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Validation of the AmpliFLP™ D1S80 PCR Amplification Kit for Forensic Casework Analysis According to TWGDAM Guidelines

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ABSTRACT: The validation of the AmpliFLP™ D1S80 PCR Amplification Kit for use in forensic casework was accomplished by performing all the relevant experiments outlined in the TWGDAM guidelines. Standard specimen and reproducibility studies were performed using organic and rapid DNA extraction techniques on both stain and liquid samples (blood, semen and saliva). Over 300 samples from three different populations (US Caucasians, African Americans and US Hispanics) were analyzed to determine allele and genotype frequencies. Purified DNA was mixed in defined ratios (ranging from unmixed DNA samples to 1:9 mixtures of 2 different DNA samples) prior to amplification to demonstrate that samples containing DNA from more than one individual can be detected and, in many cases, that the genotypes contributing to the mixture can be identified. Since casework samples frequently are exposed to environmental insults that can result in DNA degradation, purified DNA was degraded in the laboratory to analyze the effect of DNA fragment length on D1S80 amplification. It is crucial in the validation process to examine actual casework evidentiary material. This D1S80 kit can be used successfully by forensic scientists to amplify and type nonprobative evidentiary material, including bloodstains collected from crime scenes and rape kit materials collected for sexual assault cases. The D1S80 kit is specific to human DNA, and the D1S80 alleles are inherited according to the laws of Mendel. The sensitivity of the novel gel electrophoresis gel matrix allowed the PCR cycle number to be reduced to 29 cycles and the D1S80 kit sensitivity to be increased to 2.5 ng from the previous D1S80 Reagent Set specifications of 30 cycles and 5 ng, respectively.

KEYWORDS: forensic science, D1S80 amplified fragment length polymorphism (D1S80 AMP-FLP), validation, polymerase chain reaction (PCR), deoxyribonucleic acid (DNA)

Two widely used DNA typing methods for the analysis of forensic casework samples are based on RFLP [*1,2*] and PCR [*3–5*] methods. RFLP analysis involves hybridization of multi- or single-locus probes to extracted DNA following restriction enzyme digestion and gel electrophoresis. PCR-based tests use sequence-specific probes or gel electrophoresis to analyze amplified regions of DNA. One PCR-based test being used by many casework laboratories around the world involves amplification of a region of the HLA DQA1 locus followed by detection of allelic sequence polymorphisms using a reverse dot blot strip assay [*6–8*]. Amplification of fragment length polymorphisms (AMP-FLPs) provides a second class of PCR-based polymorphic markers that can be used for the analysis of biological samples. These markers contain a variable number of tandem repeat units (VNTR) between the PCR primer binding regions. Consequently, AMP-FLP typing systems are based on differences in length between alleles rather than sequence differences. Alleles are distinguished by gel electrophoresis systems using silver stain, probe hybridization or fluorescent tags for detection.

PCR amplification and typing of the D1S80 locus were first described by Kasai et al. [*9*]. In addition, they provided some sequence data confirming that the size of the repeat unit is 16 base pairs. Subsequently, Budowle et al. [*10*] described a horizontal high-resolution gel system and provided D1S80 population data indicating that this AMP-FLP marker would be suitable for forensic applications. Hochmeister et al. [*11*] obtained reliable D1S80 typing results from decomposed body remains, demonstrating that samples unlikely to give RFLP results may be analyzed using AMP-FLP markers.

Given the promise of this genetic marker as a forensic DNA typing system, a set of D1S80 reagents that would be able to meet internal QC and manufacturing requirements as well as the requirements of the forensic community were developed originally at Cetus, now Roche Molecular Systems, Inc. (RMS). To produce a reliable D1S80 kit that could be used to analyze forensic casework samples, the published reaction conditions and cycling parameters [*9*] were modified. The initial commercial product was the D1S80 Reagent Set, which included an amplification reaction mix and protocol, a 15-allele D1S80 allelic ladder and a protocol for the analysis of amplified D1S80 products using an agarose gel followed by ethidium bromide staining. Improvements were made to the D1S80 Reagent Set at RMS, which resulted in our current product, the AmpliFLP™ D1S80 PCR Amplification Kit. The present kit includes:

1) the AmpliFLP D1S80 Allelic Ladder which contains 27 D1S80 alleles and is compatible with the FBI D1S80 allelic ladder [*12*],

2) protocols for separating and detecting D1S80 alleles on a novel, high-resolution vertical gel system followed by silver staining,

3) amplification protocols for the Perkin Elmer DNA Thermal Cycler 480 (TC480) using Thin-Walled GeneAmp® Tubes and the

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GeneAMP® PCR System 9600 (GAPS 9600) using MicroAmp™ Tubes with Caps.

In addition, each of the Technical Working Group on DNA Analysis Methods (TWGDAM) Validation Guidelines [*13*] has been addressed using the D1S80 kit. Representative results obtained from validation studies are presented here.

Materials and Methods

DNA Extraction

Three different procedures were used to extract DNA for the D1S80 amplification kit validation studies. The "salting-out" procedure described by Miller et al. [*14*] was used to extract double stranded DNA from buffy coats that had been separated from red blood cells (RBCs) and serum. To lyse residual RBCs, a lysis buffer (144 mM NH_4Cl, 1 mM $NaHCO_3$) was added to the sample and the tube was inverted and incubated at room temperature for 15 mins. Following a 20 min spin at 2000 rpm, the supernatant containing lysed RBCs was discarded. Nuclei lysis buffer (10 mM Tris-HCl, pH 8, 400 mM NaCl, 1 mM EDTA) was used to resuspend the pellet; 10% SDS and Proteinase K were added and the slightly viscous suspension was incubated overnight at 37°C. The following day, saturated NaCl was added and the sample was shaken vigorously and then spun at 2500 rpm for 15 min. The solution was transferred to two clean 50 mL polypropylene tubes. Twice the volume of 100% ethanol was added to precipitate the DNA. The DNA was transferred into a polypropylene tube containing 2 mL of TE buffer (10 mM Tris-HCl, pH 8, 0.1 mM EDTA) and incubated at 37°C for at least 2 hours. Additional TE buffer or additional incubation at 37°C was used to resuspend the DNA completely. The DNA was quantitated by both UV spectroscopy and D17Z1 probe hybridization as described by Walsh et al. [*15*] using the QuantiBlot™ Human DNA Quantitation Kit (Perkin Elmer, Norwalk, CT).

Phenol/chloroform and Chelex® extraction procedures outlined in the AmpliType® User Guide [*16*] were used to extract DNA from blood, semen and saliva (extraction of liquid saliva and saliva stains was done according to the liquid blood and bloodstain protocols). The organic extraction procedure involved digestion of blood, semen and saliva with Proteinase K and separation of the nucleic acids from protein fragments using a buffered phenol/chloroform/isoamyl alcohol (24:23:1) solution. The DNA was washed and concentrated using a Centricon-100 (Amicon) ultrafiltration device. Chelex was used as a rapid procedure for DNA extraction. Both liquid and stain blood, semen and saliva samples were boiled in a 5% Chelex solution.

D1S80 PCR Amplification

The reagents contained in the AmpliFLP™ D1S80 PCR Amplification Kit (Perkin Elmer) were used to amplify extracted DNA in 50 μL reactions containing 20 μL D1S80 PCR Reaction Mix, 10 μL 5 mM $MgCl_2$, 1 drop mineral oil and 20 μL DNA sample. The reagents were added in the order listed above to Thin-Walled GeneAmp PCR Tubes unless otherwise indicated. Samples were amplified in the DNA Thermal Cycler 480 (Perkin Elmer) using the following parameters for 29 or 30 cycles: denature at 94°C for 1 min, anneal at 65°C for 1 min and extend at 72°C for 1 min. A final extension at 72°C for 10 min was added to the end of the program.

