TECHNICAL NOTE

Katherine A. Micka,[1] B.S.; Elizabeth A. Amiott,[1,2] B.S.; Tara L. Hockenberry,[2] B.S.; Cynthia J. Sprecher,[1] B.S.; Ann M. Lins,[1] B.S.; Dawn R. Rabbach,[1] A.A.S.; Jennifer A. Taylor,[1] B.S.; Jeffrey W. Bacher,[1] Ph.D.; Debra E. Glidewell,[2] B.S.; Sandra D. Gibson,[2] B.S.; Cecelia A. Crouse,[2] Ph.D.; and James W. Schumm,[1] Ph.D.

TWGDAM Validation of a Nine-Locus and a Four-Locus Fluorescent STR Multiplex System*

REFERENCE: Micka KA, Amiott EA, Hockenberry TL, Sprecher CJ, Lins AM, Rabbach DR, Taylor JA, Bacher JW, Glidewell DE, Gibson SD, Crouse CA, Schumm JW. TWGDAM validation of a nine-locus and a four-locus fluorescent STR multiplex system. J Forensic Sci 1999;44(6):1243–1257.

ABSTRACT: The *Gene Print*® PowerPlex™ 1.1/Amelogenin and FFFL Fluorescent STR Systems have been validated following the recommendations presented by the Technical Working Group on DNA Analysis Methods (TWGDAM). The PowerPlex™ 1.1/Amelogenin System supports simultaneous amplification of eight short tandem repeat loci and the Amelogenin gender identification marker. The loci D16S539, D7S820, D13S317, and D5S818 are labeled with fluorescein (FL) while the loci CSF1PO, TP0X, TH01, vWA and Amelogenin are labeled with carboxy-tetramethylrhodamine (TMR). The FFFL Multiplex System is composed of the loci F13A01, FESFPS, F13B, and LPL, each labeled with fluorescein. We have observed no overlap of alleles across loci labeled with an individual fluorescent dye. Samples of each system were amplified and labeled in a single reaction, separated by electrophoresis through a denaturing polyacrylamide gel, and amplified alleles detected using a Hitachi FMBIO® Fluorescent Scanner. Alterations from the standard amplification protocols in cycle number and annealing temperature generally produced excellent results. In experiments testing sensitivity as little as 0.2 ng of DNA template could be detected. As expected, different body fluids from the same individuals generated identical DNA profile results. Template DNA derived from bloodstrains deposited on a variety of matrix supports displayed robust amplification except for material derived from deposits on wood and Japanese orchid leaves. Mixtures of DNA templates could be interpreted with the minor component present in as little as ten percent of the total sample. Monoplex and multiplex amplifications produced identical amplified allele patterns, indicating that STR multiplex systems save template and increase efficiency in the amplification procedure without loss of quality. Analyses of genotype frequencies in African-American, Caucasian-American and Hispanic-American populations using all twelve loci were used to determine matching probabilities smaller than 1 in 1.14×10^8 and 1 in 2658 for the PowerPlex™ 1.1 and the FFFL Multiplex Systems, respectively. The matching probability achieved with the two systems combined is smaller than 1 in 3.03×10^{11}. The independence of alleles within loci was generally demonstrated by applying the exact test to demonstrate Hardy-Weinberg Equilibrium. All of the studies performed indicate that the PowerPlex™ 1.1/Amelogenin and FFFL Multiplex Systems are powerful, robust, and reliable investigative tools that can be used in the analysis of forensic samples.

KEYWORDS: forensic science, short tandem repeat, polymerase chain reaction, validation, TWGDAM, PowerPlex, FFFL, microsatellite, polymorphism, D16S539, D7S820, D13S317, D5SA818, CSF1PO, TPOX, THO1, VWA, Amelogenin, F13A01, FESFPS, F13B, LPL

The use of short tandem repeat (STR) loci in forensic and paternity laboratories (1–3) has become a preferred alternative to use of restriction fragment length polymorphism (RFLP) analysis (4). The excellent discrimination provided by RFLP analysis is offset by a number of disadvantages. For example, more sample material is required, degraded samples are less likely to provide reliable results and completion of the analytical methods consumes more time. The analysis of STR loci utilizes the polymerase chain reaction (PCR) which requires as little as 0.2 ng of template and can provide rapid results, often in a matter of hours. Another advantage of the STR approach is the ability to obtain significant power of discrimination through the use of multiplex systems.

In this work two such systems, the *GenePrint*® PowerPlex™ 1.1/Amelogenin and the *GenePrint*® FFFL Fluorescent Multiplex Systems have been validated according to the guidelines provided by the TWGDAM, Technical Working Group on DNA Analysis Methods (5). The *GenePrint*® PowerPlex™ 1.1/Amelogenin System amplifies nine loci simultaneously while the *GenePrint*® FFFL Fluorescent Multiplex System amplifies four loci at once (1–3,6–10). Performance of both STR multiplex systems under conditions set forth by TWGDAM was robust and reproducible, indicating that these systems are suitable for use in forensic analyses.

Materials and Methods

DNA Extraction and Quantification

Genomic DNA was isolated using a variety of methods. Two human genomic DNA samples (labeled DNA #1 and DNA #2) were

[1] Promega Corporation, Madison, WI.

[2] Palm Beach County Sheriff's Office Crime Lab, West Palm Beach, FL.

* Portions of this work were presented at the Eighth International Symposium on Human Identification, 17–20 September 1997, Scottsdale, Arizona and at the AAFS 50th Annual Scientific Meeting, 9–14 February 1998, San Francisco, California.

Received 12 June 1998; and in revised form 2 Dec. 1998, 15 March 1999; accepted 16 March 1999.

extracted using an organic phenol/chloroform procedure (11). The DNA from forensic specimens was extracted using the one-step organic extraction method of Comey et al. (12). Sperm DNA was separated from vaginal epithelial cells using a differential extraction protocol followed by a one-step phenol chloroform extraction. The DNA from cell lines CCRF-SB, Raji and CCRE-CEM (ATCC, Manassas, VA) was isolated using the salt precipitation method of Miller et al. (13). Extracted DNA from the cell line K562 was obtained from Promega Corporation (Madison, WI) and DNAs from the cell lines GM09947A and GM09948 were obtained from Life Technologies, Inc. (Gaithersburg, MD). Standard Reference Material 2391 used in the interlaboratory comparison was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD). With the exception of DNA#1, DNA#2, cell line DNAs and the Standard Reference Material 2391 all remaining DNA samples were concentrated using Microcon® devices (Millipore Corp., Bedford, MA). Samples were quantified using dot blot hybridization with a primate-specific alkaline phosphatase-conjugated probe complementary to the repeated sequence of locus D17Z1 as described by Waye et al. (14).

PowerPlex™ 1.1 PCR Amplification

Amplification was performed using the reagents contained in the PowerPlex™ 1.1 and Amelogenin STR Systems according to manufacturer's instructions in technical manual TMD008 (15). The amount of DNA template amplified is described for each experiment. Experiments including *Taq* DNA Polymerase (i.e., AmpliTaq™, Perkin Elmer, Foster City, CA) or AmpliTaq Gold® DNA Polymerase (Perkin Elmer, Foster City, CA), are indicated as appropriate. Unless noted, experiments using AmpliTaq Gold® were completed using Gold ST★R 10X Buffer (500 mM KCl, 100 mM Tris, 15 mM $MgCl_2$, 1% Triton, 2 mM of each dNTP, 1600 ug/mL BSA, pH 8.3, Promega Corporation, Madison, WI). The cycling conditions for the GeneAmp® PCR System 9600 Thermal Cycler were: One cycle of 96°C for 1 min; then 10 cycles of 94° for 30 s, ramp 68 s to 60°C for 30 s, ramp 50 s to 70°C for 45 s; then 20 cycles of 90° for 30 s, ramp 60 s to 60°C for 30 s, ramp 50 s to 70°C for 45 s; and then 1 cycle of 60°C for 30 min. The following cycling parameters were used with the Model 480 Thermal Cycler: One cycle of 96°C for 2 min; then 10 cycles of 94°C for 1 min, 60°C for 1 min, 70°C for 1.5 min; then 20 cycles of 90°C for 1 min, 60°C for 1 min, 70°C for 1.5 min; followed by 1 cycle at 60°C for 30 min. An incubation of 11 min at 95°C was included prior to the standard cycling protocol when AmpliTaq Gold® was employed.

FFFL PCR Amplification

The FFFL Multiplex System amplification procedure in the *GenePrint*® Fluorescent STR Systems technical manual (16) was followed as per manufacturer with the exception that Gold ST★R 10X Buffer and AmpliTaq Gold® were used in the amplification reactions. The cycling profile for the GeneAmp® PCR System 9600 Thermal Cycler was: 1 cycle at 95°C for 11 min; 1 cycle of 2 min at 96°C; 10 cycles of ramp 50 s to 94°C for 1 min, ramp 34 s to 60°C for 1 min, ramp 25 s to 70°C for 1.5 min; then 22 cycles of ramp 45 s to 90°C for 1 min, ramp 30 s to 60°C for 1 min, ramp 25 s to 70°C for 1.5 min; and then 1 cycle at 60°C for 30 min. The cycling parameters for the Model 480 Thermal Cycler were: 1 cycle at 95°C for 11 min; 1 cycle of 2 min at 96°C; 10 cycles of 94°C for 1 min, 60°C for 1 min, 70°C for 1.5 min; then 22 cycles of 90°C for 1 min, 60°C for 1 min, 70°C for 1.5 min; and then 1 cycle at 60°C for 30 min.

