TECHNICAL NOTE

Chantal J. Frégeau,[1] Ph.D.; Kathy L. Bowen,[1] B.S.; and Ron M. Fourney,[1] Ph.D.

Validation of Highly Polymorphic Fluorescent Multiplex Short Tandem Repeat Systems Using Two Generations of DNA Sequencers

REFERENCE: Frégeau CJ, Bowen KL, Fourney RM. Validation of highly polymorphic fluorescent multiplex short tandem repeat systems using two generations of DNA sequencers. J Forensic Sci 1999;44(1):133–166.

ABSTRACT: Validation studies are a crucial requirement before implementation of new genetic typing systems for clinical diagnostics or forensic identity. Two different fluorescence-based multiplex DNA profiling systems composed of amelogenin, HumD21S11 and HumFGA (referred to as multiplex 1A), and HumD3S1358, HumD21S11 and HumFGA (multiplex 1B) have been evaluated for use in forensic identification using the Applied Biosystems Model 373A and Prism™ 377 DNA Sequencers, respectively. Experiments were aimed at defining the limit of target DNA required for reliable profiling, the level of degradation that would still permit amplification of the short tandem repeat (STR) loci examined, and the robustness of each locus in the multiplexes after samples were exposed to environmental insults. In addition, the specificity of the multiplexes was demonstrated using nonhuman DNAs. Forensically relevant samples such as cigarette butts, chewing gum, fingernails and envelope flaps were processed using both an organic extraction procedure and a QIAamp protocol. DNAs and resultant multiplex STR profiles were compared. The validation of the triplex STR systems was extended to include over 140 nonprobative casework specimens and was followed with a close monitoring of initial casework (over 300 exhibits). Our results document the robustness of these multiplex STR profiling systems which, when combined with other multiplex systems, could provide a power of discrimination of approximately 0.9999.

KEYWORDS: forensic science, short tandem repeat, validation, multiplex, fluorescence, polymerase chain reaction, sequencer

Individual identification through genetic analysis is a key element in the investigation of crimes (1–3), the resolution of disputed paternity cases (4,5), the re-association of fragmented human remains following fatal accidents (airplane crash, gas explosion, bombs, fire, flood) (6–10), or verification of biological origin in potential sample mix-up in a clinical setting (11). Genetic analysis of forensic specimens or archival material using the traditional restriction fragment length polymorphism (RFLP) strategy is often limited by the quantities of material and/or degradation state of the genetic material recovered from these samples (2,3,12–14). The use of the polymerase chain reaction (PCR) with the smaller size range variable number of tandem repeat (VNTR) loci (referred to as STRs; (15)) in a multiplex fashion, combined with the highly sensitive four-color fluorescence-based detection technology, has enabled reliable identifications from challenging forensic specimens (2,9,16–23).

We reported earlier (24,25) the development of a fluorescence-based multiplex DNA profiling system comprising two highly polymorphic STR loci, HumD21S11 (26) and HumFGA (27), and the amelogenin gender determination system (28,29). In the present report, we document the extensive validation studies performed and casework experience gained using this triplex system on the Applied Biosystems Model 373A DNA Sequencer. Validation experiments were also conducted using a modified triplex comprising HumD3S1358 (30), HumD21S11 and HumFGA on the second-generation ABI Prism™ 377 DNA Sequencer. Initial experiments were designed to determine the optimal amount of target DNA for reliable amplification results on both analytical instruments. As a complement to previous reports documenting the suitability (quantity and quality) of DNA extracted from specimens under extreme environmental conditions for RFLP profiling (31–34) or AmpFLP/STR profiling (6–10,16,35–40), this paper further examined adverse conditions relevant to fluorescence-based amplification including 1) the presence of potential amplification inhibitors from substrates, 2) the level of DNA degradation induced by environmental insults, and 3) the role artificial whitening agents present in a variety of detergents may have in interfering with the detection of fluorescently labeled DNA fragments. Experiments incorporating a large number of primate and nonprimate individuals were also performed to investigate the specificity of the two multiplex systems. In addition, the multiplex STR systems were tested on simulated sexual assault cases, as well as forensically relevant samples including cigarette butts, chewing gum, fingernails and envelope flaps extracted using two different protocols. The final stage of the validation involved the use of the multiplex systems on a range of nonprobative casework items (approximately 140) to verify their reliability. Corroboration of the multiplex STR typing results with genetic profiles derived from conventional DNA technology was also established. Following the validation phase, initial

[1] Royal Canadian Mounted Police, Central Forensic Laboratory, DNA Methods and Data Base, 1200 Vanier Parkway, Ottawa, Ontario, Canada K1G 3M8.

Received 6 Nov. 1997; and in revised form 8 June 1998; accepted 25 June 1998.

cases were processed using the STR multiplexes and closely monitored for any peculiarities. The results obtained from all stages of the testing procedure and results from initial casework are described below.

Materials and Methods

Samples—The control cell lines GM9948 (41, NIST Standard Reference Material #2391 PCR-based DNA Profiling Kit) and GM9947A (41) were used as sources of pristine DNA in some experiments and also served as positive amplification controls. Liquid blood, semen, vaginal swabs, buccal swabs, saliva, scalp and pubic hair, cigarette butts, chewing gum, fingernails and envelopes used in the validation experiments herein were obtained from volunteers among laboratory personnel. Nonhuman DNA samples were kindly provided from the following sources: Metropolitan Toronto Zoo (Dr. Kay Mehren), Calgary Zoo (Dr. R. Cooper), Saskatoon Bovine Typing Laboratory (Dr. Yves Plante), Animal Resources/Health Canada (Dr. Bill Pierce), Department of Zoology/University of Western Ontario (Dr. David Ribble), Department of Zoology/University of Alberta (Dr. Curt Strobeck), Department of Biochemistry/University of British Columbia (Dr. Lorne Kirby), Department of Biochemistry/University of Alberta (Dr. Bernard Lemire), Laboratory Centre for Disease Control/Health Canada (Dr. Joan Dillon) and BiosLab (New Haven, Connecticut). Nonprobative casework items (a total of 142) were provided from the RCMP Biology Operational Section to the RCMP DNA Methods and Data Base Section as quantitated DNA extracts remaining from completed RFLP cases. Following the validation phase, casework samples (approximately 300) deemed appropriate for PCR STR analysis were examined.

