997. (A) Identifiler® kit (modified to 30 cycles of amplification) and (B) MiniFiler™ kit electropherograms. The circles in panel A identify the loci missing from the Identifiler® kit but amplified by the MiniFiler™ kit.*

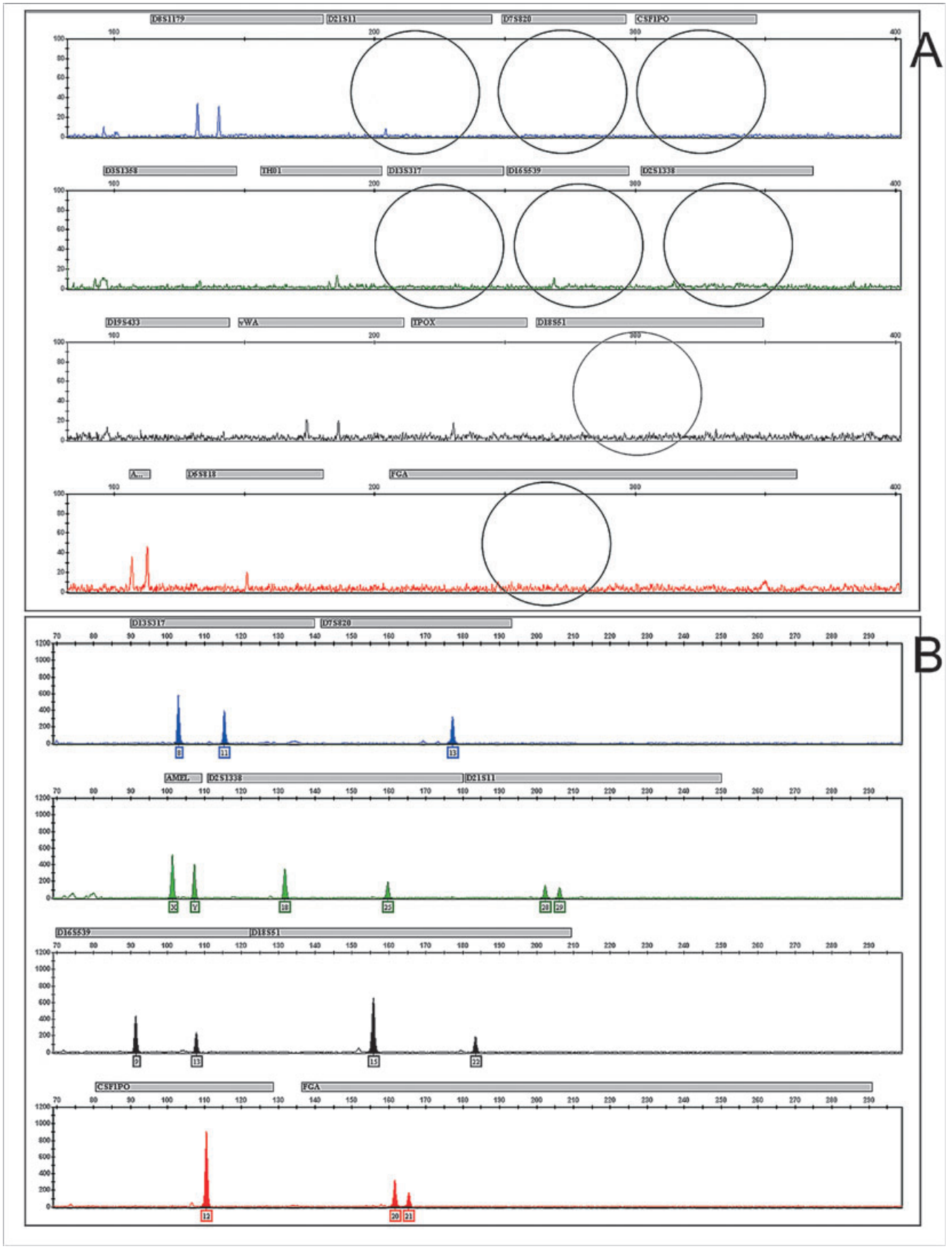

FIG. 10—*Results from the amplification of 1 ng of degraded DNA extracted from a skeletal remain. (A) Identifiler® kit and (B) MiniFiler™ kit electropherograms. The circles in panel A identify the loci missing from the Identifiler® kit but amplified by the MiniFiler™ kit.*

TABLE 6—*DNA mixture study.*

Mixture Ratio	Genotype	D13S317	D7S820	D2S1338	D21S11*	D16S539	D18S51	CSF1PO*	FGA
0:1	Major GT	11	7,12	20,23	28,31	9,10	12,15	11,12	24,26
1:0	Minor GT	12,14	8,9	20,21	28,30	12,13	17,19	10	21,22
1:1	Minor GT	12,14	8,9	21	30	12,13	17,19	10	21,22
1:1	Minor GT	12,14	8,9	21	30	12,13	17,19	10	21,22
1:3	Minor GT	12,14	8,9	21	30	12,13	17,19	10	21,22
1:3	Minor GT	12,14	8,9	21	30	12,13	17,19	10	21,22
1:7	Minor GT	12,14	8,9	21	30	12,13	17,19	10	21,22
1:7	Minor GT	12,14	8,9	21	30	12,13	17,19	10	21,22
1:10	Minor GT	12,14	8,9	21	30	12,13	17,19	10	21,22
1:10	Minor GT	12,14	8,9	21	30	12,13	17,19	10	21,22
1:15	Minor GT	12,14	8,9	21	30	12,13	17,19	10	21,22
1:15	Minor GT	12,14	9	21	30	12,13	19	10	21,22

Major GT, major component genotype; Minor GT, minor component genotype.

Minor component allele calls at nonoverlapping STR loci from duplicate mixture amplifications. Detected genotype of minor component using a peak amplitude threshold of 50 RFU.

*Stutter peak present, which may mask allele.

TABLE 7—*Peak height ratio for eight loci obtained from genotyped population samples (input DNA = 500 pg).*

Allele	Number of Observations (*n*)	Mean	Median	SD	Minimum	Maximum
CSF1PO	781	87.9	89.3	29.8	57.9	100
D2S1338	911	87.0	88.8	33.8	52.3	100
D7S820	820	88.1	89.7	29.4	58.4	100
D13S317	733	88.4	90.4	35.0	50.5	100
D16S539	804	87.5	89.1	38.1	46.1	100
D18S51	906	87.9	89.2	31.7	55.1	100
D21S11	856	88.2	90.0	37.4	47.1	100
FGA	904	88.0	89.4	32.6	53.9	100

Peak height ratios were determined only for those heterozygous samples with peak heights >200 RFUs.

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