For amplification of samples in the GAPS 9600, MicroAmp Tubes with Caps were used. The GAPS 9600 PCR reaction set up was identical to the TC480 reaction set up except that no oil was added to the tubes. The GAPS 9600 cycling parameters for 29 or 30 cycles are: denature at 95°C for 15 s, anneal at 66°C for 15 s and extend at 72°C for 40 s. A final extension at 72°C for 10 min was added to the end of the program.

Gel Electrophoresis and Allele Detection

D1S80 amplification products were analyzed using the GeneAmp® Detection Gel High Resolution Gel Concentrate and Loading Buffer Kit (Perkin Elmer) with the GIBCO BRL Model SA32 apparatus (0.8 mm comb and spacers) and GelBond (Perkin Elmer).

The gels were cast and run in 0.5X TBE buffer (44.5 mM Tris-borate, 1 mM EDTA). Five microliters of amplified product were combined with 1 μL of a sucrose loading buffer containing bromophenol blue and xylene cyanol dyes. The 6 μL of sample/loading buffer mixture was loaded on the gel and run at 800 to 1000 volts using a Pharmacia ECPS 3000/150 power supply on the constant voltage setting until the xylene cyanol dye migrated 23 cm into the gel. Under these conditions, D1S80 alleles containing 40 and 41 repeat units (785 bp and 801 bp, respectively) should be separated by at least 2 mm.

Once electrophoresis was complete, the D1S80 amplified products and the 27-allele D1S80 Allelic Ladder were visualized by staining with silver as follows. The gel apparatus was disassembled and the gel (affixed to GelBond) was transferred to a clean, glass dish containing 40% methanol and placed on an orbital shaker for at least 10 min. The solution was poured off and 160 mM nitric acid was added for 6 min with shaking. After a water rinse, the gel was submerged in 12 mM silver nitrate for 20 min with shaking. Two 5 min water washes followed. Chilled development solution was added (280 mM sodium carbonate with 0.0185% formaldehyde) and allowed to rotate until the desired band intensity was achieved (usually 6 to 10 minutes; the band intensity and background staining will increase upon drying of gel). The reaction was stopped with 100 mM citric acid. The gels were dried at 80°C for one hour in a Model 583 gel dryer (Bio-Rad, Richmond CA). Photography was performed either before or after drying using a Polaroid camera with Polaroid Type 55 film over a white light box.

Results

The Results section is organized according to the Validation portion of the TWGDAM "Guidelines for a Quality Assurance Program for DNA Analysis" [*13*]. Sections 4.1.5, 4.2 and 4.4 of the Guidelines describe studies that should be included in the validation process for new PCR-based genetic marker systems. The DNA amplifications for all the validation studies used the AmpliFLP D1S80 PCR Amplification Kit (D1S80 kit). Product analysis was performed on GeneAmp Detection Gels (Detection Gels) using the D1S80 Allelic Ladder comprised of 27 amplified D1S80 alleles to allow for accurate and reliable typing of amplified D1S80 products.

Section 4.2 of the Guidelines outlines the characteristics of the marker locus that must be determined and documented: the locus has been mapped to chromosome 1p [*17*], the primer sequences are published [*9*], and the marker contains a VNTR length polymorphism in which the size of the repeat unit is 16 base pairs [*9*]. All of the applicable guidelines in Section 4.4 have been addressed as well at RMS and some of them are presented as follows. The remaining relevant studies described in the Guidelines are detailed below.

Studies 4.1.5.1 and 4.1.5.4—Standard Specimens and Reproducibility

Blood, semen and saliva were collected from volunteers (five men, four women) at RMS. Stains were made on sterile cotton cloth from the fluids and both the stains and remaining aliquots of liquid were frozen at −20°C. For the Standard Specimens Study (4.1.5.1), samples were extracted by both Chelex and phenol/chloroform procedures (AmpliType User Guide, Sample Preparation sections [*16*]). Amplification was performed in the GAPS 9600 on the extracted stains following the protocol in the Materials and Methods section using 30 cycles. D1S80 PCR products amplified from all extracted tissues from one individual were run on a Detection Gel and are shown in Fig. 1*A*. As expected, all tissues from the same individual yielded the same D1S80 type (24,31) from both Chelex and phenol/chloroform extracted samples.

To demonstrate that liquid and dried samples from an individual yield the same D1S80 type (Reproducibility Study 4.1.5.4), Chelex and phenol/chloroform extractions of stain and liquid samples from six of the nine individuals were amplified and typed according to the Materials and Methods section using 30 cycles. The liquid and dried samples from each individual yielded the same D1S80 type whether the DNA was extracted with Chelex (Fig. 1*B*) or phenol/chloroform (Fig. 1*C*).

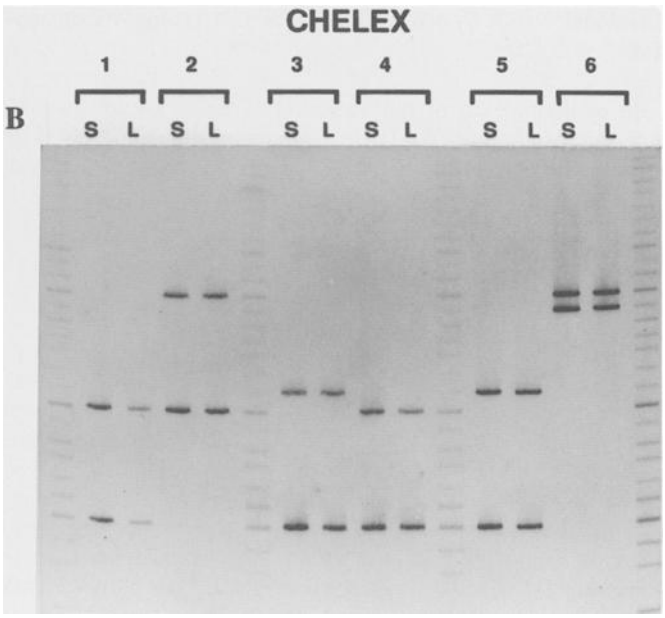


FIG. 1*B*—*Bloodstains (S) and liquid blood (L) from 6 individuals were Chelex extracted following the protocol in the AmpliType User Guide and amplified according to the Materials and Methods section. Amplified products were run on a Detection Gel followed by silver staining. Unmarked lanes contain the D1S80 Allelic Ladder.*

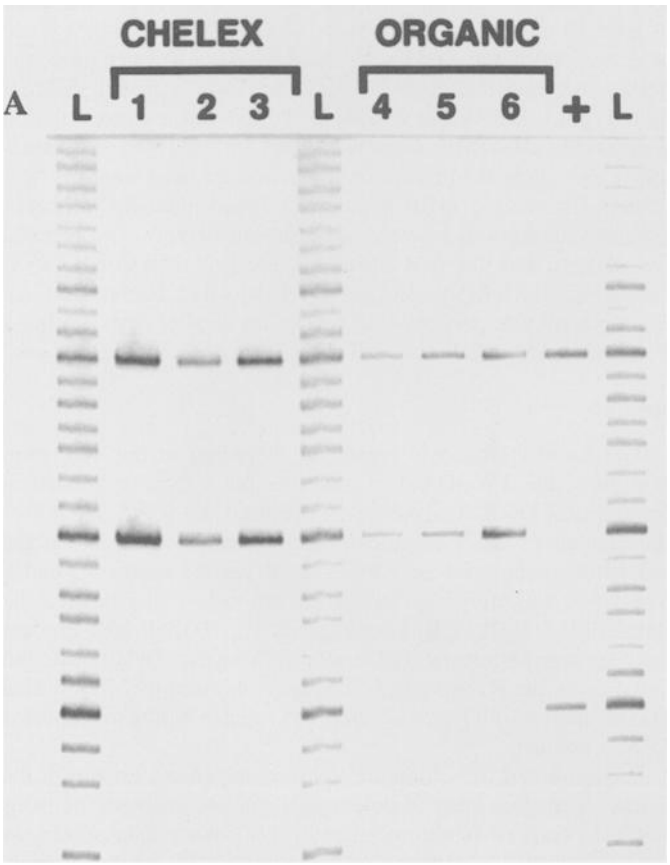


FIG. 1*A*—*D1S80 typing of different tissues. DNA extracted from blood (lanes 1 and 4), saliva (lanes 2 and 5) and semen stains (lanes 3 and 6) from one individual was amplified and typed as described in the Materials and Methods section. Each tissue/fluid yielded the same D1S80 type (24,31). The AmpliFLP D1S80 Allelic Ladder was run in lanes labelled "L."*