DNA Extraction—The control cell line GM9948 and all other human samples including casework specimens and blood from some animals used in the validation studies were subjected to standard DNA extraction protocols using organic solvents and ethanol precipitation (RCMP Methods Manual, 1990). Differential extractions were also performed on specimens containing semen to separate the female cell component (referred to as F2 fraction) from the male cell component (F3 fraction) (RCMP Methods Manual, 1990).

DNA Quantitation—Quantitation of human genomic DNA in biological samples was determined using the slot blot hybridization procedure developed by Waye and colleagues (42) and the D17Z1 α-satellite probe. Nonhuman DNA samples were quantified by UV spectrophotometry.

Agarose Gel Electrophoresis—DNA samples included in the environmental studies and degradation studies were separated electrophoretically on 0.8% agarose 1X Tris-Borate-EDTA gels (1XTBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA) to check for the presence of high molecular weight genomic DNA. Gels were stained with ethidium bromide (0.5 μg/mL in 1XTBE).

PCR Primers—Two multiplex STR systems were evaluated in this study. The triplex 1A was comprised of the highly polymorphic STR loci HumD21S11 and HumFGA and the gender determination system, amelogenin. The triplex 1B consisted of three discriminatory STR loci, HumD3S1358, HumD21S11 and HumFGA. Triplex 1A profiles were analyzed on the ABI 373A DNA Sequencer and triplex 1B profiles, on the ABI Prism™ 377 DNA Sequencer. The primer sequences for amelogenin and each STR locus examined, along with the fluorochromes utilized to tag the primers, are provided in Table 1. Also included in the table are the amplicon size ranges for the gender determination system and each STR locus as estimated on the ABI 373A DNA Sequencer or the ABI Prism™ 377 DNA Sequencer and the corresponding allele designations according to Möller et al. (43), Urquhart et al. (44), Barber et al. (45) and Lazaruk et al. (46).

Amplification Conditions—For the triplex 1A, the amplification cocktail consisted of 8 to 10 ng of genomic DNA (or as otherwise

TABLE 1—*Multiplex STR systems used in present study.*

Triplex 1A analysed on the ABI 373A DNA Sequencer		Fluorochrome (5′ Label)	Amplicon size range (bases)*	Allele designation
Amelogenin [44, 45]	Forward: 5′-CCC TGG GCT CTG TAA AGA ATA GTG-3′ Reverse: 5′-ATC AGA GCT TAA ACT GGG AAG CTG-3′	JOE or FAM	106–112	X, Y
HumFGA [44, 45]	Forward: 5′-GTG ATG ATG GCA TTT CTA TCC-3′ Reverse: 5′-CTT CTG TAT TTC CCA TAG CTT CTA TC-3′	TMR	203–244	17–30.2
HumD21S11 [44]	Forward: 5′-TGT ATC TAT ATG TGT ATA TGT ATG TGT-3′ Reverse: 5′-GAT AAC AGA ATG TTT TTC TAT CCT TTG-3′	VIC/R6G	251–288	24–38
Triplex 1B analysed on the ABI Prism™ 377 DNA Sequencer				
HumD3S1358 [46]	Forward: modified by ABI (proprietary) Reverse: modified by ABI (proprietary)	TMR	112–156	12–20
HumD21S11 [44]	Forward: 5′-GTG ATG ATG GCA TTT CTA TCC-3′ Reverse: 5′-CTT CTG TAT TTC CCA TAG CTT CTA TC-3′	FAM	198–246	24.2–38.2 3.2–4
HumFGA [44]	Forward: 5′-TGT ATC TAT ATG TGT ATA TGT ATG TGT-3′ Reverse: 5′-GAT AAC AGA ATG TTT TTC TAT CCT TTG-3′	JOE	256–300	17–28

* Allele size ranges as estimated on the ABI 373A DNA Sequencer.
† Allele designations according to Möller et al. (43).
‡ Allele designations according to Urquhart et al. (44).
§ Allele designations according to Barber et al. (45).
¶ Allele designations according to Lazaruk et al. (46).

specified in the Results and Discussion section or in the tables), 0.05 μM (0.8 pmoles) of each amelogenin primer, 0.20 μM (3 pmoles) of each HumD21S11 primer, 0.24 μM (3.6 pmoles) of each HumFGA primer, 117 μM of each dNTP, 1 U of Ampli*Taq*® DNA polymerase (Perkin Elmer, Montréal, Québec), 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% (w/v) gelatin and 0.16 μg/μL of bovine serum albumin in a 15 μL final reaction volume. The amplification parameters for this triplex system were: denaturation at 95°C, 45 s, annealing at 60°C, 30 s and extension at 72°C, 30 s for 28 cycles (unless otherwise noted) in a Perkin Elmer GeneAmp 9600 thermal cycler. The amplification reactions were maintained at 4°C prior to analysis.

For the triplex 1B, the amplification cocktail consisted of 5 ng of genomic DNA (or as otherwise specified in the Results and Discussion section or in the tables), 0.08 μM (1.2 pmoles) of each HumD3S1358 primer, 0.25 μM (4 pmoles) of each HumD21S11 primer, 0.31 μM (5 pmoles) of each HumFGA primer, 109 μM of each dNTP, 1 U of Ampli*Taq*® DNA polymerase (Perkin Elmer, Montréal, Québec), 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% (w/v) gelatin and 0.16 μg/μL of bovine serum albumin in a 16 μL final reaction volume. The amplification parameters for this triplex system were: denaturation at 95°C, 45 s; annealing at 60°C, 30 s and extension at 72°C, 30 s for 30 cycles (unless otherwise noted), followed by a 30 min extension at 60°C in a Perkin Elmer GeneAmp 9600 thermal cycler. The amplification reactions were left overnight in the dark at room temperature (22°C) prior to analysis.

Microcon-100 Column Purification—Casework DNA extracts that failed to amplify or amplified very weakly or partially (i.e., provided a profile for amelogenin only or for one of the two STR systems in the multiplex) were further purified using Microcon-100 microconcentrators (cutoff of 100 basepairs or 300 bases for DNA/RNA; Amicon, Inc., Beverly, MA) following recommendations from the manufacturer.