Gel Preparation, Electrophoresis, and Detection

Two methods of gel preparation were employed during the validation study. One method is a technique developed at Promega Corporation that allows for the reuse of gels using the following protocol (17). A stock solution of acrylamide was prepared as 4% acrylamide:bis (19:1), 7 M urea, 0.5X TBE (0.1 M Tris base, 1 mM EDTA, 44 mM boric acid at a pH 8.2). The glass plates were prepared by treating both plates with γ-methacryloxypropyltrimethoxysilane (bind silane) (Sigma, St. Louis, MO). A 4% denaturing polyacrylamide gel was then poured using 0.4 mm spacers and a 0.4 mm 30 well square tooth comb (Owl Scientific, Woburn, MA) and allowed to polymerize for a minimum of 1 h. The gel was then placed on an SA43 vertical electrophoresis apparatus (Life Technologies, Inc., Gaithersburg, MD) and the gels were prerun at 60 W until a plate temperature of 45° to 50°C was obtained, (i.e., approximately 30–45 min). Amplified products were prepared either including or not including the Fluorescent Ladder (CXR), 60–400 Bases (Promega Corporation, Madison, WI) as a molecular weight standard. The samples were prepared for electrophoresis by mixing 2 μL of the amplified sample or allelic ladder with 1 μL of the Fluorescent Ladder CXR and 3 μL of Bromophenol Blue Loading Solution (95% formamide, 0.05% bromophenol blue, 10 mM NaOH) (Promega Corporation, Madison, WI). The samples were then denatured at 95°C for 2 min and then chilled on ice for 2 min. Three μL of the denatured samples were then loaded onto the gel and the gel was run at 60 W for 1 h. The PowerPlex™ 1.1/Amelogenin or FFFL allelic ladders were routinely included on each gel. Post-electrophoresis, amplified products were detected using the Hitachi FMBIO® II Fluorescent Scanner (Hitachi Software Engineering, Ltd., So. San Francisco, CA). If the gels were to be reused the same day the electrophoresis tank buffer was replaced and the samples were removed from the gel by reverse electrophoresis for an additional 15–30 min longer than the previous forward electrophoresis time. This concurrently serves as the prerun for the next gel run. If the gels were stored overnight, paper towels saturated with a solution of 0.5X TBE with 7M urea were placed over the well area and the bottom of the gel then covered with plastic wrap. The gels were reused up to four times with similar gel resolution being observed in all runs (17).

The second method of gel preparation utilized the 4.5% R³™ Precast Gels (Hitachi Software Engineering, Ltd., So. San Francisco, CA) and entailed the same sample preparation method as mentioned above. These gels were not subjected to pre-electrophoresis and were run at 30 W for 90 min. All precast gels were scanned using the Hitachi FMBIO® 100 or II Fluorescent Scanner (Hitachi Software Engineering, Ltd., So. San Francisco, CA). Results were analyzed either manually or using the STaR Call™ Allele Calling Software available with the Hitachi FMBIO® Fluorescent Scanner.

Stutter Band Determination

Analysis of the percent stutter for each of the 12 loci (18,19) was conducted using 1 ng of DNA from each of 20 human samples. The samples were then separated on an ABI PRISM™ 377 DNA Sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, CA) and analyzed using the GeneScan® Analysis Software. To calculate percent stutter, the peak height of the stutter band (i.e., that fragment observed four bases smaller than the major fragment) was divided by the peak height generated by the true allele, then multiplied by 100.

Results and Discussion

The following studies were performed according to guidelines defined by TWGDAM (5) to validate the PowerPlex™ 1.1/Amelogenin and FFFL Multiplex Systems. Unless otherwise stated, results described were obtained with both multiplex systems. Numerical headings refer to specific validation sections within the TWGDAM Guideline Manual.

4.1.5.1 Standard Specimens and 4.1.5.4 Reproducibility

Identical genotypes were obtained from the amplified products of DNA samples obtained from blood and semen from the same individual. Similarly, identical genotypes were obtained from liquid blood and dried bloodstains prepared from the same individuals. Five paired sets were compared in each experiment. For the PowerPlex™ 1.1/Amelogenin System, 2.5 ng of each template was amplified and for the FFFL Multiplex System, 1.2 ng of each template was amplified (data not shown).

4.1.5.2 Consistency

A standard set of eight DNA templates available from the National Institute of Standards and Technology (NIST) and DNA from cell lines K562, GM09947A, and GM09948 were amplified in two separate laboratories using 2 ng template with each multiplex. Alleles were identified visually or employing the STaR Call™ Allele Calling Software available with the Hitachi FMBIO® II Fluorescent Scanner. Both laboratories obtained identical genotypes and similar amplified fragment intensities compared to standard amounts of allelic ladder for all loci in each sample (data not shown).

4.1.5.3 Population Studies

Allele frequencies for three different population groups (African-American, Caucasian-American, Hispanic-American) were studied using the 12 loci present in the PowerPlex™ 1.1/Amelogenin and FFFL Multiplex Systems. These data, and the corresponding matching probabilities, have been previously reported (20). The matching probabilities for each multiplex and the two multiplexes combined (i.e., 12 loci) are displayed in Table 1. The exact test for independence of alleles within individuals revealed only one significant deviation from independence in the 36 data sets evaluated (20), i.e., the D13S317 locus in the Caucasian-American database. As 1.8 deviations would be expected on average in 36 evaluations, this result is consistent with no departure from Hardy-Weinberg equilibrium for the 12 loci tested.

4.1.5.5 Mixed Specimen Studies

Two DNA samples (DNA #1 and DNA #2) were mixed in the following ratios for a final template amount of 2 ng: 100:0, 97.5:2.5, 95:5, 90:10, 80:20, 50:50, 20:80, 10:90, 5:95, 2.5:97.5, and 0:100. Following amplification and gel separation of the amplified fragments, specific amplified fragments derived from each sample were generally observed in proportion to the presence of that DNA sample in the mixture (Fig. 1). In general, the band(s) contributed by the minor component could be routinely detected at all 12 loci when present as 20% of the mixture. A mixture was interpretable at some loci with only 10% of the minor component present in the mixture. This result is consistent with the sensitivity of the systems defined at approximately 0.2 ng in section 4.1.5.10, below.

4.1.5.6 Environmental Studies

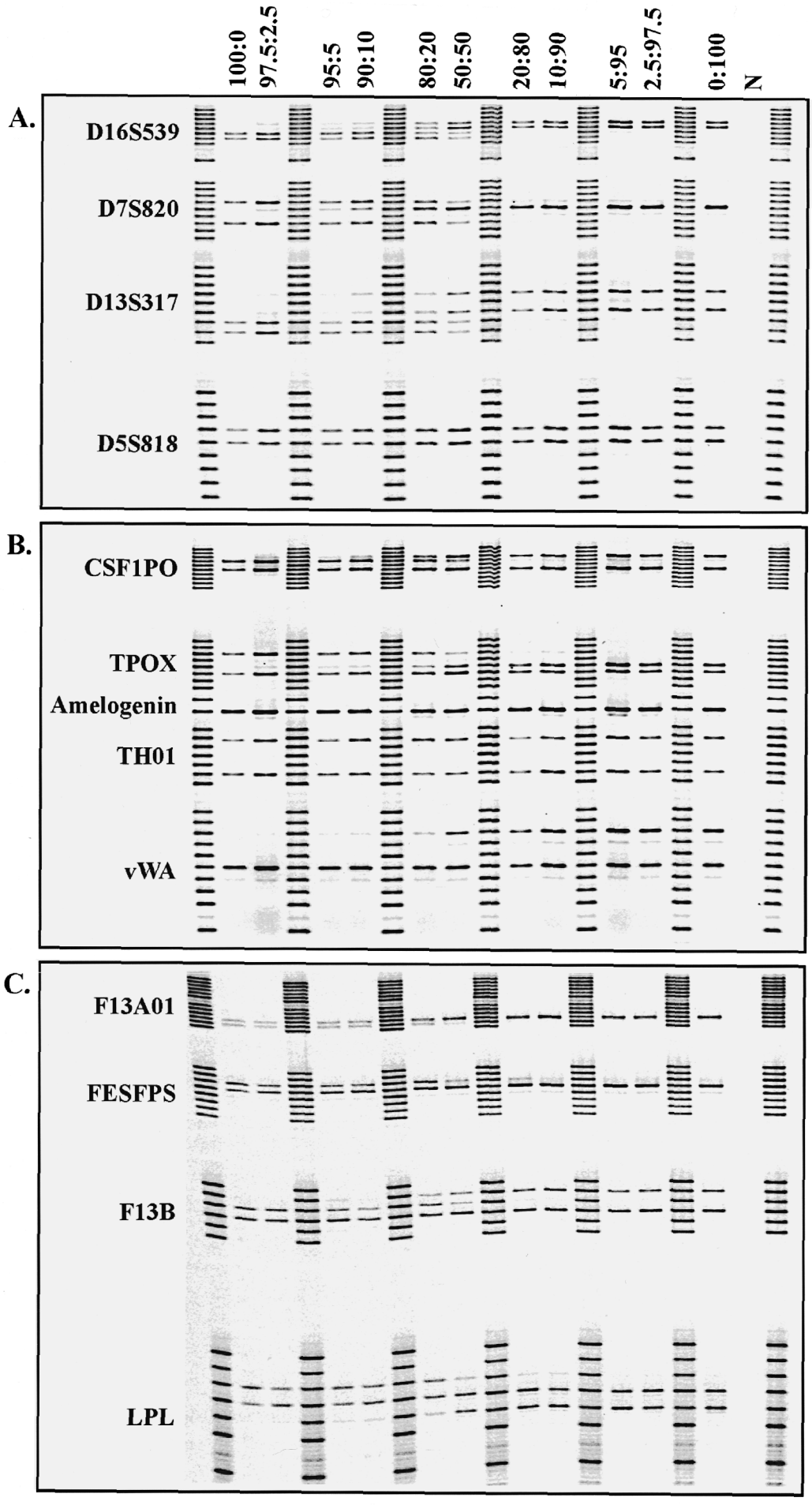
DNA samples derived from bloodstains exposed to sunlight and the environment were evaluated using both the PowerPlex™ 1.1/Amelogenin and FFFL Multiplex Systems. To reproduce ultraviolet exposure (UV) and humid conditions that can affect the integrity of forensic samples, bloodstains from a single known donor were prepared on white cotton cloth and placed (along with unstained negative controls) in a glass container near a canal during mid-summer in southern Florida. The glass lid of the container was perforated so that the samples were exposed to both UV and changes in humidity. Daily environmental conditions of temperature, rainfall, and humidity were recorded throughout the experiment and samples were collected at time intervals of 0 (positive control sample), 1, 3, 5, 7, 14, and 28 days. Approximately 0.5 to 1.5 ng of template DNA isolated from the bloodstains was used for amplification. Samples collected at 0 days through 7 days had identical genotypes and similar product yield at each locus in both systems (Fig. 2). After 14 days of environmental exposure, decreased yield was observed for the larger amplification products (i.e., the loci CSF1PO, D16S539, and F13A01), suggesting degradation or damage to the DNA derived from these samples. No amplification result was observed for any locus of either multiplex system with the DNA extracted from bloodstains collected after 28 days of environmental exposure.