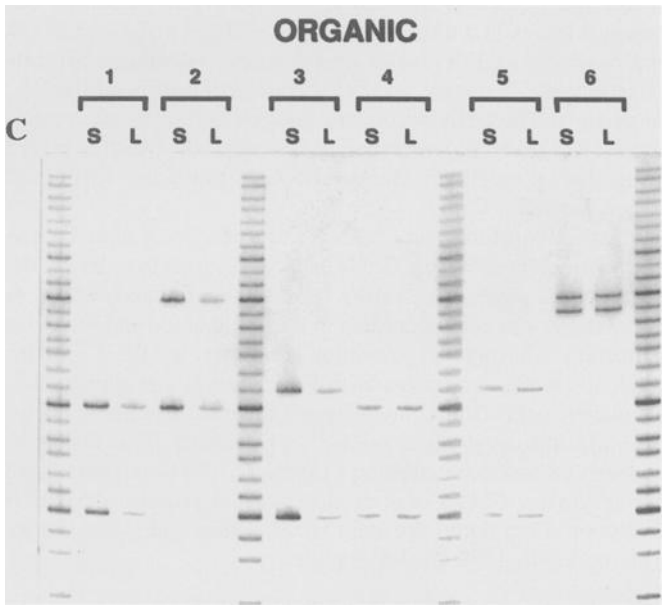


FIG. 1*C*—*Saliva stains (S) and liquid saliva (L) from 6 individuals were phenol/chloroform extracted following the AmpliType User Guide protocol for the organic DNA extraction from blood (Section 3.1.2). Samples were amplified and typed according to the Materials and Methods section. Unmarked lanes contain the D1S80 Allelic Ladder.*

Study 4.1.5.3—Population Studies

DNA samples from randomly selected individuals from US Caucasian (98), African American (117) and US Hispanic (107) populations were amplified and typed as described in the Materials and Methods section. The population study samples were provided by Roche Biomedical Laboratories and were collected from across

the United States (that is, no specific regional groups are represented). US Hispanic individuals were identified by last name. These samples have been used for AmpliType HLA DQA1 and PM population studies and are described in Helmuth et al. [*18*].

The allele frequencies shown in Fig. 2 were used to calculate expected D1S80 genotype frequencies assuming Hardy-Weinberg equilibrium. Comparisons of the expected frequencies to the observed genotype frequencies indicate that the D1S80 system is in Hardy-Weinberg equilibrium and that no systematic typing errors are introduced by this method of amplification and typing (manuscript in preparation).

An interesting feature of the D1S80 allele frequency distribution observed with other AMP-FLP systems [*19–21*] is that in US Caucasian and US Hispanic populations, two alleles (D1S80 18 and 24) have a combined allele frequency of 60% and are represented in approximately 83% of individuals. The alleles are more evenly distributed in the African American population, which is reflected in the higher P_d value for this group (0.98) relative to the P_d value for the Caucasian and Hispanic groups (0.94 and 0.95, respectively). A more detailed analysis of the D1S80 population data will be presented in a separate manuscript.

Study 4.1.5.5—Mixed Specimen Studies

Samples containing fluids from more than one individual are encountered frequently by forensic casework laboratories. Consequently, it is important to use typing tests that are able to detect mixtures. In addition, laboratories need to develop guidelines for interpreting and reporting results obtained from mixed samples.

One approach to evaluate the ability of the D1S80 system to detect mixtures is to mix purified DNA samples of known D1S80 type in defined ratios prior to amplification. The D1S80 products resulting from the amplification of a mixture containing an 18,18 homozygous DNA and an 18,24 heterozygous DNA are shown in Fig. 3*A*. These two DNAs were chosen because they contain the most common D1S80 alleles and are likely to be encountered in casework samples. When the 18,24 DNA is present as $^1/_{10}$ (0.5 ng) of the total DNA in the reaction, the 24 allele band can be detected and is significantly less intense than the 18 allele band (lane 6). The 24 allele band also is noticeably less intense than the 18 allele band when the 18,24 DNA is present as $^1/_4$ (1.25 ng) and $^1/_2$ (2.5 ng) of the total DNA in the reaction (lanes 5 and 4, respectively).

The results of a separate mixture study using DNA samples that do not contain any shared alleles (17,28 and 24,37) are shown in Fig. 3*B*. When either DNA sample is present as only 1/10 (0.5 ng) of the total DNA in the reaction, a mixture can be detected (lanes 2 and 6) and the alleles can be sorted and assigned to two individuals based on band intensity differences. When the DNA samples are present in equal amounts (2.5 ng each), again a mixture is readily detected (lane 4). However, the alleles cannot be sorted because the intensities of the four bands are too similar.

Study 4.1.5.6—Environmental Studies

Several approaches can be taken to address the Environmental Studies validation guideline. One approach is to degrade purified DNA with DNase I for increasing amounts of time to generate a series of DNA fragments decreasing in average size (Fig. 4*A*). The series of degraded DNA samples can be amplified and typed to assess the effect of degradation on the ability of this D1S80 system to yield a reliable type. It has been observed that certain levels of DNA degradation or PCR inhibitors can affect AMP-FLP typing results by enhancing preferential amplification of the