Analysis of Amplification Products—Analysis of the triplex 1A fluorescent amplified products was performed as follows: to 3 μL (or as specified in the tables) aliquots of the PCR reaction were added 4 μL of denaturing loading buffer (5 mg/mL blue dextran, 8M urea, 1X TBE) and 0.5 μL of ABI Genescan™ 2500 ROX internal lane standard. Following denaturation at 90°C for 5 min, the entire aliquot of each sample was loaded on a 6% (19:1) acrylamide:bisacrylamide gel containing 8 M urea, (12 cm well-to-read glass plate format) that had been prerun at constant voltage (1500 V) for 30 min and equilibrated to 45 to 50°C. Electrophoresis was conducted for 3.5 h at constant voltage (1200 V) in 1X TBE using an ABI 373A DNA Sequencer with the photomultiplier tube capacity set at 800 V and the laser at 20 mW. Allele sizes were determined using the ABI 672 Analysis version 1.2.1 software and the local Southern size calling method.

Analysis of the triplex 1B fluorescent amplified products was performed as follows: to 1.5 μL aliquots of the PCR reaction were added 4.5 μL of denaturing loading buffer (20 mg/mL blue dextran, 7.3M urea, 2X TBE, 20 mM EDTA) and 0.5 μL of ABI Genescan™ 350 ROX internal lane standard. Following denaturation at 95°C for 2 to 3 min, samples were snap-cooled in ice-cold water, then 1.5 μL aliquots were loaded on a 4% (19:1) acrylamide:bisacrylamide gel containing 6 M urea (36 cm well-to-read glass plate format) which had been prerun at constant voltage (1000 V) for 30 min and equilibrated to 51°C. Electrophoresis was conducted for 2 h at constant voltage (3000 V) in 1X TBE using an ABI Prism™ 377 DNA Sequencer with the laser at 40 mW. Allele sizes were determined using the GeneScan Analysis version 2.0.2 software and the local Southern size calling method.

PCR products were only scored as alleles if 1) the peak was located in the appropriate size range of a given STR locus, 2) the height of the peak (measured on an arbitrary scale using Genescan 672 software, version 1.2.1 or 2.0.2) was greater or equal to 20 fluorescent units (FU), and 3) the ratio of the highest background peak, in the size range of the locus, over the putative allele peak was ≤40%.

Validation Experiments

Sensitivity Studies—In order to define the lower limit of template DNA to use for reliable triplex 1A profiling on the ABI Model 373A DNA Sequencer, GM9948 control cell line DNA was serially diluted from 10 ng to 0.005 ng and amplified under conditions detailed above using 28, 32 or 34 cycles. A similar titration experiment was carried out to test the sensitivity of the ABI Prism™ 377 DNA Sequencer using the triplex 1B system. In this case, serial dilutions from the control cell line GM9948 were prepared (10 ng down to 0.04 ng) and amplified using 28, 30 and 32 cycles.

Environmental Studies

Substrate Study—Blood from one individual (125 μL) was deposited on cotton, cotton/polyester blend material, black leather, blue denim, newspaper, glass, linoleum floor tile and nylon rug, then air-dried and stored at −70°C until processed for DNA extraction.

Temperature, Moisture and Substrate Study—Blood from one individual (125 μL) was deposited on two sets of the following substrates: cotton, cotton/polyester blend material, black leather, blue denim, newspaper, glass, linoleum floor tile and nylon rug. One set of substrates was air-dried overnight. The second set was prepared fresh the next day. Both sets of substrates were subjected (for 30 min) to 37°C or 56°C in an incubator or 100°C or 200°C in an oven, then allowed to cool and air-dry at room temperature. The substrates were stored at −70°C until processed for DNA extraction.

Stability of DNA Extracts Left at Room Temperature for Various Lengths of Time—Liquid blood from one individual was processed for DNA extraction following procedures outlined in this section. This individual's DNA along with those of two control cell lines (GM9947A and GM9948) were left on the bench, at room temperature for various periods of time (6 h, 1 to 3 days, 1 to 4 weeks), then stored at 4°C until all samples could be processed simultaneously for PCR.

Stability of DNA from Semen and Bloodstains Following Long- and Short-Term Exposures to Sunlight and Darkness—Bloodstains (60 μL) and semen stains (40 μL) from one individual were prepared on washed cotton cloth. The stains were divided into two portions; one set was stored at room temperature in the light on the laboratory bench (fluorescent lighting and filtered sunlight from a window), i.e., was exposed to the diurnal cycle of sunlight and darkness, while the other set was maintained at room temperature

exclusively in the dark. Aliquots of the stains were removed at various times (1 day; 6, 12, 18 and 24 months) and were stored at −70°C for approximately two years prior to extraction. Bloodstain and semen stain controls were maintained at three different temperatures (4°C, −20°C and −70°C) over the entire time period.

Detergent Study—In order to determine the level of potential background fluorescence generated by artificial whitening agents found in detergents, pieces of white cotton cloth were treated with Ultratide (powder), Javex (powder), Ivory Snow, Spray and Wash, Liquid Tide and Fleecy softener under normal washing conditions, then blood from two individuals was deposited separately on the washed pieces. Stains were air-dried, then processed for DNA extraction.

Soiled DNA—A bloodstain prepared on cotton was treated simultaneously with a mixture of gasoline, motor oil, dirt, bleach, and 10% SDS to mimic extensive wearing of garments. The stain was extracted and the DNA was subjected to PCR.

Degradation Studies

Boiling, Freezing, Thawing—Seven micrograms of male control cell line DNA (GM9948) was diluted in 350 μL of TE buffer to make up a final concentration of 20 ng/μL. The DNA was then subjected to repeated cycles of boiling, freezing and thawing for the duration specified in Table 2. Aliquots of 5 μL (100 ng) were taken out following each cycle and stored at −70°C until all samples could be processed simultaneously for PCR.

Sonication—Eight micrograms of control cell line DNA (GM9948) was diluted in 400 μL of TE buffer to make up a final concentration of 20 ng/μL. The tube containing the DNA was placed in an ice bath, attached to a sonicator probe (Bronwill Biosonik III) and subjected to ultrasounds at intensity 50 for different time spans (2, 4, 8, 10, 20, 30, 40, 50, 60, 90 and 120 s). An aliquot of 5 μL (100 ng) was taken out following each session and frozen at −70°C until ready for PCR processing.