4.1.5.7 Matrix Studies

Possible degradation of sample material or inhibition of amplification due to contact with various matrix supports was tested. Liquid blood was deposited on a variety of cloth materials (100% cotton, 100% polyester, 50% polyester, 100% nylon, 100% rayon, a 65% polyester/35% cotton blend, an acetate/lycra blend, pantyhose, leather, light blue denim and dark blue denim) and common household materials (carpet, sponge, cardboard, plastic wrap, bandage, latex, rubber, vehicle paint, dry wall, metal, paper bag, plastic bag, and a plastic jug). In addition, liquid blood was added to a cotton cloth treated with various substances (bleach, Tide®, Tilex®, diazanon, alkaline, vinegar, soil, grass) and to various plant materials (wood and the leaves of a Japanese orchid and dead sea grape). DNA was also extracted from saliva deposited on an envelope and stamp, respectively. Approximately 1-2 ng of DNA isolated from the various bloodstains or saliva was used for each amplification. Complete and correct DNA pro-

TABLE 1—*Matching probability*.

	African-American	Caucasian-American	Hispanic-American
PowerPlex™ System	1 in 2.74×10^8	1 in 1.14×10^8	1 in 1.45×10^8
FFFL Multiplex	1 in 16802	1 in 2658	1 in 3276
All 12 Loci	1 in 4.61×10^{12}	1 in 3.03×10^{11}	1 in 4.75×10^{11}


100:0
97.5:2.5
95:5
90:10
80:20
50:50
20:80
10:90
5:95
2.5:97.5
0:100
N
A.
D16S539
D7S820
D13S317
D5S818
B.
CSF1PO
TPOX
Amelogenin
TH01
vWA
C.
F13A01
FESFPS
F13B
LPL

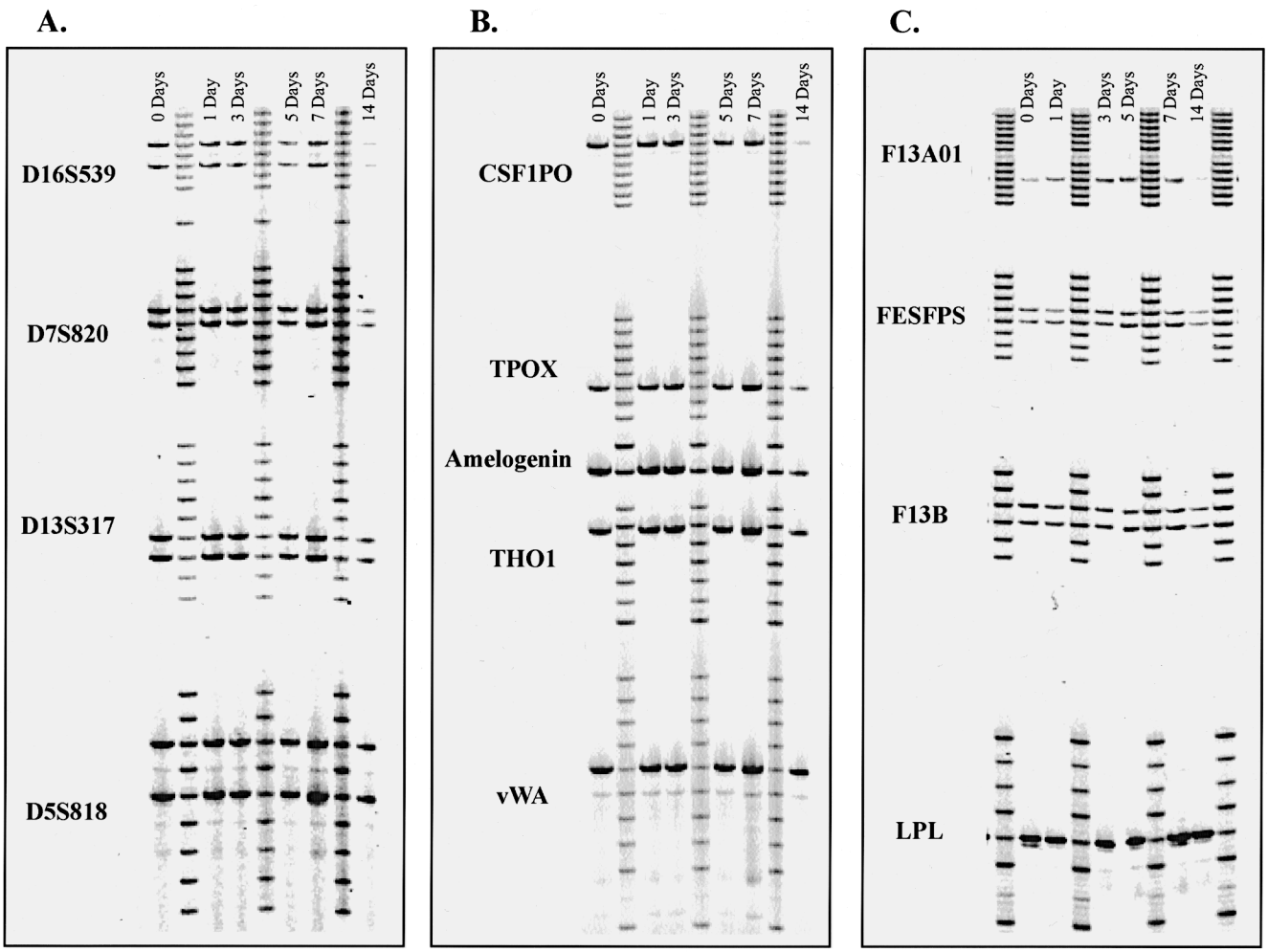


FIG. 2—*Effects of environmental exposure on bloodstains over time. Multiplex amplification and electrophoresis is shown for DNA derived from bloodstains exposed to sunlight and environmental conditions for 0, 1, 3, 5, 7, and 14 days. No amplification products were detected at 28 days of environmental exposure. Amplifications were performed in the GeneAmp® PCR System 9600 Thermal Cycler using AmpliTaq Gold® and Gold ST★R Buffer. The amplified products were separated by electrophoresis in a 4.5% denaturing polyacrylamide gel and detected using the Hitachi FMBIO® 100 Fluorescent Scanner. Panel A displays the 505 nm scan which reveals the fluorescein-labeled products of the PowerPlex™ 1.1/Amelogenin System. Panel B displays the 585 nm scan which reveals the TMR-labeled products of the PowerPlex™ 1.1/Amelogenin System. Panel C is a 505 nm scan which displays the fluorescein-labeled products of the FFFL Multiplex System. Allelic Ladders were subjected to electrophoresis adjacent to each sample for their respective systems.*

files were obtained from nearly all matrix substrates. The following exceptions were observed. Results suggest that degradation of template or inhibition of PCR affected amplification of higher molecular weight fragments (loci CSF1PO, D16S539, and F13A01) when the blood samples were in contact with wood and Japanese orchid leaves. Also, PCR inhibition was observed when blood was mixed with soil as evidenced by reduced amplified product yield for the loci CSF1PO, D16S539, D7S820 and D13S317 in the PowerPlex™ 1.1/Amelogenin System and the loci FESFPS and F13B in the FFFL Multiplex System (data not shown).

Amplification of DNA recovered from the stamp and envelope

FIG. 1—*Mixed specimen studies. DNA Samples 1 and 2 were mixed in the following ratios of DNA#1:DNA#2; 100:0, 97.5:2.5, 95:5, 90:10, 80:20, 50:50, 20:80, 10:90, 5:95, 2.5:97.5, and 0:100. A negative control (containing no DNA template) is in the lane marked N. The corresponding allelic ladders are included in every third lane. The PowerPlex™ 1.1/Amelogenin amplifications were performed with AmpliTaq Gold®, GeneAmp® PCR Buffer, and the GeneAmp® PCR System 9600 Thermal Cycler as described. Amplifications with the FFFL System were completed in the GeneAmp® 9600 Thermal Cycler using AmpliTaq Gold® and Gold ST★R Buffer. All amplified products were separated by electrophoresis in a 4% denaturing polyacrylamide gel and detected using the Hitachi FMBIO® II Fluorescent Scanner. Panel A displays the 505 nm scan of the PowerPlex™ 1.1/Amelogenin System revealing the fluorescein-labeled products of four loci. Panel B displays the 585 nm scan revealing the TMR-labeled products of five loci. Panel C displays the 505 nm scan of the FFFL Multiplex System revealing the fluorescein-labeled products. A 650 nm scan illustrating the CXR-labeled ILS used for size determination is not shown.*

was attempted using only the PowerPlex™ 1.1/Amelogenin System due to low DNA template recovery (less than 0.15 ng/uL). For the envelope sample, a very weak signal was observed for products of the loci CSF1PO, TPOX, TH01, vWA, and Amelogenin, while those of other loci were not detected. No amplification products were detected from the stamp sample.

4.1.5.8 Nonprobative Evidence

Four nonprobative cases were examined using the PowerPlex™ 1.1/Amelogenin and FFFL Multiplex Systems. The amount of template DNA amplified in all cases ranged from 0.15 ng to 5 ng. All cases had previously been analyzed with the AmpliType® PM and DQA1 PCR Amplification Kit and the GenePrint™ STR Multiplex System CSF1PO, TPOX, and TH01. All initial interpretations for inclusions were maintained after PowerPlex™ 1.1/Amelogenin and FFFL Multiplex System analysis.

Case 1 was a homicide that included a victim reference standard, a suspect reference standard, a bloodstain on a hat and a bloodstain found on a pair of jeans belonging to the suspect (Fig. 3). The DNA profile obtained from the hat and suspect's jeans was consistent with the DNA profile from the victim at all twelve genetic markers. The suspect was excluded as a contributor of either of the bloodstains.

Case 2 was a homicide that included a victim reference standard, a suspect reference standard, and two swabs taken from a trail of blood found at the crime scene. The two blood swabs matched the suspect reference sample profile at all PowerPlex™ 1.1/Amelogenin System loci and all FFFL Multiplex System loci (data not shown).

Case 3 included a victim reference standard, a suspect reference standard, and semen found on a towel from a sexual battery offense. The genotype profile from the semen stain matched the suspect reference standard at all 12 STR loci and the locus Amelogenin, thereby including the suspect as the probable source of the stain on the towel. The profile from the semen stain was not consistent with the genotype profile of the victim (data not shown).