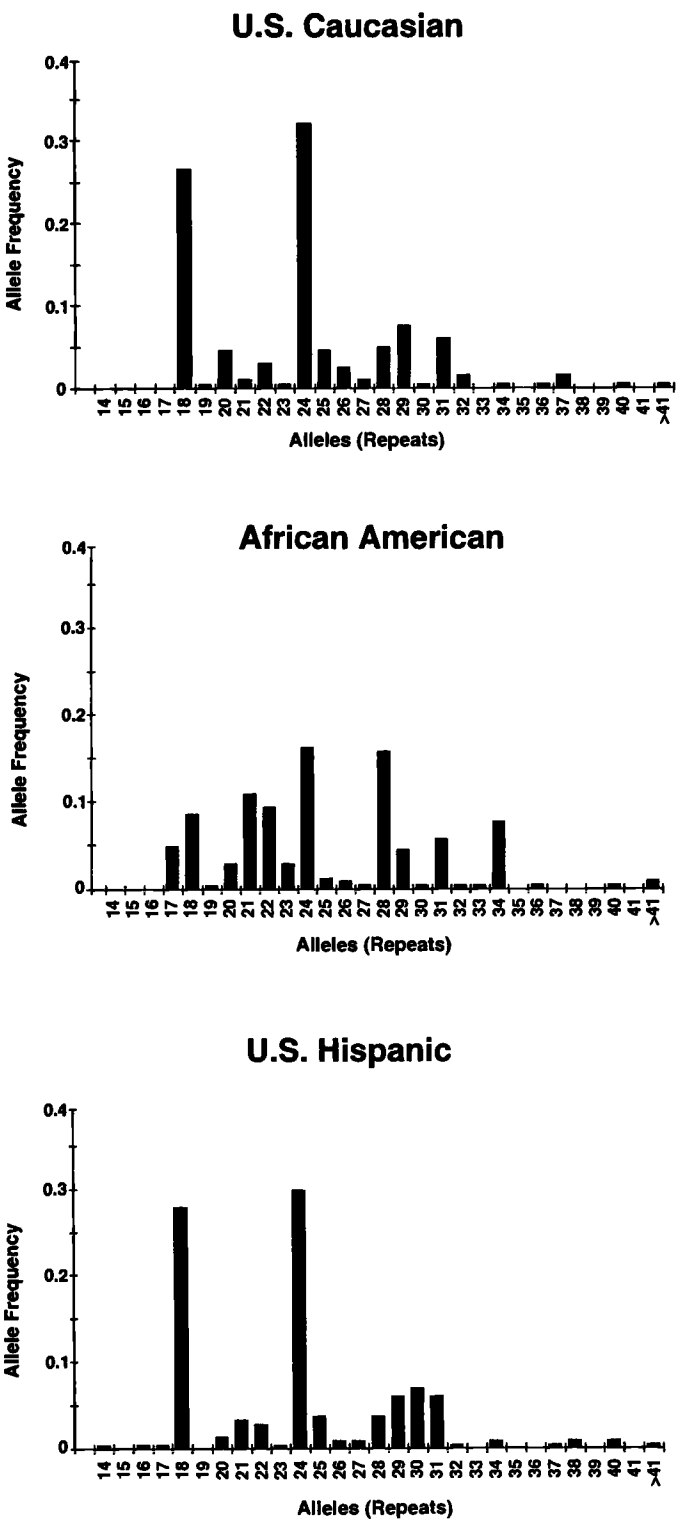


FIG. 2—*D1S80 allele frequency distributions for three population groups. D1S80 genotypes were determined from 98 US Caucasian, 117 African American and 107 US Hispanic samples obtained from random individuals and provided by Roche Biomedical Laboratories (RBL).* P_d values calculated from these data are 0.94 for Caucasians, 0.95 for Hispanics and 0.98 for African Americans.

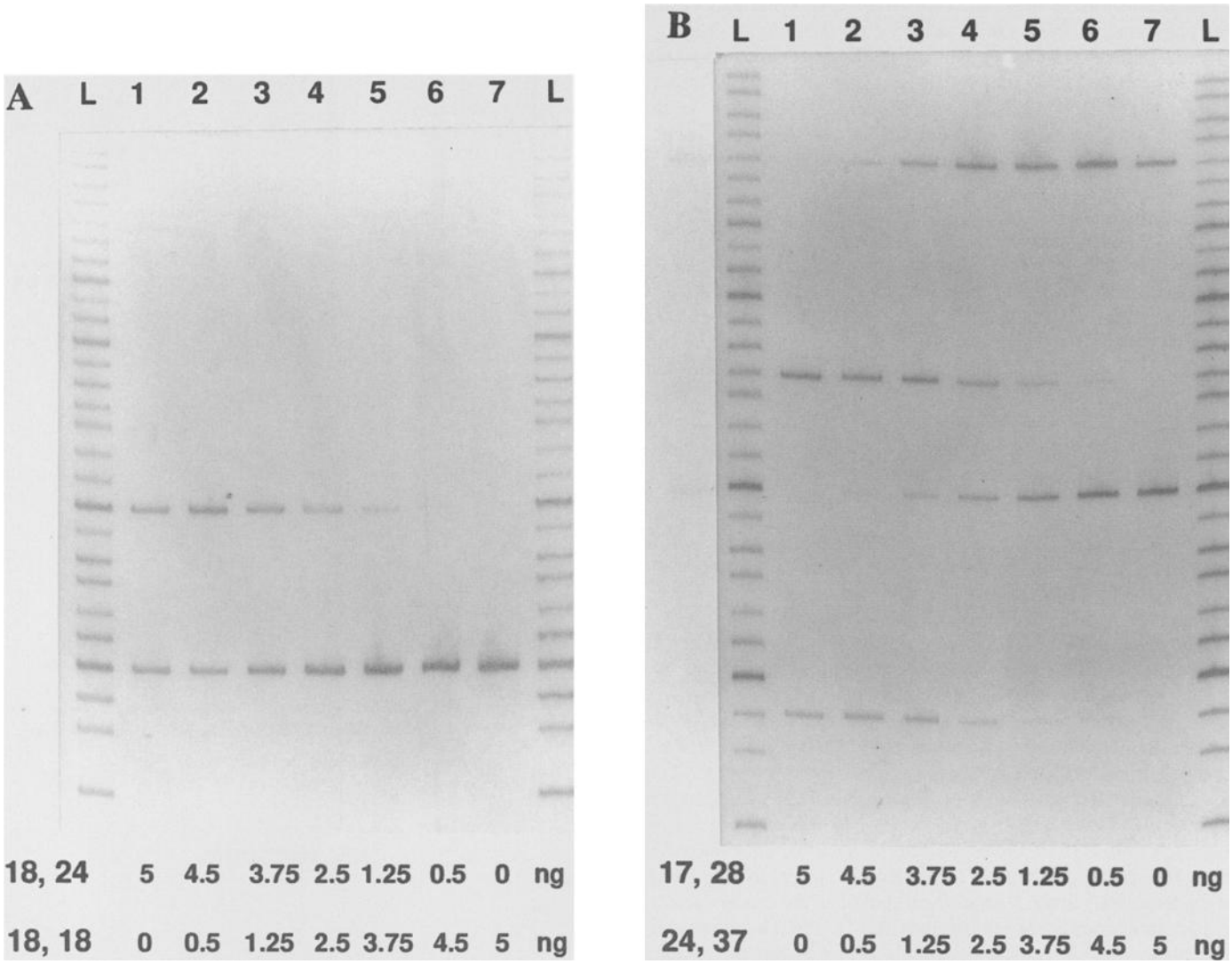


FIG. 3—*D1S80 amplification of purified DNAs mixed in defined ratios. Two purified DNAs that share an allele* (A) *and two DNAs that do not share an allele* (B) *were mixed as defined below each lane prior to amplification. The total amount of DNA added to each reaction was 5 ng. D1S80 amplification and typing were performed as described in the Materials and Methods section. The D1S80 Allelic Ladder was run in lanes labelled "L".*

smaller allele [*22*]. Typing results obtained from partially degraded DNA samples may be ambiguous in that they can be indistinguishable from results obtained from certain mixtures of DNAs (for example, 18,18 DNA mixed with 18,24 DNA—Fig. 3*A*). In some instances, the DNA may be degraded to the extent that only the smaller of widely disparate alleles is amplified. Degraded DNA studies have been repeated here with a sample containing D1S80 alleles 17 and 28 (417 bp and 593 bp, respectively). The PCR products were analyzed as described in the Materials and Methods section, and the results in Fig. 4*B* indicate that the intensity of both alleles decreases at approximately the same rate. While it may be possible to detect a trace of a band for only one of the alleles at the 12′ timepoint, the signal is so faint that no call should be made. The molecular weight of the DNA fragments from the 12′ timepoint range from approximately 1300 bp to 500 bp with the majority near 800 bp. The effects of other environmental insults can be assessed by carefully monitored studies such as those described by Comey and Budowle [*6*].