Nonhuman DNA Study—The species specificity of HumD3S1358, HumD21S11, HumFGA and amelogenin primers was examined in a large survey of animals, and microorganisms from the vaginal flora using different amounts of DNA (i.e., 5, 10, 50 and 100 ng). The triplex 1A amplicons were analyzed on the ABI 373A DNA Sequencer, and the triplex 1B amplified products on the ABI Prism™ 377 DNA Sequencer. Many representatives from the Higher Primates (8 gorillas, 8 orangutans and 5 chimpanzees) were evaluated in order to obtain information regarding the extent of STR polymorphism in these animals. Many members of the Old World monkeys group were also included in the study (8 Cynomulgus monkeys, 2 African green monkeys, 4 Rhesus monkeys and 1 macaque). One representative from the New World monkeys group was examined (marmoset). The 24 nonprimate species tested and number of specimens included: dog [3], cat [2], pig [8], cow [2], horse [2], sheep [3], mouse [4], hamster [1], lemming [2], deer [3], moose [2], goat [3], bison [1], elk [1], caribou [1], bighorn [1], whale [2], dolphin [1], penguin [2], shark [1], perch [1], marlin [2], kangaroo [1] and ostrich [2]. An effort was made to include animals representing both genders to examine the potential use of amelogenin for discriminating male and female animals. The 14 bacterial and yeast strains examined included: *Bacteroides vulgatis, Bacteroides fragilis, Bacteroides thetaiotaomicon, Streptococcus agalactiae, Streptococcus intermedius, Clostridium perfringens, Gardnerella vaginalis, Lactobacillus acidophilus, Lactobacillus fermentum, Peptostreptococcus asaccharolyticus, Candida albicans, Saccharomyces cerevisiae, Escherichia coli DH5* and *Escherichia coli HB101.* The amount of DNA amplified was equivalent to that used in standard casework assays (i.e., 5 ng for the model 377 instrument and 10 ng for the model 373A instrument). Fifty and 100 ng of template DNA were also amplified to simulate extreme situations of contamination.

Miscellaneous Samples—A series of miscellaneous samples

TABLE 2—*Degradation study: boiling, freezing, and thawing.*

Sample Aliquot*	Exposure time to boiling water (step 1)	Freeze time (step 2)	Thaw (step 3)	Total exposure time to boiling water	Total number of freeze-thaw cycles
1	30 sec	1 min	✓	30 sec	1
1-2	30 sec	30 min	✓	1 min	2
1-3	30 sec	2 min	✓	1.5 min	3
1-4	30 sec	5 min	✓	2 min	4
1-5	30 sec	15 min	✓	2.5 min	5
1-6	30 sec	1 min	✓	3 min	6
1-7	30 sec	5 min	✓	3.5 min	7
1-8	30 sec	1 min	✓	4 min	8
1-9	1 min	1 min	✓	5 min	9
1-10	5 min	1 min	✓	10 min	10
2-1	5 min	10 min	✓	5 min	1
3-1	10 min	10 min	✓	10 min	1

*Sample 1 was subjected to ten cycles of freezing and thawing whereas samples 2 and 3 were subjected to only one freeze-thaw cycle each.

were processed using the QIAamp Blood Kit (Qiagen Inc., Chatsworth, CA) following recommendations from the manufacturers and a standard organic extraction procedure (RCMP Methods Manual, 1990). These samples included cigarette butts (fresh samples), chewing gum (fresh samples, two types: EXCEL [Wrigley's, Canada] and EXTRA [Wrigley's, Canada]), fingernails (fresh clippings and 2-year-old clippings stored at room temperature) and envelopes (freshly sealed with saliva and 2-year-old samples kept at room temperature; three types and three different colors of paper: brown, gray/green, white). DNA extracts were then processed for PCR using the triplex 1A system.

Simulated Sexual Assault Cases—Mock sexual assault cases were developed from vaginal swabs (with and without menstrual blood) obtained from two female donors and semen from three different male donors chosen at random among the laboratory personnel. Semen (left at −20°C and thawed before use, 60 μL) was deposited on each vaginal swab, which was then air dried before being subjected to a differential extraction protocol.

Casework Samples—To test the practical application of both the triplexes 1A and 1B, DNA extracts remaining from completed RFLP cases were processed using the multiplex STR systems. In total, 142 miscellaneous samples representing 24 cases (12 sexual assaults, 8 homicides, 1 impaired driving causing death, 1 break and enter, 1 impaired driving and 1 paternity following rape) were examined. In addition, 19 full cases including 7 sexual assaults, 9 homicides, 1 armed robbery and 2 missing persons, representing a total of 232 samples, were processed using the STR-1A system. Fourteen actual PCR cases (70 samples) were examined using the STR-1B system. DNA from all specimens were extracted following protocols detailed in this section and were further purified on Microcon-100 size-exclusion columns. A total of 8 to 9.5 ng of DNA was used for the STR-1A typing system with 28 cycles of amplification when analyzed on the ABI 373A DNA Sequencer. A total of 5 ng of DNA was used for the STR-1B typing system with 30 cycles of amplification for analysis on the ABI Prism™ 377 DNA Sequencer. In instances where very limited amounts of genetic material were available, a minimum of 1 ng of DNA was used.

Results and Discussion

Sensitivity Studies

As shown in Table 3 and Fig. 1 (panels A to C), when using the ABI Model 373A DNA Sequencer and the color scheme selected for STR-1A primers, the GM9948 heterozygous genetic profile was detected using 0.32 ng to 10 ng of genomic DNA and 28 cycles of amplification. Using larger PCR aliquots for gel analysis (i.e, using 12 μL instead of 3 μL) allowed detection of profiles with as little as 0.16 ng, 0.08 ng and 0.04 ng of DNA (see panels D to F). In these instances, the fluorescence background was not significantly enhanced but an extra peak migrating at 80 bases, i.e., before the first amelogenin fragment, was noted. Both HumD21S11 and HumFGA alleles as well as the X and Y homologues of amelogenin were clearly noted in the electropherograms with peak heights above the arbitrary threshold of 20 fluorescent units (FU); the smallest peak height observed was 27 FU for HumFGA. Although a complete profile could be detected with these low amounts of template DNA (0.16 ng to 0.04 ng) and 28 cycles, differences in heterozygote peak heights were noted for amelogenin, HumD21S11 and HumFGA (see Table 3). Unequal amplification of individual alleles at HumD21S11 and HumFGA and amelogenin was also noted for 0.32 ng and 0.63 ng of template DNA due to stochastic effects. The degree of signal variation between the two alleles making up a heterozygous profile for DNA concentrations from 0.63 ng down to 0.04 ng ranged from −40 to +19% (lowest differential −3%) for amelogenin, −31 to +43% (lowest differential −11%) for HumD21S11 and −38 to −11% (lowest differential 11%) for HumFGA. When smaller amounts of template DNA were amplified (≤0.02 ng), alleles from amelogenin and HumD21S11 began to drop out, resulting in false homozygosity. In these cases, the entire HumFGA locus failed to amplify.