Case 4 was a sexual battery case that included victim and sus-

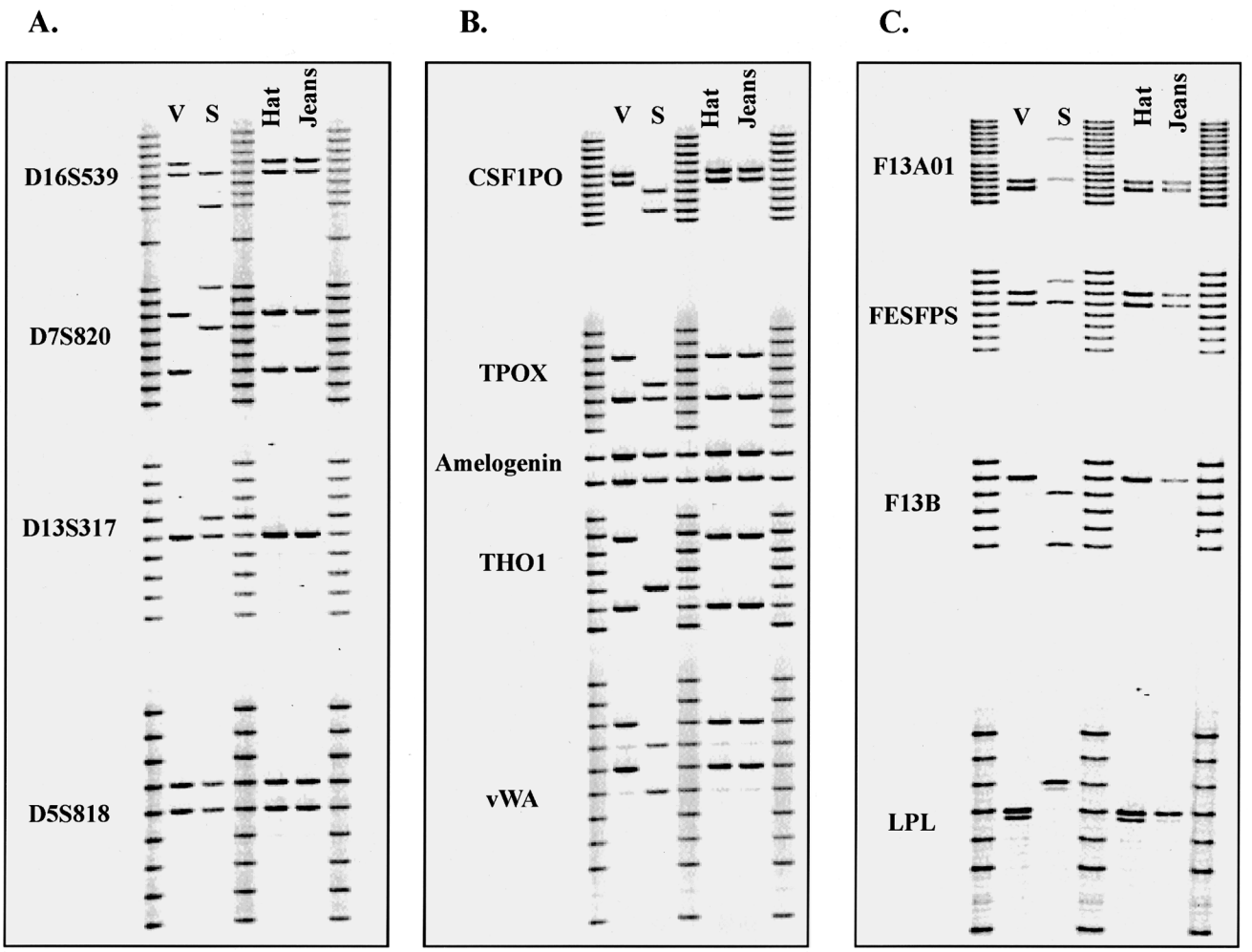


FIG. 3—*Nonprobative Case 1. Amplification and electrophoresis results from a nonprobative homicide case are displayed. In each panel, the first two sample lanes represent the victim (V) and suspect (S) reference standards, respectively, and the remaining sample lanes show the results from evidentiary bloodstains found on a hat and a pair of jeans belonging to the suspect. Amplifications were performed in the GeneAmp® PCR System 9600 Thermal Cycler using AmpliTaq Gold® and Gold ST★R Buffer. The PowerPlex™ 1.1/Amelogenin System amplified products were separated by electrophoresis in a 4.5% denaturing polyacrylamide gel and detected using the Hitachi FMBIO® 100 Fluorescent Scanner. The FFFL Multiplex System amplified products were separated by electrophoresis in a 4% denaturing polyacrylamide gel and detected using the Hitachi FMBIO® II Fluorescent Scanner. Panel A displays the fluorescein-labeled products of the PowerPlex™ 1.1/Amelogenin System. Panel B displays the TMR-labeled products of the PowerPlex™ 1.1/Amelogenin System. Panel C displays the fluorescein-labeled products of the FFFL Multiplex System.*

pect reference standards and a vaginal swab taken from the victim (Fig. 4). A differential extraction was performed on the vaginal swab to separate the sample into epithelial and sperm fractions. The profile obtained from the epithelial fraction of the vaginal swab indicates a mixture of more than one DNA source. Upon evaluation it was determined that each allele present in the epithelial fraction was consistent with a mixture of the genotypes from the victim and suspect reference samples. The DNA profile from the sperm fraction is consistent with the suspect at all twelve genetic markers. In addition, extremely weak bands are visible in the CSF1PO, TPOX, THO1 and vWA loci that are consistent with the genotype profile of the victim, suggesting that a very small concentration of epithelial DNA remained in the sperm fraction of the sample.

4.1.5.9 Nonhuman Studies

Several nonhuman sources of DNA were examined to determine the species specificity of the PowerPlex™ 1.1 and FFFL Multiplex Systems as well as the gender identification locus Amelogenin. Primate sources included: Rhesus monkey, Orangutan (4 sources), Chimpanzee (4 sources), and Gorilla (4 sources). Non-Primate sources included: dog, cat, pig, chicken, cow, horse, deer, mouse, rat, rabbit, raccoon, ferret, fox, lynx, otter, macaw, toad, frog, snake, shark, dolphin, white marlin, damsel fish and two species of bacteria (*Pseudomonas fluorescens* and *Enterobacter aerogenes*). All primate sources produced amplification products at several loci with both systems and Amelogenin. No detectable amplification products were observed for any of the non-primate species with the PowerPlex™ 1.1 or FFFL Multiplex Systems with the exception of rabbit DNA which produced an amplified fragment below the F13B locus. Also, some non-primate species (dog, raccoon, fox, ferret, and lynx) produced detectable fragments with the Amelogenin gender identification marker (data not shown). These results are consistent with previous studies of nonhuman species using the STR loci CSF1PO, TPOX, TH01, FESFPS, vWA and F13A01 described by Crouse et al. (21).

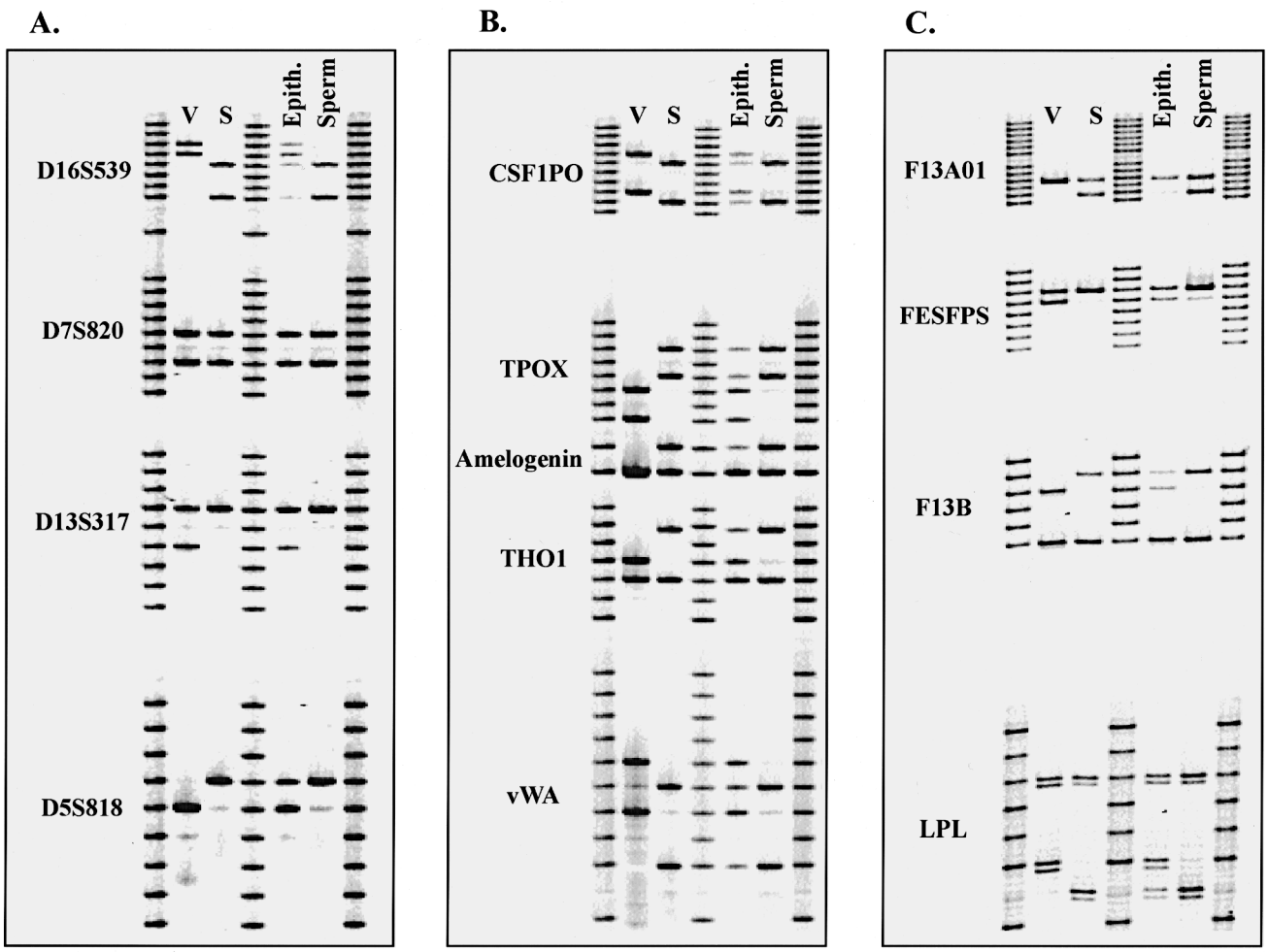


FIG. 4—*Nonprobative Case 4. Amplification and electrophoresis results from a nonprobative sexual battery case are illustrated. In each panel, labeled sample lanes represent the victim and suspect reference standards and the epithelial and sperm fractions differentially extracted from a vaginal swab, respectively. Amplifications and separations were performed as described in Fig. 3 for each system, respectively. Panel A displays the fluorescein-labeled products of the PowerPlex™ 1.1/Amelogenin System. Panel B displays the TMR-labeled products of the PowerPlex™ 1.1/Amelogenin System. Panel C displays the fluorescein-labeled products of the FFFL Multiplex System. Corresponding allelic ladders were subjected to electrophoresis adjacent to each sample for their respective systems. Note: The FFFL Allelic Ladder does not contain an LPL allele '8' but this allele can be assigned using the ILS and the STaRCall™ Allele Calling Software.*

4.1.5.10 Minimum Sample

The PowerPlex™ 1.1/Amelogenin Multiplex System was used to amplify DNA from cell lines CCRF-SB and Raji (Fig. 5). The amount of DNA template amplified was 25 ng, 10 ng, 5 ng, 2 ng, 1 ng, 0.5 ng, 0.2 ng, or 0.1 ng. Resulting profiles for each DNA template remained constant with varied amounts of template. Stutter bands (i.e., significantly less intense fragments which migrate one repeat length below the major fragment(s)) (18,19), were observed at several loci when 25 ng of template was amplified. Amplifications of samples with low DNA template amounts tend to reduce the intensity of the stutter bands. The amount of stutter fragment observed (as a fraction of the primary amplification product) differs among the loci and generally increases with the number of repeat units present in different alleles of a particular locus (22). Table 2 defines the average, highest and lowest fraction of stutter observed for alleles in each individual locus. When using these multiplex systems, the phenomenon of stutter must be considered in the development of interpretation guidelines for allele designation and analysis of mixed samples.