Studies 4.1.5.7, 4.1.5.8—Matrix Study and Nonprobative Evidence Study

Two of the most important studies in the validation process are the Matrix Study (examination of body fluids on commonly encountered substances) and the Nonprobative Evidence Study (examination of actual casework evidentiary material). To perform these validation studies, evidentiary bloodstains deposited on a variety of substrates were obtained from D. Gregonis of the San Bernardino Sheriff's Department. The evidence samples and the corresponding reference samples were provided in a blind study format. Most samples were phenol/chloroform extracted; a few

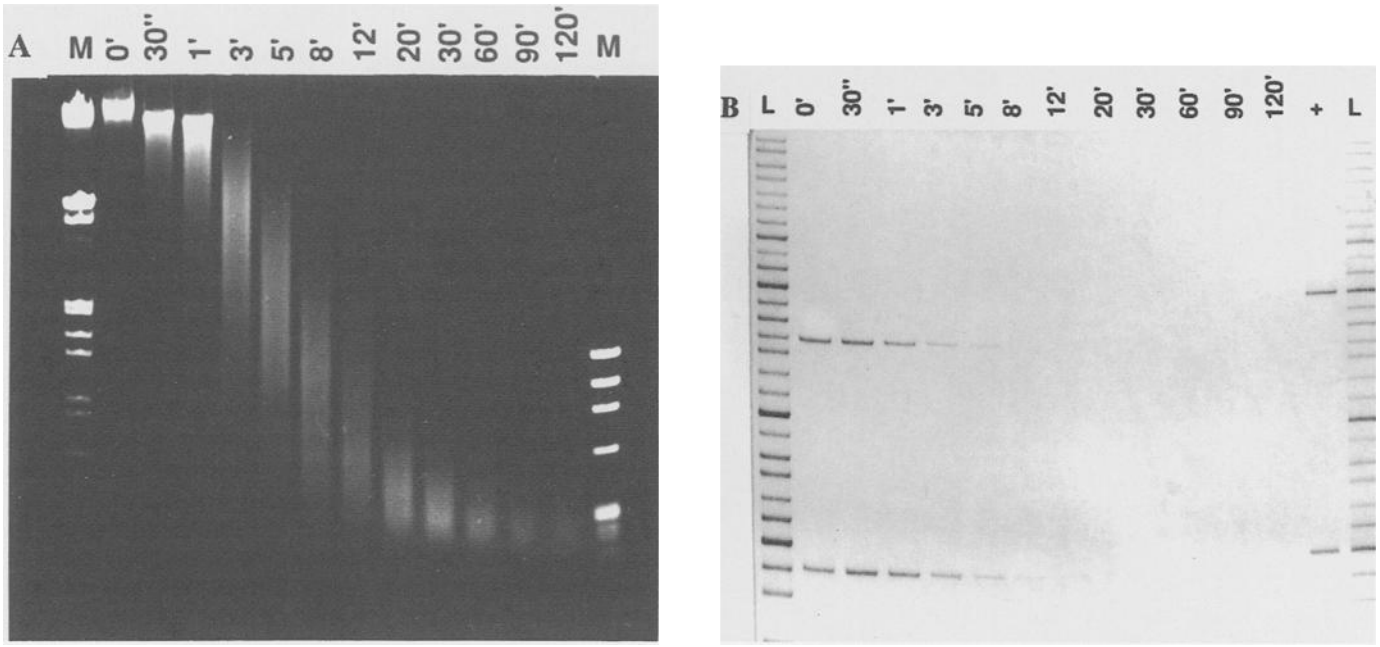


FIG. 4—*D1S80 typing of degraded DNA samples.* A) *High molecular weight human genomic DNA (0.1 mg/mL) was degraded with 1 × 10^{-4} DNase I for increasing amounts of time as indicated above each lane and run on a 1% agarose gel in 1X TAE buffer. The molecular weight marker (M) in the first lane is λ DNA digested with Hind III and EcoRI. The visible bands range in size from approximately 21200 bp to 950 bp. The molecular weight marker (M) in the last lane is PhiX174 DNA digested with Hae III. The visible bands range in size from approximately 1350 bp to 300 bp.* B) *Five nanograms of DNA from each degradation timepoint were amplified in the TC480 and run on a Detection Gel as described in the Material and Methods section.*

were extracted with Chelex. The phenol/chloroform extracted samples were evaluated on DNA yield gels (Fig. 5*A*).

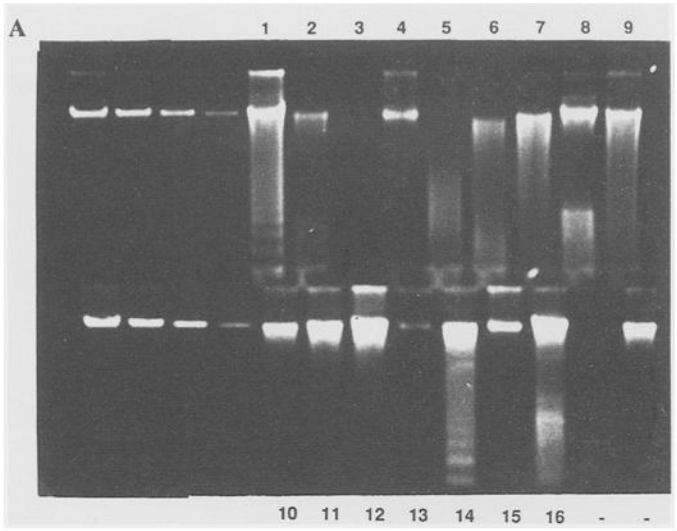
Forty five samples were amplified and typed as described in the Materials and Methods section and all but nine samples produced typeable D1S80 bands. The D1S80 results obtained from 15 samples are shown in Fig. 5*B,* which includes six of the nine non-typeable samples. The non-typeable samples were reamplified with an additional 5U or 10U AmpliTaq® DNA Polymerase added to the PCR reaction mixture. The addition of extra Taq DNA polymerase resulted in D1S80 types for 6 of the 9 previously non-typeable samples, five of which are shown in Fig. 5*C* (samples 6, 8, 10, 13, and 16). These samples most likely contained Taq enzyme inhibitors. Thus, the AmpliFLP D1S80 kit yielded typing results for 42 of the 45 actual casework samples, and the evidence samples were successfully paired with their respective reference samples. The three non-typeable samples (extractions from blue denim, a leaf and cotton cloth) were unsuccessful in AmpliType® PM and gender typing also (R. Reynolds and N. Fildes, unpublished observations), presumably because these samples contained potent or excessive PCR inhibitors or DNA that is too degraded to support amplification.

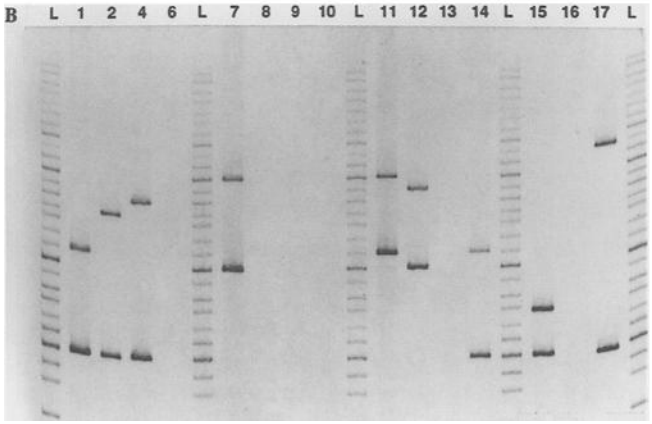
To compare the performance of the TC480 and the GAPS 9600, seven of these nonprobative evidence samples were amplified in both instruments (data not shown). The same D1S80 types for each sample were obtained from both instruments and the relative band intensities within each sample were the same (that is, no increase in preferential amplification relative to the other instrument).

To assess the performance of this D1S80 kit with actual sexual assault samples, two "no suspect" rape kits were obtained from the Oakland, CA, Police Department Crime Laboratory. Among the samples present in the kits were vaginal swabs and reference buccal swabs from the victim. These samples were extracted with Chelex according to the AmpliType User Guide [*16*], amplified for 29 cycles and typed according to the protocols in the Materials and Methods section. Rape kit A in Fig. 6 shows D1S80 PCR products amplified from the female epithelial cell fraction, the sperm cell fraction (apparently mixed with residual epithelial cell DNA), and the extracted buccal swab reference material. There are four D1S80 alleles observed in the sperm cell fraction, and two of the bands (alleles 28 and 33) are significantly darker than the other two. Using the D1S80 type obtained from the female reference material, it is possible to assign allele types present in the sperm cell fraction to the victim and assailant, assuming there was only one sperm donor, because the lighter bands correspond to the victim's D1S80 type (alleles 24, 31). Rape kit B in Fig. 6 also shows D1S80 PCR products amplified from the epithelial cell fraction, the sperm cell fraction, and the extracted buccal swab reference material. The sperm cell fraction does not appear to contain a mixture. However, in this example, the victim and assailant share an allele ("21").