Increasing the number of cycles to 32 and 34 augmented the total product yield and allowed detection of complete profiles using 0.08 ng and 0.16 ng of DNA, respectively [using a 3 μL aliquot of the 15 μL PCR (see Table 3)]. Therefore, by increasing the number of amplification cycles, there was less advantage to sample concentration when minimal amounts of template DNA were encountered. Partial GM9948 profiles (amelogenin and HumD21S11) were obtained using even lower amounts of template DNA (such as 0.04–0.02 ng) at both cycle numbers, but significant differences in heterozygote peak height ratios were observed (−47% to −3% for amelogenin, −40% to +69% for HumD21S11 alleles). In addition, a reduction of specificity reflected by an increase in nonspecific DNA fragments was noted (data not shown).

Table 3 and Fig. 2 summarize the detection limits of the ABI Prism™ 377 DNA Sequencer for the triplex STR-1B system. Complete profiles were obtained using 28 cycles of amplification and as little as 0.32 ng of template DNA. Allele peaks at HumD21S11 and the two amelogenin fragments were clearly observed with peak heights above the arbitrary threshold of 20 FU. The HumFGA alleles were detected with intensities of 22 and 20 FU using 0.32 ng of input DNA (Fig. 2, panel A). Lower quantities of input DNA (<0.32 ng) contributed to locus dropout. When larger aliquots of the PCR products were used for analysis (i.e., 6 μL instead of 1.5 μL), fluorescent signal intensities were enhanced and the HumFGA allele peak heights (at 0.32 ng DNA) were no longer borderline values (Fig. 2, panel B). This signal enhancement using larger aliquots of the PCR products was not noted when 0.16 ng and 0.08 ng of DNA were used for amplification (Fig. 2, panel F). Although authentic peak sizes were detected in the allele size range for HumD21S11, extraneous bands were also seen and significant imbalance between heterozygote peak heights was observed due to stochastic effects.

An additional two cycles of amplification increased the total yield of products which was reflected by higher peak heights when panels 2A and 2C, 2B and 2D, 2E and 2G, 2F and 2H were compared. These amplification conditions permitted detection of complete triplex 1B profiles from 0.16 ng of template DNA using a 1.5 μL aliquot of the 16 μL PCR (Table 3 and Fig. 2, compare panels E and G). A few nonspecific bands were also observed on the electropherograms. Although the two extraneous blue bands migrated outside the allele size range for amelogenin and HumD21S11, the green peak sized at 260 bases was within the allele size range designated for HumFGA. Using a fourfold larger aliquot of the PCR enhanced the fluorescence signal intensities by a factor of three to four, without any significant increase in the fluorescence background (compare panels 2C and 2D, 2G and 2H). Therefore, using a larger aliquot of PCR for samples that show borderline peak height values could have a significant impact on successful STR typing analysis. This would be particularly relevant to laboratories that use a higher arbitrary cutoff threshold limit for scoring alleles (i.e., >50 FU, see Refs 16 and 18). Using 30 cycles

TABLE 3—*DNA template titration studies using the multiplex STR systems 1A and 1B on the ABI Model 373A and Prism™ 377 sequencers.*

STR Multiplex	Input DNA (ng)	Number of Cycles	Aliquots of PCR Product for analysis (µL)	Amelogenin			[illegible]			[illegible]			[illegible]		
				Peak Height of 106 base Fragment	Peak Height of 112 base Fragment	% Peak Height 112/106 Fragment	Peak Height of Allele 1	Peak Height of Allele 2	% Peak Height A2/A1	Peak Height of Allele 1	Peak Height of Allele 2	% Peak Height A2/A1	Peak Height of Allele 1	Peak Height of Allele 2	% Peak Height A2/A1
1A	10	28	[illegible]	653	808	[illegible]				[illegible]	[illegible]	91	1436	[illegible]	[illegible]
	5		[illegible]	[illegible]	[illegible]	81				[illegible]	[illegible]	[illegible]	628	[illegible]	[illegible]
	2.5		[illegible]	[illegible]	228	78				800	[illegible]	[illegible]	521	[illegible]	[illegible]
	1.25		[illegible]	126	102	105				[illegible]	126	[illegible]	[illegible]	[illegible]	[illegible]
	0.63		[illegible]	[illegible]	41	65				[illegible]	170	[illegible]	[illegible]	[illegible]	[illegible]
	0.32		[illegible]	25	21	84				[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	51
	0.16		[illegible]	ND	ND	-				[illegible]	52	[illegible]	[illegible]	[illegible]	[illegible]
	0.08		[illegible]	ND	ND	-				25	[illegible]	[illegible]	ND	ND	
	0.04		[illegible]	ND	ND	-				17	[illegible]	[illegible]	[illegible]	ND	
	0.02		[illegible]	ND	ND	-				[illegible]	ND	-	ND	ND	-
	0.01		[illegible]	ND	ND	-				ND	ND	-	ND	ND	-
	0.005		[illegible]	ND	ND	-				ND	ND	-	ND	ND	-
1B	10	[illegible]	[illegible]	[illegible]	1322	80				[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	90
	5		[illegible]	[illegible]	[illegible]	87				2218	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	2.5		[illegible]	[illegible]	[illegible]	[illegible]				[illegible]	888	[illegible]	[illegible]	288	[illegible]
	1.25		[illegible]	[illegible]	[illegible]	[illegible]				429	[illegible]	86	[illegible]	[illegible]	71
	0.63		2	[illegible]	[illegible]	[illegible]				[illegible]	[illegible]	[illegible]	232	[illegible]	72
	0.32		[illegible]	[illegible]	[illegible]	[illegible]				221	[illegible]	100	[illegible]	[illegible]	[illegible]
	0.16		2	[illegible]	146	[illegible]				[illegible]	[illegible]	80	[illegible]	21	[illegible]
	0.08		2	[illegible]	[illegible]	[illegible]				108	[illegible]	[illegible]	[illegible]	27	[illegible]
	0.04		[illegible]	[illegible]	[illegible]	[illegible]				[illegible]	[illegible]	[illegible]	45	25	[illegible]
	0.02		[illegible]	[illegible]	ND	-				[illegible]	ND		ND	ND	-
	0.01		[illegible]	ND	[illegible]					28	ND		ND	ND	-
	0.005		[illegible]	ND	ND					[illegible]	ND	-	ND	ND	-