The phenomenon of non-template directed terminal nucleotide addition (23,24) has been previously described for the vWA locus when *Taq* DNA Polymerase was employed in the PCR amplification. The incomplete nature of this process can lead to production of two bands, one with the added nucleotide and one without. With the PowerPlex™ 1.1/Amelogenin System, this phenomenon has been minimized by primer design and the inclusion of the final 30 min extension at 60°C (22) in the amplification protocol. These adjustments increase the frequency of full terminal nucleotide addition to nearly 100%.

With the PowerPlex™ 1.1/Amelogenin Multiplex System, an artifact band of approximately 208 bases can be observed between the TH01 and Amelogenin allelic ladders when *Taq* DNA Polymerase is used (Fig. 5). This band is generally seen only when more than 2 ng of DNA template is amplified. When AmpliTaq Gold® is

TABLE 2—*Stutter observed as a percentage of associated full-length amplified allele.*

Locus	Average (%)	Highest (%)	Lowest (%)
CSF1PO	4.1	9.5	0.0
TPOX	2.5	5.6	0.0
THO1	1.7	5.2	0.0
vWA	6.6	11.4	3.7
D16S539	5.1	8.6	1.7
D7S820	3.7	8.2	1.6
D13S317	4.8	7.5	2.8
D5S818	6.1	9.0	0.0
F13A01	1.1	9.7	0.0
FESFPS	3.6	10.0	0.0
F13B	2.6	7.7	0.0
LPL	5.3	15.0	1.7

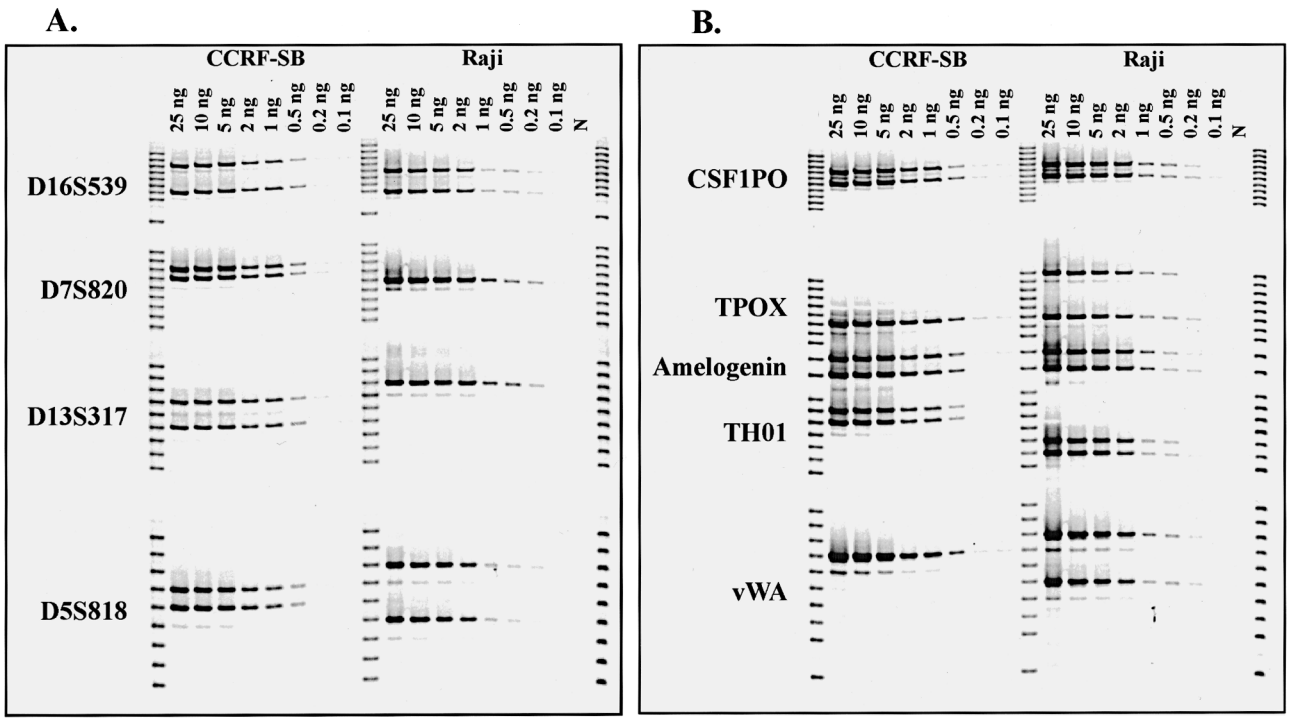


FIG. 5—*Sensitivity of the GenePrint® PowerPlex™ 1.1/Amelogenin System. DNAs from cell lines CCRF-SB and Raji were amplified with the PowerPlex™ 1.1/Amelogenin System using 25, 10, 5, 2, 1, 0.5, 0.2, or 0.1ng template, respectively. The lane marked N contains the negative control (i.e., no template). Amplifications were performed with Taq DNA Polymerase, STR Buffer, and the GeneAmp® PCR System 9600 Thermal Cycler as described. Lanes 1, 10, and 20 of each panel include allelic ladders for the loci defined to the left of the respective panel. All amplified products were separated by electrophoresis in 4% denaturing polyacrylamide gels and detected using the Hitachi FMBIO® II Fluorescent Scanner. Panel A displays the 505 nm scan of the PowerPlex™ 1.1/Amelogenin System revealing the fluorescein-labeled products of four loci. Panel B displays the 585 nm scan revealing the TMR-labeled products of five loci. The 650 nm scan illustrating the CXR-labeled ILS used for size determination is not shown.*

used, this artifact is visible only when more than 5 ng of template is used (data not shown).

The FFFL Multiplex System was analyzed using DNA isolated from cell lines CCRE-CEM and K562 (Fig. 6). The amount of DNA amplified was 25 ng, 10 ng, 5 ng, 2 ng, 1 ng, 0.5 ng, 0.2 ng, or 0.1 ng with each template. The generated profiles remained constant from 25−0.2 ng template in each case although some intensity differences between alleles within a locus were observed (e.g., F13B). Stutter bands were again more prominent with increasing amounts of template. Ranges of stutter observed are also listed for these loci in Table 2.

With 1–25 ng of template, the partial absence of a non-template directed nucleotide addition was observed for the locus LPL. Primer design and the inclusion of the final 30 min extension at 60°C in the amplification protocol have not completely eliminated this feature of the LPL locus.

4.2.1 Inheritance

Mendelian inheritance has been demonstrated by Lins et al. (20) by following allele transfer in more than 300 mother-child pairs for each locus. In the combined study of 12 loci involving 3838 mother-child inheritances, only one deviation from inheritance (i.e., mutation) was observed. This occurred in an African-American pair at the D13S317 locus.

4.4.1.3 Amplification—PCR Reaction Components and Thermocycling Parameters

The PCR reaction conditions for the PowerPlex™ 1.1/Amelogenin and FFFL Multiplex Systems have been developed by a reiterative process of primer design and protocol modification. Separate thermal cycling parameters were established for the Model 480 and the GeneAmp® PCR 9600 Thermal Cyclers. The effectiveness of the defined protocols has been demonstrated by evaluating kit performance when key parameters are varied.

We evaluated the effects of varying the enzyme concentration in amplification reactions using each multiplex system. These tests were performed because some forensic samples either contain amplification inhibitors or very small amounts of DNA template and one way to increase the yield of amplification product under these circumstances is to increase the amount of enzyme included in the reaction mixture. The standard protocol recommends 1 Unit of AmpliTaq Gold® per sample when using the FFFL Multiplex System and 2.25 Units of *Taq* DNA Polymerase or AmpliTaq Gold® per sample when using the PowerPlex™ 1.1/Amelogenin System. When the amount of enzyme was varied from 0.5 times to 5.0 times the recommended amount of enzyme with either multiplex system, the yield of amplification products decreased or increased accordingly (Fig. 7). No allele dropout was noted with either system and no artifact bands were observed with the PowerPlex™ 1.1/Amelogenin System. Some light artifact bands were observed at approximately 280, 255, and 218 base pairs with 5X AmpliTaq Gold® when using 1, 2 and 5 ng of DNA template with the FFFL system (data not shown). Amplification products of the PowerPlex™ 1.1/Amelogenin System were similar when employing *Taq* DNA Polymerase and AmpliTaq Gold®.

A second method used in forensic laboratories to deal with samples which are difficult to amplify, is to add the protein BSA to the amplification buffer (12). With standard reference samples, we did not observe significant effects from the use of this additive (Fig. 7). We have matched each enzyme and buffer with the appropriate multiplex system and amplification protocol. For example, the best performance for the FFFL Multiplex System is achieved using GoldST★R 10X Buffer (pH 8.3, containing BSA) in combination with the AmpliTaq Gold® DNA Polymerase. The PowerPlex™ 1.1/Amelogenin System works well with either the STR 10X Buffer (pH 9.0, no BSA) and *Taq* DNA Polymerase combination or the GoldST★R 10X Buffer and AmpliTaq Gold® Polymerase combination, with minor performance differences described throughout this text.