Study 4.1.5.9—Nonhuman Studies

A panel of nonhuman DNAs (100 ng) was amplified and analyzed under the standard D1S80 conditions described in the Materials and Methods section using 30 cycles. As expected, amplified product was observed from gorilla, chimp and orangutan DNA, but only the gorilla products were in the size range of the D1S80 Allelic Ladder (data not shown). No products were observed from samples containing bacterial or yeast DNA (50 ng and 100 ng, respectively), which are more likely to be present in routine casework samples than animal fluids (data not shown).





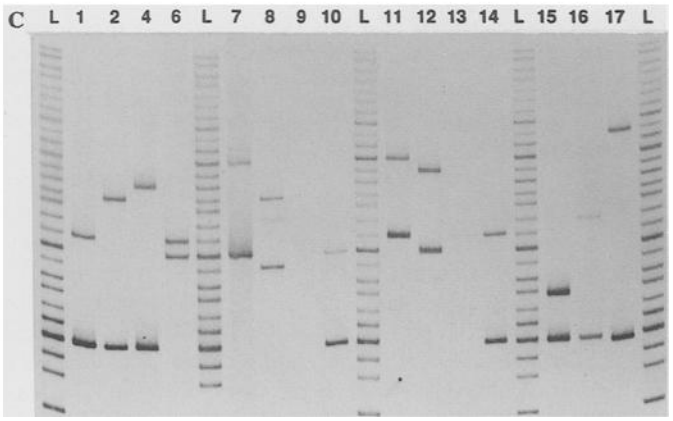


FIG. 5—*D1S80 typing of nonprobative casework samples.* A) *DNA was extracted from nonprobative casework bloodstains deposited on the following substrates: 1. nylon/polyester blanket batting; 2. cotton sheet; 3. denim (blue); 4. cotton cloth; 5. leaf; 6. throw rug; 7. trilobal nylon carpet; 8. dacron carpet; 9. grease/soil; 10. denim (blue); 11. nylon seat cover; 12. wooden chair; 13. paint from car; 14. cotton thermal shirt; 15. galvanized roof; 16. trilobal nylon carpet; 17. cotton cloth; 18. cotton/acrylic shirt; 19–23. cotton cloth; 24. wet rock; 25. cotton/polyester shirt; 26. cotton t-shirt; 27–45. cotton cloth. Sixteen of the extracted samples were run on this 1% agarose/1 × TAE yield gel. The first four lanes contain 200, 100, 50 and 20 ng of purified DNA. The remaining lanes contain 5 μL of extracted DNA from the samples listed.* B) *Fifteen nonprobative evidence samples were amplified and typed as described in the Materials and Methods section. Samples 1, 2, 4 and 6–16 were described in A. Sample 17 is a reference bloodstain. Samples 3 and 5 (blue denim and leaf, respectively) did not amplify and were not included on the gel.* C) *To the six samples described in B that did not amplify, an additional five units of AmpliTaq DNA Polymerase were added to the reaction mixture. The D1S80 PCR products were detected on a Detection Gel, and only Sample 9 remained untypeable. (Sample 13 was amplified very weakly and is barely visible on the gel in this figure.) Sample 9 was successfully amplified after 10 units of Taq DNA Polymerase were added to the reaction mixture (not shown). Lanes labelled "L" contain the D1S80 Allelic Ladder.*

Studies 4.1.5.10 and 4.4.1.4—Minimum Sample Study and Cycle Number Study

AMP-FLP markers are particularly sensitive to the amount of input DNA and the cycle number. Too much input DNA and too many cycles can lead to extraneous bands, preferential amplification of the smaller allele, and "thick" product bands that may not be typed unambiguously. After the silver-stained GeneAmp Detection Gel system for D1S80 typing was adopted over the significantly less sensitive agarose system, the sensitivity and cycle number aspects of the assay described in the D1S80 Reagent Set had to be readdressed.

To address Study 4.1.5.10, a DNA sample was serially diluted and used to set up duplicate D1S80 reactions containing from 10 ng to 0.16 ng per reaction. One set of reactions was amplified in the TC480 for 29 cycles and the other set was amplified for 30 cycles (Fig. 7). D1S80 products amplified from 300 pg were visible after 29 and 30 cycles of amplification. While D1S80 products

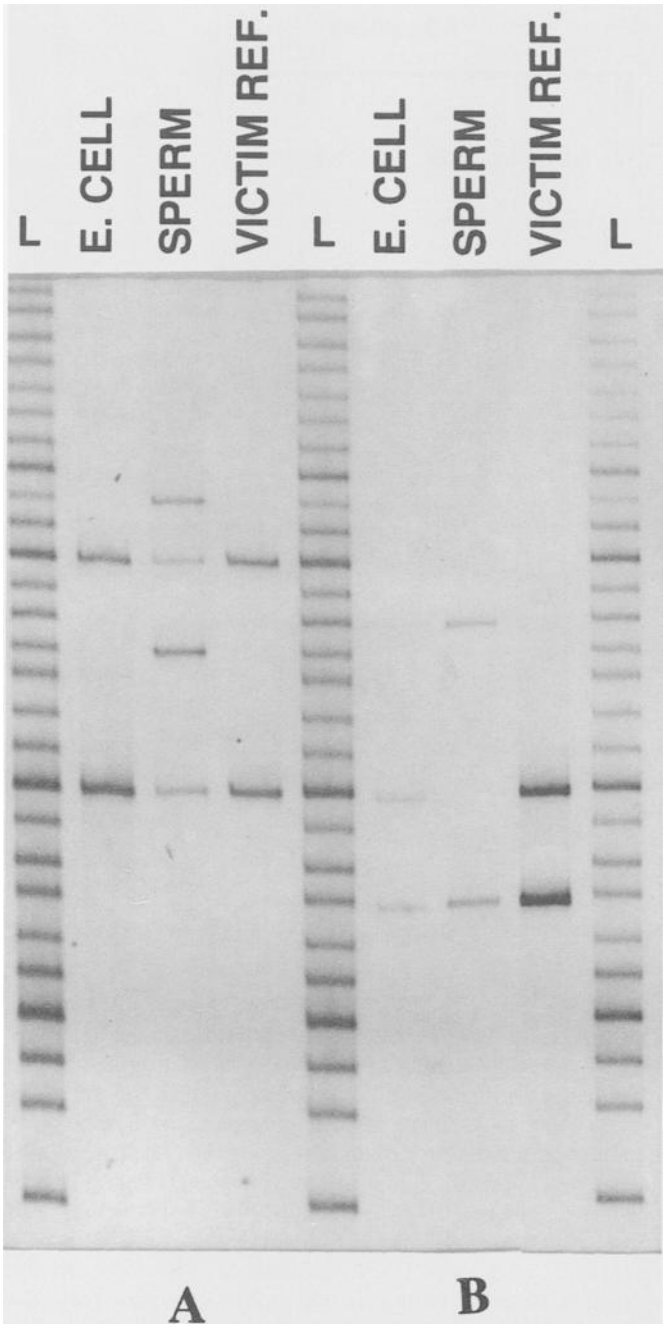


FIG. 6—*D1S80 typing of two "no suspect" sexual assault cases. Vaginal swabs from two sexual assault kits were differentially extracted with Chelex to produce an epithelial cell fraction and a sperm cell fraction. In addition, a buccal swab reference sample from the victim was also Chelex extracted. The samples were amplified for 29 cycles and typed on a Detection Gel as described in the Materials and Methods section. Kit A shows a mixture in the sperm cell fraction in which the two lighter bands (24 and 31) correspond to the victim's D1S80 type. The darker bands in the sperm cell fraction, presumably corresponding to the assailant, are 28 and 33. From Kit B, there is no apparent mixture in the sperm cell fraction.*

can frequently be detected from less than 1 ng input DNA using either 29 or 30 cycles, RMS chose to guarantee a reliable typing result from 2.5 ng purified input DNA at 29 cycles in both PCR instrument systems.