TABLE 3—*Continued.*

TABLE 3—*Continued.*

III	10	74	15				>35	11	85	1620	1158	50	368	245	81
	5		15				297	341	172	872	764	84	169	155	97
	2.5		15				46	13	109	85	66	55	51	81	124
	1.25		15				48	64	51	129	79	147	69	84	76
	0.63		6				212	75	93	86	267	56	149	157	85
	0.31		6				In	68	>5	142	88	56	61	>	81
	0.16		6				90	<1	-	110	26	35	22	ND	
	0.08		6				<1	<1		37	26	19	21	ND	
	0.01		6				<1	<1		ND	ND		ND	ND	
V	6	50	15				455	1455	96	1547	1380	57	326	525	81
	3		15				81	43	8	111	63	91	132	193	63
	1.25		15				61	22	95	118	653	86	258	155	82
	0.63		15				59	51	86	211	264	54	93	71	82
	0.31		15				101	75	73	182	214	174	111	87	78
	0.32		15				85	56	66	95	150	144	75	82	117
	0.16		15				17	85	38	61	89	57	46	46	83
	0.08		15				23	<1		30	ND	-	ND	ND	-
	0.01		15				ND	ND		ND	ND	-	ND	ND	-
IV	10	50	15				496	495	60	1113	268	84	624	570	-
	5		15				155	25	81	129	710	84	133	76	19
	2.5		15				630	262	87	684	613	84	255	228	58
	1.25		15				125	28	63	222	165	74	79	62	75
	0.63		6				512	423	24	516	615	17	525	226	76
	0.32		6				236	140	66	262	379	43	196	329	173
	0.16		6				162	244	51	102	135	76	145	121	54
	0.08		6				77	ND	-	61	74	34	33	28	32
	0.01		6				ND	ND	-	ND	ND	-	ND	ND	-
VI	10	52	5				2,251	3,513	86	1711	1902	87	3,067	3,287	58
	5		15				2445	2707	82	3160	2760	87	1241	1,652	116
	2.5		15				1750	1553	91	3113	2706	87	1350	1,159	77
	1.25		15				515	591	98	1,055	759	72	597	657	62
	0.63		6				87	87	101	1015	144	48	164	86	75
	0.32		6				86	74	81	113	97	55	113	69	47
	0.16		6				ND	ND	-	25	ND	-	71	ND	-
	0.08		6				ND	ND	-	ND	ND		ND	ND	-
	0.01		6				ND	ND		ND	ND		ND	ND	
VII	10	52	5				2,719	3,011	91	1,762	1,753	118	2,956	2,211	58
	5		15				2,013	1986	86	3,205	2,983	87	1,642	1,482	116
	2.5		15				1,231	1,124	92	2,170	2,006	88	1,264	1,170	-
	1.25		15				458	479	92	787	820	71	562	522	83
	0.63		7				79	81	101	971	999	53	271	298	76
	0.32		8				372	295	80	862	825	80	377	219	117
	0.16		8				ND	37		78	67	86	217	39	116
	0.08		6				ND	ND		ND	ND		ND	15	
	0.01		6				ND	ND		51	25	44	ND	ND	

* FU = fluorescent units. Alleles were scored only if their peak height was > 70 FU.