We investigated the fidelity of the amplification protocols to perturbation of the annealing temperature recommended for each multiplex system. It is important to perform these tests because thermal cycler performance can vary with regard to temperature precision and ramp times even when calibrated according to the instrument manufacturer's recommendations. In these experiments, annealing temperatures of 56°C, 58°C, 59°C, 60°C, 61°C, 62°C, and 64°C were evaluated with 60°C being the standard procedure. Two DNA samples (strain GM09947A and DNA#1) were evaluated using 2 ng, 1 ng, 0.5 ng, and 0.2 ng of each template in each assay. Figure 8 illustrates that the same alleles and no new artifacts, other than the previously mentioned 208 base band, are observed at any of the tested annealing temperatures using the PowerPlex™ 1.1/Amelogenin Multiplex System in combination with *Taq* DNA Polymerase. All predicted alleles for all twelve loci were observed regardless of temperature except that the product yield for the locus D13S317 was reduced at 62°C and notably diminished at 64°C. In addition, in samples from female individuals, an extra Amelogenin related fragment is sometimes observed between the Amelogenin and TH01 allelic ladders. The FFFL Multiplex System performed well in the range of 58°C to 61°C, with diminution of the FESFPS product when annealing temperatures exceeding 61°C were employed (Fig. 9). No extraneous bands were observed with the FFFL multiplex system regardless of temperature.

Modification of primer concentrations used to amplify each multiplex system was investigated using the recommended amount of primer and 0.5 to 2 times the recommended primer concentration. All other amplification parameters were kept constant. As expected, amplification product yields increased or decreased with corresponding increases or decreases in primer concentrations employed (data not shown). Identical DNA profiles were observed with all primer concentrations tested when using 0.2 ng to 2 ng of template.

4.4.1.4 Amplification—Number of Cycles

In theory, varying the cycle number in an amplification protocol could have deleterious effects due either to allele dropout as sensitivity is lost at lower cycle number or production of unacceptable levels of artifact bands at higher cycle number. With both the PowerPlex™ 1.1/Amelogenin or the FFFL Multiplex System, it was observed that product yield correlates with cycle number, but that artifacts are not generated by this protocol deviation (Fig. 10). The recommended number of cycles for the PowerPlex™ 1.1/Amelogenin multiplex system is 30, while that for the FFFL Multiplex System is 32. Decreasing the cycle number by as much as three cycles still allowed detection of 1ng of template or less, while increasing cycle number by two cycles generated more product at each and every fragment without introducing artifact fragments.

4.4.1.5 Amplification—Differential and Preferential Amplification

Differential amplification is defined as the difference in the amount of amplified product detected among the loci present

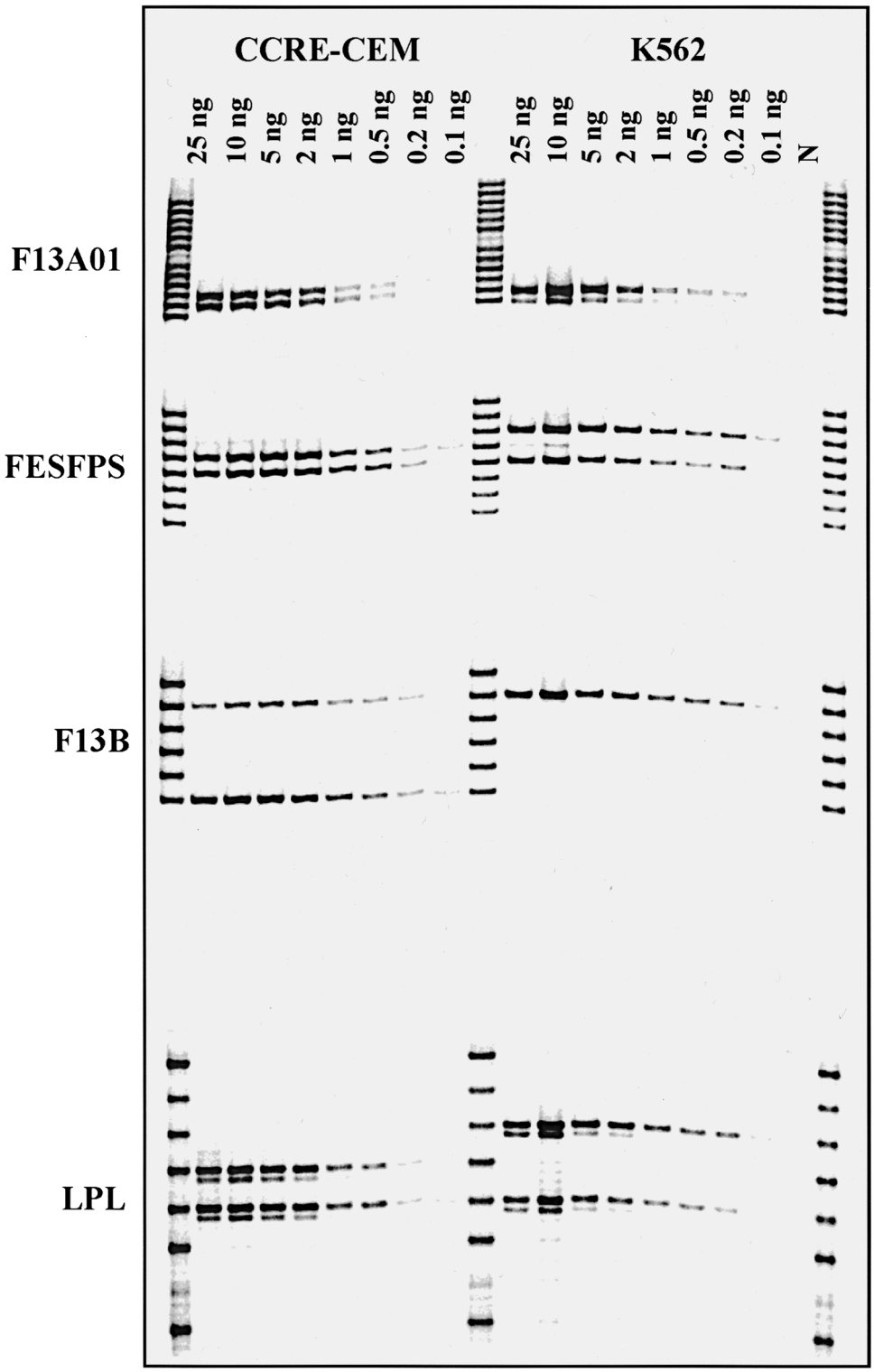


FIG. 6—*Sensitivity of the GenePrint® FFFL System. DNAs from the cell lines CCRE-CEM and K562 were amplified with the FFFL Multiplex System using 25, 10, 5, 2, 1, 0.5, 0.2, or 0.1 ng template, respectively. The lane marked N displays the negative control, (i.e., no template). Amplifications were performed in the GeneAmp® PCR System Thermal Cycler 9600 with AmpliTaq Gold® and Gold ST★R Buffer as described. Lanes 1, 10, and 20 display the FFFL Multiplex allelic ladder. All amplified products were separated by electrophoresis in a 4% denaturing polyacrylamide gel and detected using a 505 nm scan with the Hitachi FMBIO® II Fluorescent Scanner. A 650 nm scan revealing the CXR-labeled ILS used for size determination is not shown.*

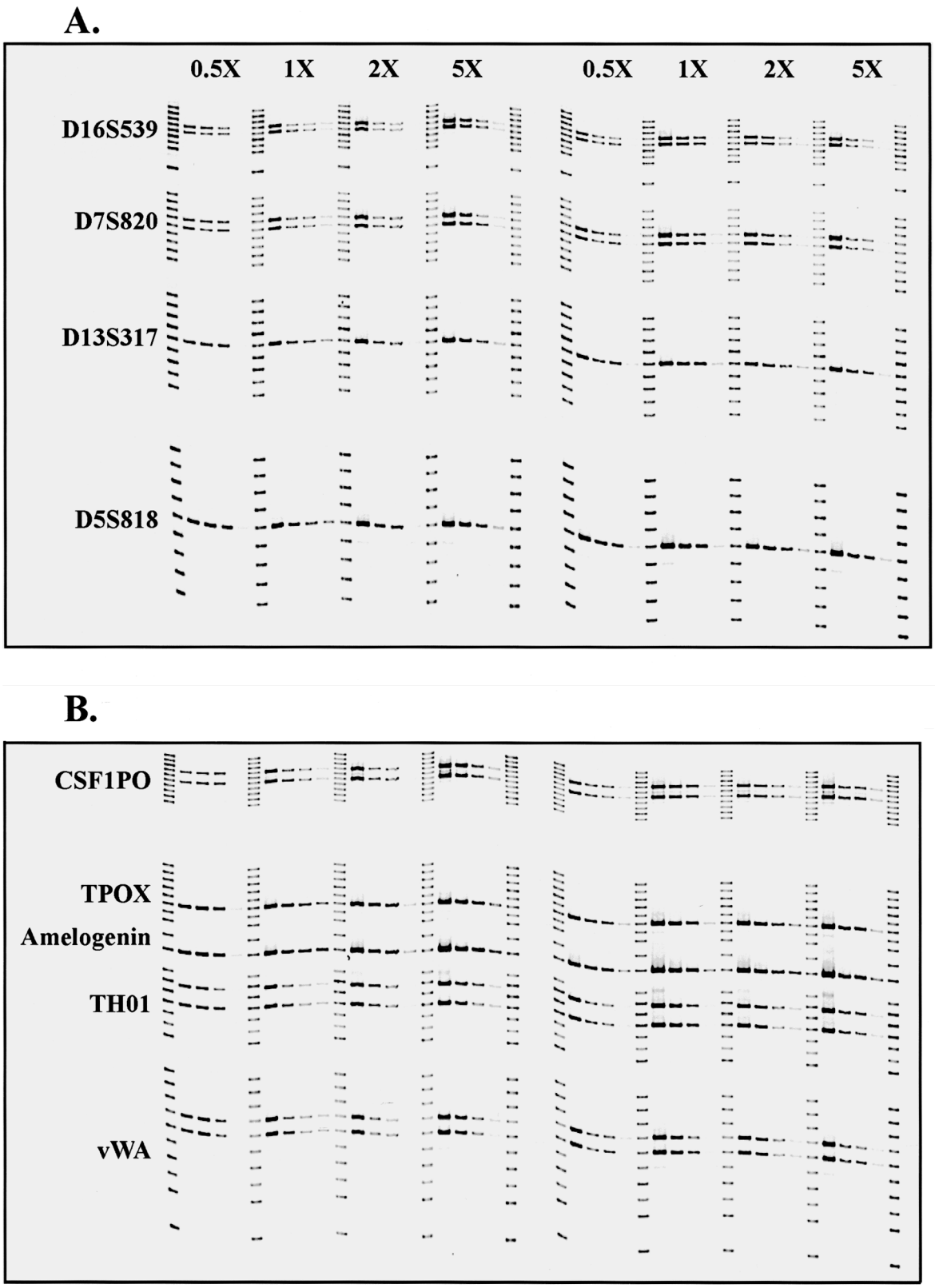


FIG. 7—*Variation of the amount of Taq DNA Polymerase and BSA used to amplify the GenePrint® PowerPlex™ 1.1/Amelogenin System. Amplifications were performed in the GeneAmp® 9600 Thermal Cycler with varying amounts of Taq DNA Polymerase. The recommended amount of Taq DNA Polymerase (labeled 1X) is 2.25 units. Amplifications were also performed with 0.5X, 2X or 5X this amount of Taq DNA Polymerase. DNA from the cell line GM09947A was amplified with the PowerPlex™ 1.1/Amelogenin System using 2, 1, 0.5, or 0.2 ng template. Each set of four samples is flanked by allelic ladders for the loci as defined on the left side of the gel and the samples were loaded from highest to lowest amount. The amount of Taq DNA Polymerase used in a set is indicated above the lanes. The left set of lanes labeled with 0.5X to 5X had no BSA present in the reaction while the right set of lanes labeled 0.5X to 5X used an equivalent buffer containing 160 μg/mL of BSA. The amplified products were separated and detected as described in Fig. 5. Panel A displays the 505 nm scan of the PowerPlex™ 1.1/Amelogenin System revealing the fluorescein-labeled products. Panel B displays the 585 nm scan revealing the TMR-labeled products.*