The gel in Fig. 7 shows the D1S80 typing results obtained from decreasing amounts of input DNA after 29 and 30 cycles of amplification in the TC480. In Fig. 8, D1S80 products amplified from 2 and 5 ng of two input DNAs after 29 cycles of amplification in the TC480 and GAPS 9600 instruments are compared. The D1S80 alleles contained in both DNA samples can be typed unambiguously from 2 ng of DNA amplified in both instruments. The yield of PCR product amplified in the GAPS 9600 tends to be slightly lower than the TC480 but in no way compromises the ability to obtain a reliable result. These amplification and typing results support the decision to reduce the cycle number from 30 to 29 for both instruments and to increase the AmpliFLP D1S80 Kit sensitivity claim from 5 ng to 2.5 ng.

Study 4.2.1 Inheritance Study

Extracted DNA samples from a CEPH family (Centre d'Etude du Polymorphisme Humain) consisting of maternal and paternal grandparents, parents (mother: 22,24; father: 18,29) and 11 offspring, were amplified according to the Materials and Methods section using 29 cycles. As expected, only the four possible D1S80 genotypes predicted by the laws of Mendel (18,22; 18,24; 22,29; 24,29) were present in the offspring (data not shown). These results are consistent with previously published family studies using RFLP analysis [*17*] and PCR amplification [*9*] of the D1S80 locus.

Guideline 4.4.2.1—Characterization without Hybridization

This guideline states that "appropriate standards for assessing the alleles shall be established." With AMP-FLP markers, the most relevant and accurate standard is a ladder containing a collection of the marker's alleles. RMS created a D1S80 allelic ladder that contains 27 alleles (alleles 14 and 16–41) and covers over 98% of observed common alleles (Fig. 2). D1S80 alleles for which variants have been observed are represented in the ladder by the most common form of the allele. The bands in this ladder have the same mobility as the corresponding bands in the FBI D1S80 ladder, (S. Baechtel, personal communication) which has been used to collect D1S80 population data from around the world. The FBI and RBL organizations generously contributed samples for the construction of this ladder. Alleles containing greater than 41 repeat units cannot be typed accurately with this or any other known ladder. However, the individual amplified evidence and reference samples may be mixed prior to gel electrophoresis to determine if they contain the same or indistinguishable alleles (single >41 band) or different alleles (two >41 bands).

The ladder allele designations correspond to the number of tandem repeat units present in each amplified D1S80 allele. The AmpliFLP D1S80 Allelic Ladder has been designed such that allele bands 18, 24, 31 and 34 are somewhat darker than the other bands. These alleles include the most common alleles across the three population groups (see Study 4.1.5.3), and the darker bands aid the analysis of amplified samples whether an imaging system or a manual approach is used.

Discussion

The D1S80 PCR Amplification and Detection Gel Kits are simple to use, and the silver stain detection method uses inexpensive, common laboratory reagents. The D1S80 Allelic Ladder containing 27 alleles allows samples to be typed reliably and accurately. Using these kits, the D1S80 assay is rapid (amplification, electrophoresis and detection are easily accomplished in one day), sensitive (2.5 ng input DNA) and versatile (can be performed in both the TC480 and GAPS 9600 instruments). The results presented in this paper also demonstrate that this D1S80 system

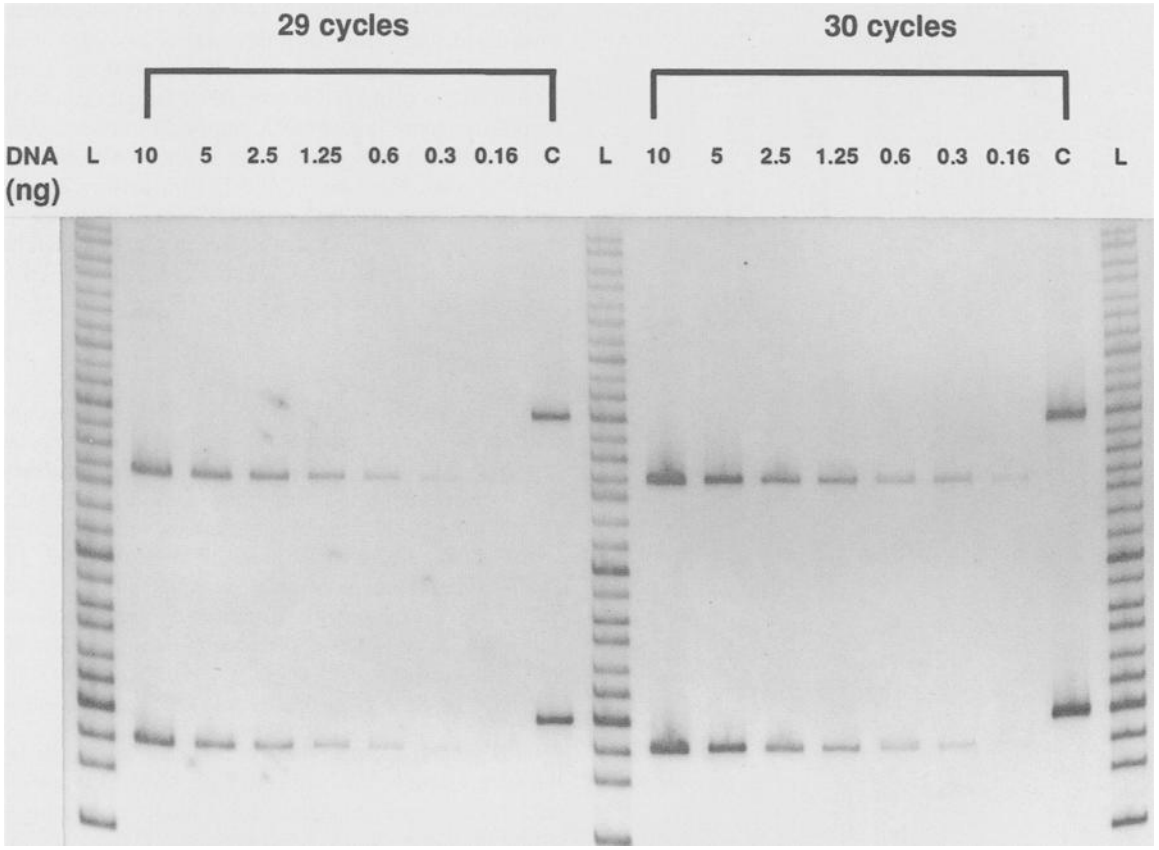


FIG. 7—*Determination of minimum sample required to obtain reliable D1S80 typing results. D1S80 products amplified from a range of input DNA quantities (indicated above each lane) in the TC480 for 29 and 30 cycles were run on a Detection Gel as described in the Materials and Methods section. The D1S80 products in lanes labelled "C" were amplified from 10 ng of the control DNA provided in the D1S80 Kit (alleles 18 and 31). The D1S80 Allelic Ladder was run in lanes labelled "L".*

is robust and suitable for forensic DNA typing. However, since the range of input DNA observed to yield the most specific D1S80 PCR products is narrow (2.5 to 10 ng), it is important to determine the quantity of DNA in the sample prior to amplification (for example, using the D17Z1 probe-based QuantiBlot kit). Amplification of too much DNA may produce non-specific amplification products, which could interfere with mixture interpretation, or "thick" bands on the gel, which cannot be sized accurately. Amplification of too little DNA may not yield a result, or in some cases may yield only a very faint single band from a heterozygous sample.