† SDR LS = satisfied validation criteria only when the ABI Model 377A Sequencer

‡ % = 0.01% amplified products were analyzed on the ABI Prism® 377 Sequencer

ND = peak not detected

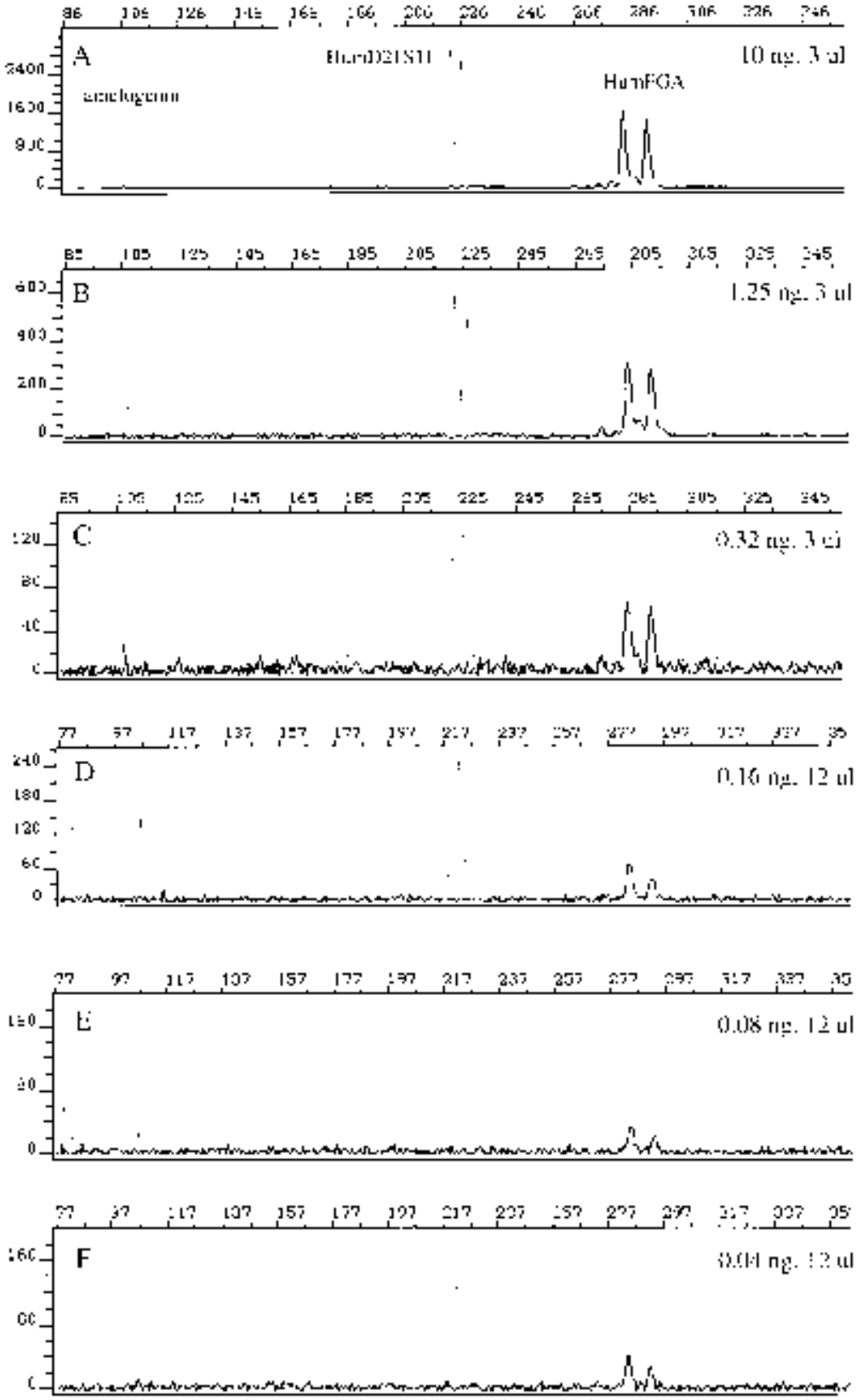
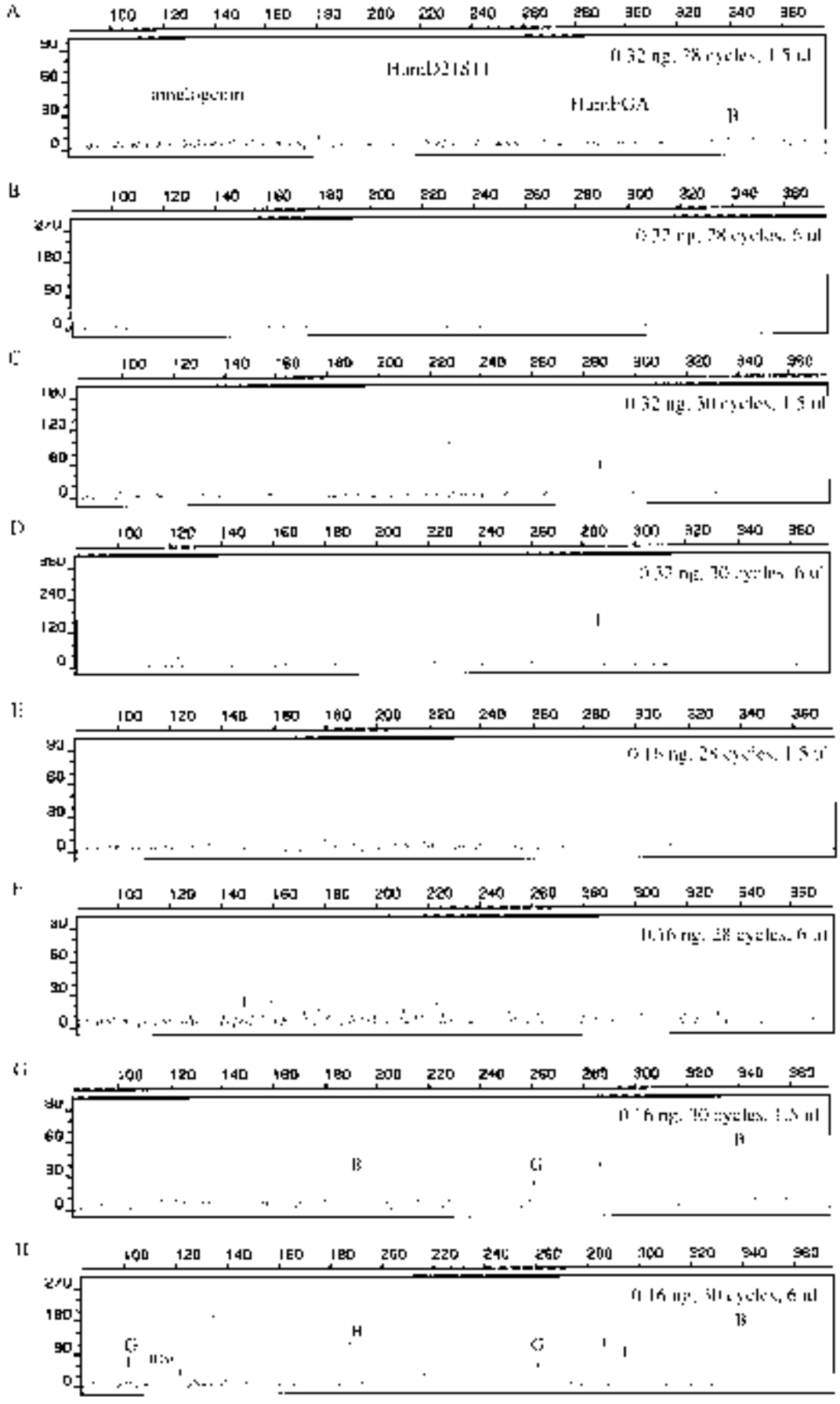


FIG. 1—*Detection limits of STR-1A amplicons using the ABI Model 373A DNA Sequencer. GM9948 control cell line DNA was serially diluted from 10 ng to 0.005 ng and amplified under conditions detailed in Materials and Methods, using 28 cycles of amplification. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 2500 ROX using the ABI 672 Analysis version 1.2.1 software. (A) 10 ng of template DNA, 3 μL of a 15 μL PCR used for analysis; (B) 1.25 ng, 3 μL; (C) 0.32 ng, 3 μL; (D) 0.16 ng, 12 μL; (E) 0.08 ng, 12 μL; (F) 0.04 ng, 12 μL. Amelogenin primers were labeled with JOE (green), HumD21S11 primers with JOE and HumFGA primers with TAMRA (yellow).*


A
0.32 ng, 28 cycles, 1.5 ul
Amelogenin
Hum_D21S11
Hum_FGA
B
B
0.32 ng, 28 cycles, 6 ul
C
0.32 ng, 30 cycles, 1.5 ul
D
0.32 ng, 30 cycles, 6 ul
E
0.16 ng, 28 cycles, 1.5 ul
F
0.16 ng, 28 cycles, 6 ul
G
0.16 ng, 30 cycles, 1.5 ul
B
G
H
0.16 ng, 30 cycles, 6 ul
B
G
H

of amplification and a larger aliquot of PCR for analysis did not improve the profiling results obtained with 0.08 ng of template DNA. Unreliable and partial profiles for HumD21S11 and HumFGA were observed, with major imbalances between heterozygote peak heights (Table 3).