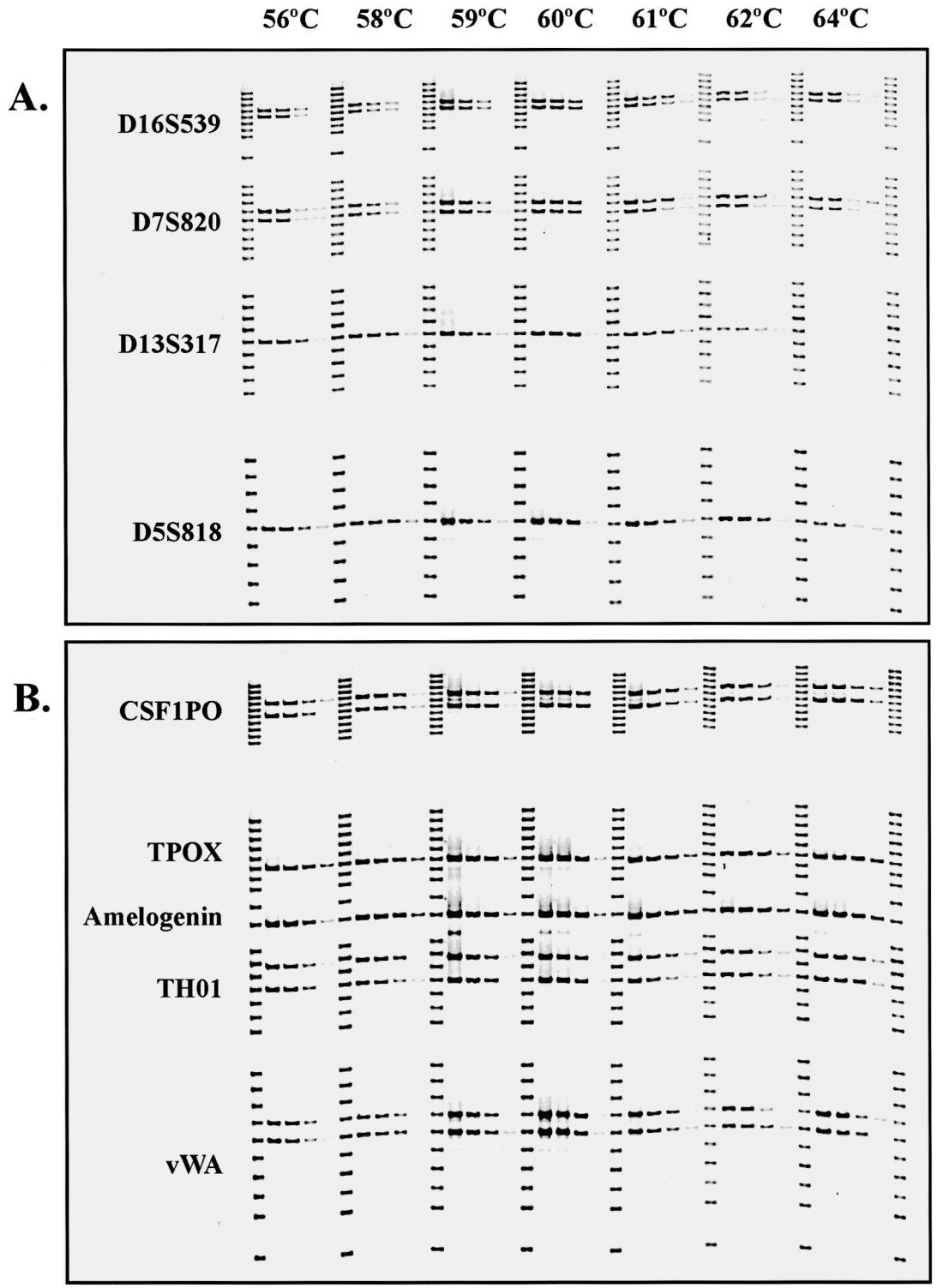


FIG. 8—*Variation of the annealing temperature for amplification of the GenePrint® PowerPlex™ 1.1/Amelogenin System. DNA from cell line GM09947A was amplified using 2, 1, 0.5 or 0.2 ng template in the GeneAmp® 9600 Thermal Cycler with Taq DNA Polymerase. The results for seven different annealing temperatures are demonstrated. Each set is shown from the highest to lowest amount of DNA and is separated by a lane containing allelic ladder. The temperature used is displayed above each set. The amplified products were separated and detected as described in Fig. 5. Panel A displays the fluorescein-labeled products of the PowerPlex™ 1.1/Amelogenin System. Panel B displays the TMR-labeled products of the PowerPlex™ 1.1/Amelogenin System.*

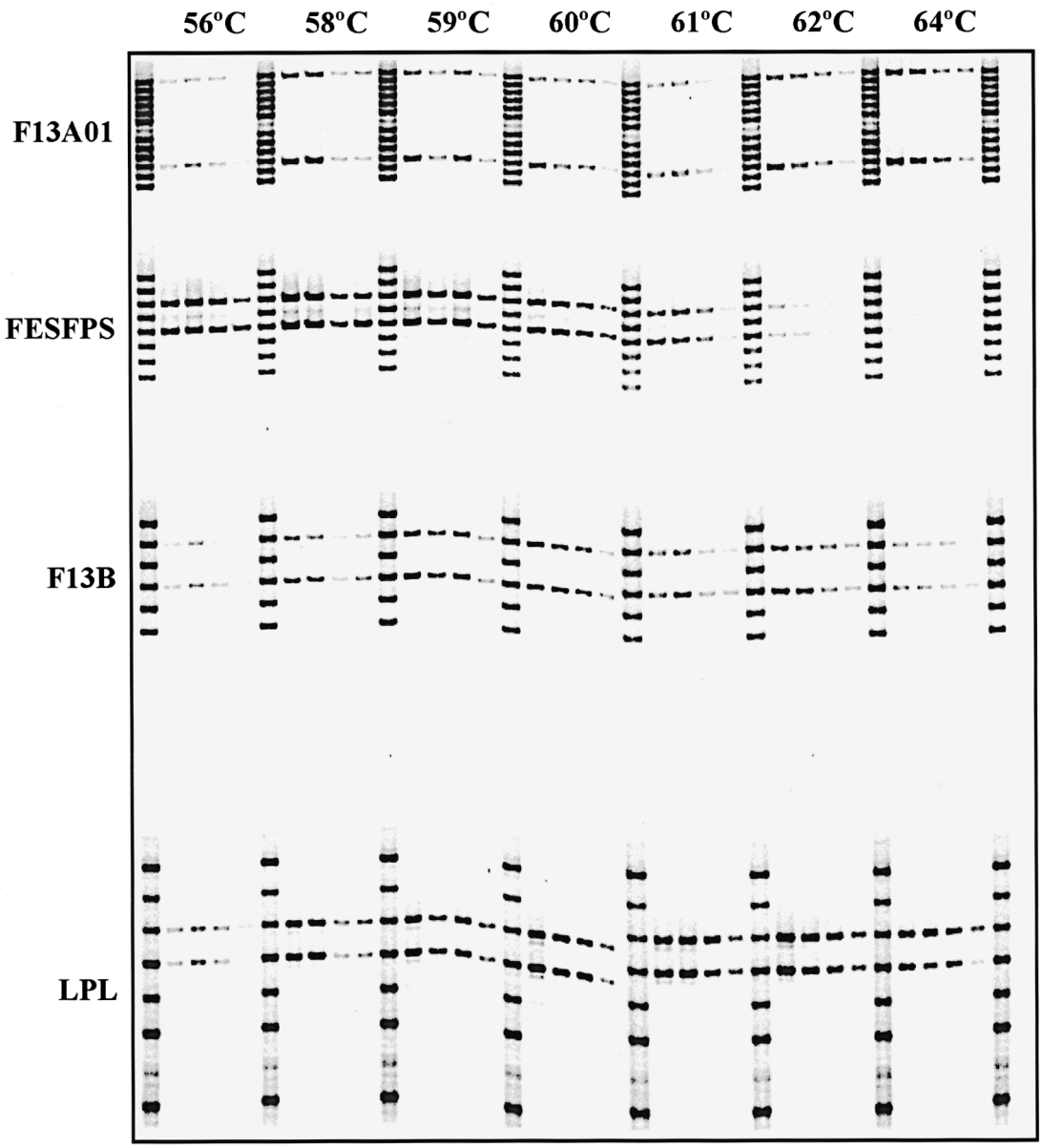


FIG. 9—*Variation of the annealing temperature for amplification of the GenePrint® FFFL System. DNA from cell line GM09947A was amplified using 2, 1, 0.5, or 0.2 ng template in the GeneAmp® 9600 Thermal Cycler with AmpliTaq Gold® DNA Polymerase and Gold ST★R Buffer. The results for seven different annealing temperatures are demonstrated. Each set is shown from the highest to lowest concentration of DNA and is separated by a lane containing allelic ladder. The annealing temperature employed is displayed above each set. The amplified products were separated by electrophoresis in a 4% denaturing polyacrylamide gel and detected using a 505 nm scan with the Hitachi FMBIO® II Fluorescent Scanner.*

within a multiplex system. Preferential amplification refers to differences in product yield between individual alleles within a single locus in heterozygous individuals.