The experiments outlined in the TWGDAM Guidelines were designed to address challenges frequently encountered by forensic scientists. It is often necessary to type and identify several fluid or tissue samples from a crime scene. Studies 4.1.5.1 and 4.1.5.4 clearly demonstrate that not only will different tissues from a single individual result in the same D1S80 type, but a liquid and stain sample from one individual will yield consistent D1S80 results. The method of extraction, either Chelex or phenol/chloroform, appears to have no influence on the outcome of the D1S80 result. However, if a sample is likely to contain degraded DNA, organic extraction may be preferable since the boiling step in the Chelex extraction protocol may further degrade the DNA. Chelex extraction may be used for victim and suspect reference samples collected under controlled conditions because it is faster and less hazardous than organic extraction.

Forensic scientists frequently must handle samples containing DNA from more than one individual, such as sexual assault samples. To evaluate the ability of the D1S80 system to detect and distinguish mixtures, carefully controlled experiments in which known quantities of DNA from homozygous and heterozygous samples were mixed and coamplified. The results indicate that when a homozygous sample is mixed with a heterozygous sample where one allele is shared, the unique allele is significantly less intense than the shared allele when the heterozygous sample is present at $^1/_{10}$ of the total DNA. A difference in intensity can also be detected in mixture ratios of $^1/_4$ and $^1/_2$. However, these particular mixtures need to be interpreted carefully. For example, it is possible that the result did not arise from a DNA mixture but rather from amplification of degraded DNA where the smaller allele was preferentially amplified over the larger allele. In mixed samples containing two heterozygous samples with no shared alleles, mixtures can be easily detected. However, assigning specific alleles to individuals is dependent upon the ratio of the DNAs being mixed. The relative quantities of the two DNAs must be sufficiently different to result in significantly unequal band intensities. The two sets of results from the rape kit samples demonstrate that mixtures can be detected and, when the appropriate reference samples are included, be resolved when there are no shared alleles.

Another challenge faced by forensic scientists is the analysis of samples containing degraded DNA. DNA degradation is a major concern in the amplification of AMP-FLPs as severely degraded samples may produce uneven band intensities between large and small alleles. Multiple environmental factors such as exposure to

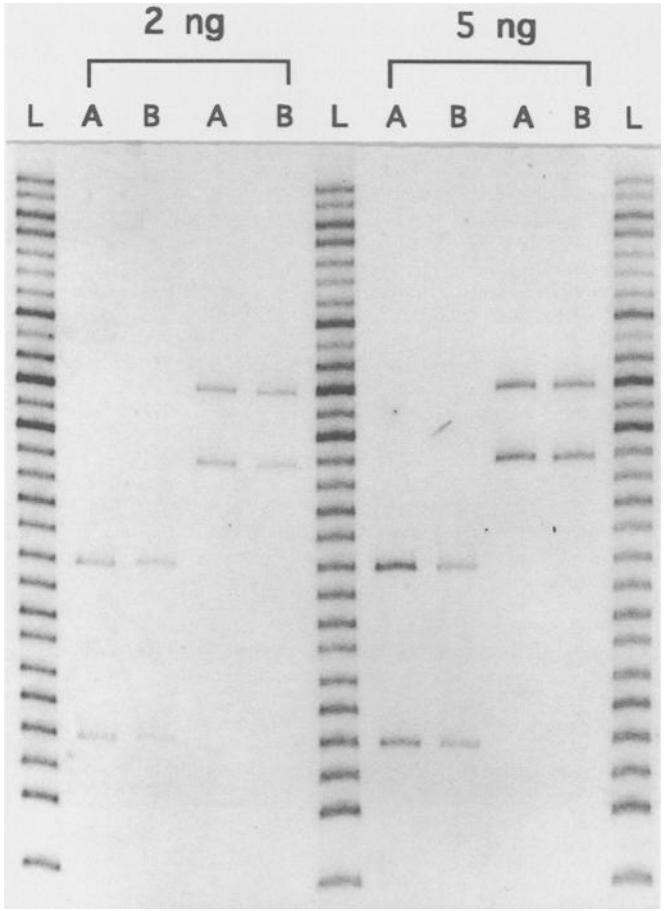


FIG. 8—*Determination of cycle number required to obtain reliable D1S80 typing results. D1S80 products amplified from 2 and 5 ng of two purified DNA samples in the TC480 and the GAPS 9600 for 29 cycles were run on a Detection Gel as described in the Materials and Methods section. For each pair of lanes, the TC480 PCR products are in the lanes labelled "A" and the GAPS 9600 PCR products are in the lanes labelled "B". An early version of the D1S80 Allelic Ladder was run in lanes labelled "L". This version contains all 27 alleles but the intensities of bands 18, 24, 31, and 34 were not adjusted as described in Guideline 4.4.2.1.*

soil, dyes, or high temperatures can contribute to the degradation of DNA in casework samples. Purified DNA degraded with DNase I for increasing amounts of time can be used to mimic and evaluate the effects of degradation on DNA typing systems. For the D1S80 study, a DNA sample with widely disparate alleles (17 and 28) was chosen to increase the potential for preferential amplification of the smaller allele. The results using silver stained gels indicated that the PCR product bands decreased in intensity at approximately the same rate. Consequently, it is unlikely that incorrect typing results will be obtained from degraded DNA using this D1S80 kit. However, as with all DNA typing systems, it is important to make an independent assessment of DNA quality when possible and to understand the limits of the amplification system. The results in Fig. 4 indicate that although the majority of the DNA fragments were greater in length than the larger D1S80 allele (allele 28; 593 bp), no typeable result was obtained. Frequently a result can be obtained from degraded DNA by adding additional DNA to the PCR (R. Reynolds, unpublished observations). However, a useful guideline may be that samples likely to contain degraded DNA should first be analyzed by systems involving relatively smaller PCR amplified regions (for example, ~100–300 bp), particularly if the sample is very limited.

Because the quality of a DNA sample can be compromised by the substrate on which the sample is deposited, it is essential in the validation procedure to demonstrate that body fluids deposited on commonly encountered substances will be accurately typed using this D1S80 kit. Out of 45 actual casework and reference bloodstains deposited on a wide variety of substrates, 42 were correctly typed and matched to their corresponding reference sample. No D1S80 type was obtained for three of the 45 samples. This lack of amplification is most likely due to excessive or potent inhibitors or highly degraded DNA, particularly since these samples could not be amplified using the AmpliType PM system (in which the largest PCR product is only 242 bp). Six of the 42 typed samples required additional AmpliTaq DNA polymerase to overcome inhibitors present in the samples. Re-amplification of samples 10 and 16 using additional Taq DNA polymerase resulted in preferential amplification of the smaller allele in both samples. One cause of preferential amplification of AMP-FLP alleles is limiting amounts of active Taq DNA polymerase [*22*], and it is possible these two samples contain a level of inhibitor(s) that inactivates some but not all of the Taq DNA polymerase added to the reactions. The source of the faint third band in re-amplified sample 8 is not known; AmpliType PM and HLA DQA1 typing of this sample did not reveal a mixture.

In summary, the AmpliFLP D1S80 Kit combined with the GeneAmp Detection Gel can be used successfully to amplify and type many different kinds of forensic casework samples, provided the extracted DNA is amplifiable. As with any forensic DNA analysis system, it is desirable to know as much as possible about the sample prior to analysis (for example, amount of DNA, state of degradation). However, the results clearly demonstrate that even compromised DNA samples can produce accurate and reliable typing results using this D1S80 system.

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