The Hitachi FMBIO® 100 or II Fluorescent Scanner allows measurement of relative fluorescent units (RFU) for all alleles displayed within a single fluorophor. Thus, the fluorescein-labeled and TMR-labeled portions of the multicolor PowerPlex™ 1.1/Amelogenin Multiplex System can be displayed and evaluated independently. When comparing amplified products labeled with different dyes, the key element of balance is loss of true signal over background signal (i.e., signal observed due to the instrument, gel plates, etc.) using similar amounts of template in a DNA sample titration. This may mean that the RFU generated with one dye may not be the same as another. In the case of the PowerPlex™ 1.1 System, the RFU for the TMR-labeled loci are generally higher than those with the fluorescein-labeled loci. The fluorescent scanner allows modulation of the visual display to compensate for these consistent differences in RFU due to the dyes and their associated detection efficiencies. When these effects were taken into account with the PowerPlex™ 1.1/Amelogenin Multiplex System, we observed a loss of relative sensitivity at all loci as the amounts of template employed were decreased below 1 ng (data not shown).

4.4.1.6 Amplification—Single Locus versus Multilocus Amplification

Each of the 12 loci included in the PowerPlex™ 1.1/Amelogenin and FFFL Multiplex Systems were amplified as monoplex systems.

Raji cell line DNA was used for the loci CSF1PO, TPOX, TH01, DNA from the cell line CCRF-SB was used for the D16S539, D7S820, D13S317 and D5S818 loci and K562 cell line DNA was amplified for the F13A01, FESFPS, F13B and LPL loci. In all cases, amplification with monoplex and multiplex systems produced the same genotypes (data not shown). Coamplification of loci has the advantage of minimizing the amount of sample material and sample handling required to perform analyses.

4.4.2.1 Amplification—Standards for Direct Characterization

Allelic ladders (25,26) make ideal standards for STR detection. These standards are comprised of collections of most or all of the alleles which are observed at a particular locus in the population at large. As such, in most cases, they have identical sequence and size when compared with the unknown alleles being evaluated. This offers several advantages over other types of size markers. For example, when employing a fluorescently-labeled primer during amplification both the standard (i.e., components of the allelic ladder) and the amplified unknown DNA template are modified in identical fashion. No electrophoresis migration anomalies will result, as they could if two different labels or DNA sequences were used for standards and unknowns. The same is true for modifications in the constitution of gel matrices, variation of electrophoresis buffers, and many other variables which may be introduced either across detection formats or due to differences in individual laboratory procedures.

The sequence of each fragment contained in each allelic ladder of each multiplex system has been determined (20). We originally selected and developed the loci included in the PowerPlex™ 1.1 and FFFL Multiplex Systems because they reveal very few common sequence variants other than those resulting from changes in the number of tetranucleotide repeat sequences present at the respective loci. In fact, only two loci display length microvariants (i.e., length variants due to changes of one or two bases rather than the four base repeat units) with a frequency greater than 1 in 1000 individuals in the population. These microvariants include the locus TH01 allele 9.3 (27) which contains a one base deletion in the seventh copy of the AATG repeat sequence of what would otherwise be 10 copies of the repeat, and the locus F13A01 allele 3.2 (28) which contains a two base deletion immediately distal to the 4 copies of the AAAG repeat observed in this allele. Rarer length microvariants have been observed in several loci (29). Sequence variants (i.e., due to base substitutions of one or more bases rather than changes in the number of four base repeat units) have been described for several loci including vWA (30), and D13S317 (20).

While allelic ladders provide the greatest confidence in supporting appropriate allele designations, commonly observed gel migration differences of identical samples across an individual gel may

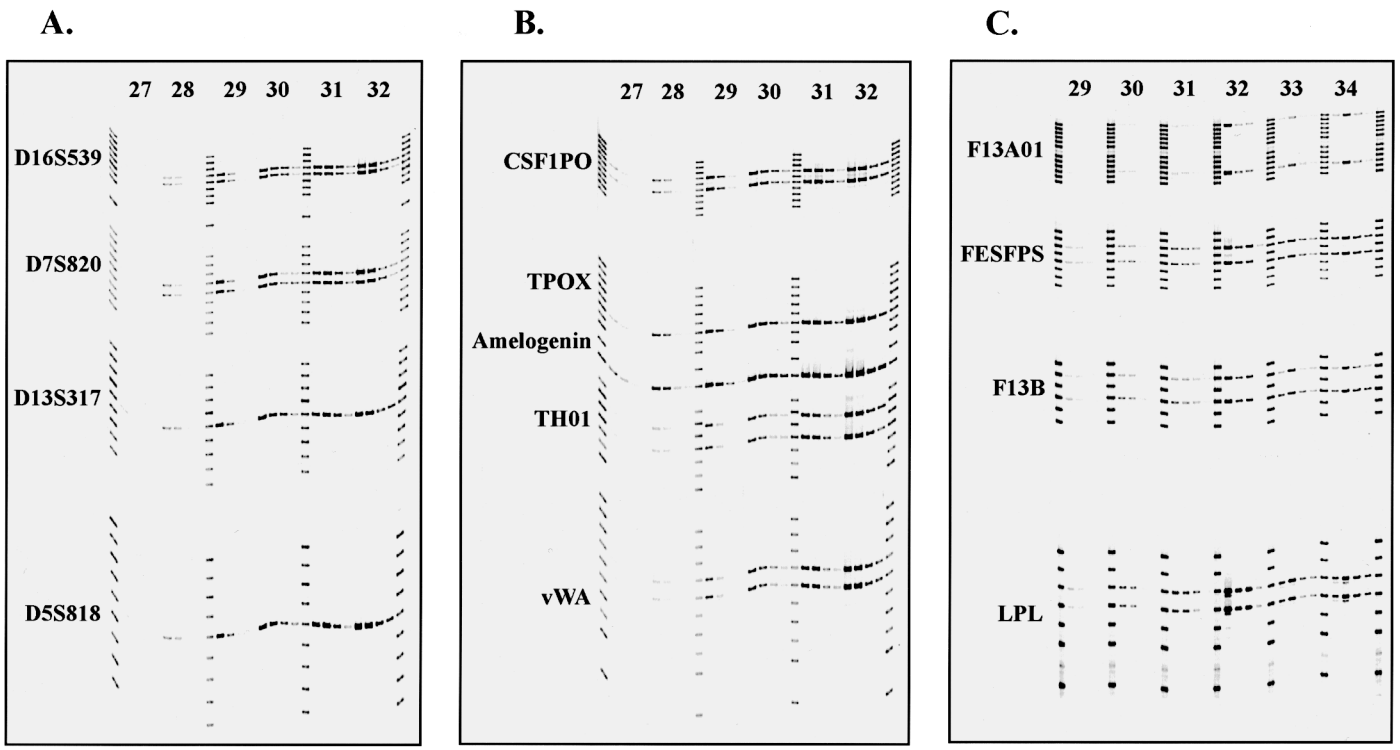


FIG. 10—*Variation of cycle number with amplification of the GenePrint® PowerPlex™ 1.1/Amelogenin and FFFL Systems. DNA from cell line GM09947A was amplified using 2, 1, 0.5, or 0.2 ng template with the PowerPlex™ 1.1/Amelogenin or FFFL STR Systems. Amplifications with the PowerPlex™ 1.1/Amelogenin System were performed in the GeneAmp® 9600 Thermal Cycler with Taq DNA Polymerase for 27, 28, 29, 30, 31, or 32 cycles. The recommended cycle number is 30. Amplifications with the FFFL System were completed in the GeneAmp® 9600 Thermal Cycler with AmpliTaq Gold® and Gold ST★R Buffer for 29, 30, 31, 32, 33, or 34 cycles. The recommended cycle number is 32. All amplified products were separated by electrophoresis in a 4% denaturing polyacrylamide gel and detected using the Hitachi FMBIO® II Fluorescent Scanner. Panel A displays the fluorescein-labeled products of the loci D16S539, D7S820, D13S317 and D5S818 of the PowerPlex™ 1.1/Amelogenin System. Panel B displays the TMR-labeled products of the loci CSF1PO, TPOX, TH01, vWA and Amelogenin. Panel C displays the fluorescein-labeled products of the loci F13A01, FESFPS, F13B and LPL of the FFFL Multiplex System. For Panels A and B, lanes 1, 10, 19, and 28 contain allelic ladders for each locus defined to the left of the panel. For Panel C, lanes 1, 6, 11, 16, 21, 26, and 31 include the allelic ladders for the loci defined on the left side of the panel.*

decrease the accuracy of designating alleles either by eye or using instrument software. Thus, inclusion of an internal lane standard (ILS), within each lane assists in monitoring for this effect.

The Fluorescent Ladder (CXR), 60–400 Bases is an ILS that consists of 16 fragments that are 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, and 400 bases, respectively. The ILS is labeled with carboxy-X-rhodamine, a fluorescent dye, which distinguishes it from the amplified products and allelic ladder fragments. Thus, it can be subjected to electrophoresis in every sample and allelic ladder lane. The STaR Call™ Allele Calling Software included with the Hitachi FMBIO®II Fluorescent Scanner or the Genotyper® Software which can be added to the Applied Biosystems PRISM™ 310 Genetic Analyzer and ABI PRISM™ DNA Sequencers can use the information provided by the ILS to compensate for any electrophoresis migration effects. This particular ILS is an extremely accurate tool as the larger fragments contain the sequence of the smaller fragments virtually eliminating the chance of migration artifacts due to sequence variation among the fragments. Also, since the bands are evenly spaced the sample alleles will never be more than 13 bases away from an ILS sizing fragment. Thus, inclusion of the ILS in each lane and the inclusion of an allelic ladder on each gel in one or more lanes provides a confident and precise means of allele designation.

Conclusion

The PowerPlex™ 1.1/Amelogenin and FFFL Multiplex Systems are powerful investigative tools available to forensic laboratories. The results of the TWGDAM validation studies presented here demonstrate the reliability, reproducibility, robust nature, and high discrimination power of these systems. The systems provide sensitive and accurate results even when the amplification parameters are stressed or forensic samples are exposed to extreme conditions. The high level of discrimination power alleviates the need for additional testing of most samples. In combination with appropriate validation of general laboratory procedures, these systems provide a reliable method of forensic casework analysis.

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Additional information and reprint requests:
Elizabeth Amiott
Research Scientist
Promega Corporation
2800 Woods Hollow Road
Madison, WI 53711-5399