Profiling results obtained using 32 cycles of amplification indicated that there was no benefit in using an extra two cycles. The limit for reliable STR-1B analysis appeared to be 0.32 ng of template DNA and a 1.5 μL aliquot of the 16 μL PCR. The amplification of lower amounts of template DNA (≤0.16 ng) resulted in allele or locus dropout problems for HumD3S1358, HumD21S11 and HumFGA (Table 3). Larger aliquots of the amplification reactions did not improve the profiling results for these low amounts of DNA.

Our sensitivity studies revealed that alleles derived from both STR-1A and STR-1B triplex systems are reliably detected with balanced signals for the two locus-specific alleles of an heterozygous individual on the ABI DNA sequencers using 1 ng of template DNA and 28 cycles of amplification. STR profiles can still be obtained using 0.63 ng or 0.32 ng but unequal amplification may occur between individual alleles at a given locus due to stochastic effects. This becomes more evident with decreasing amounts of DNA. Employing larger aliquots of the PCR for analysis and/or a higher number of amplification cycles allowed detection of reliable STR-1A and STR-1B profiles at low template DNA concentrations (0.16 ng). Amplification of minute amounts of DNA (0.08 ng or 0.04 ng) produced unreliable and partial profiles with major differences in heterozygote peak heights due to stochastic effects.

Similar results have been recorded by investigators using other STR loci (HumvWA, HumTHO1, HumF13A1 and Humfes/fps) under different amplification conditions but using the same fluorescence-based detection instruments (16,18,23,47,48).

Environmental Studies

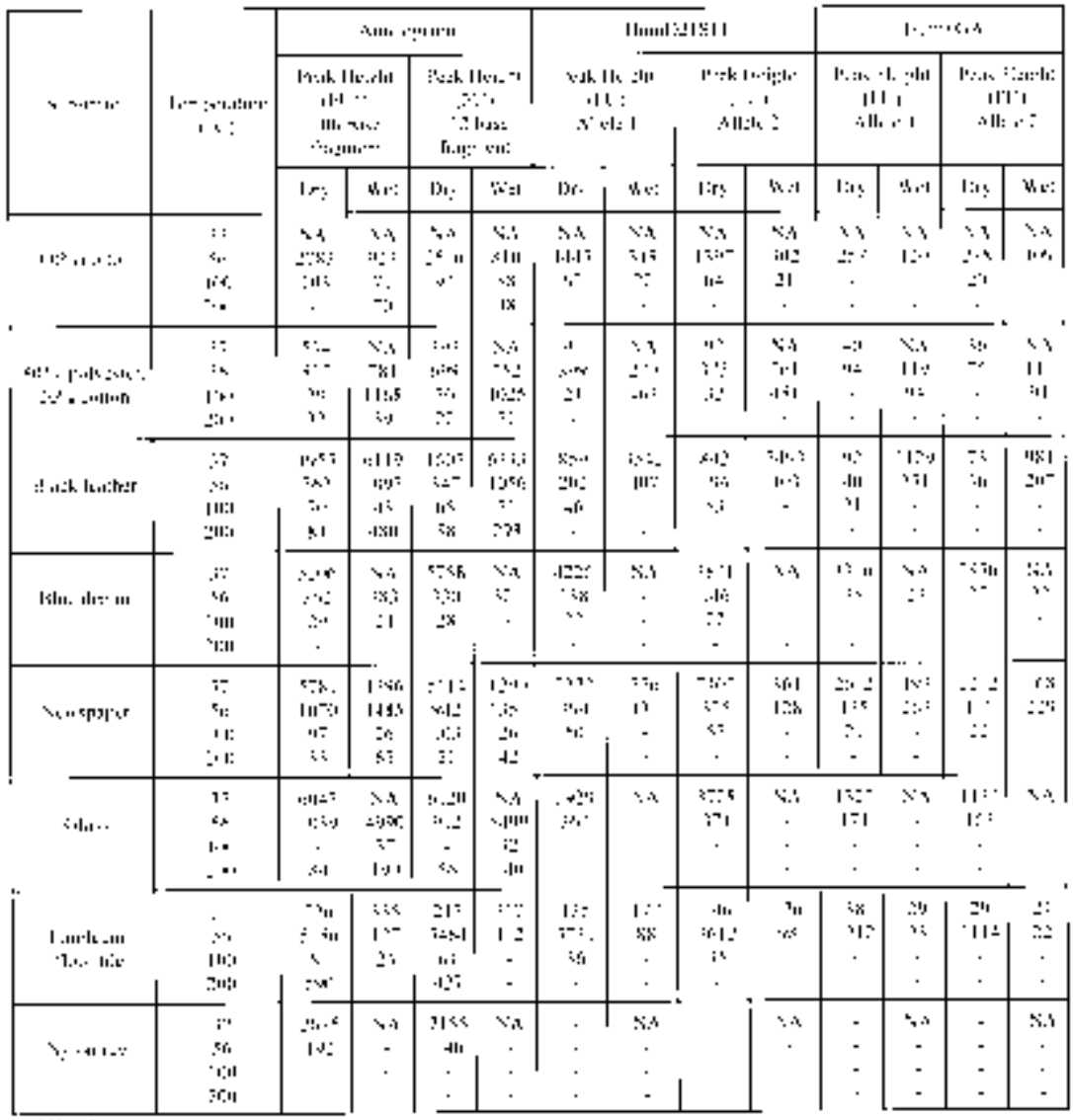
A number of experiments were carried out as a complement to previous studies describing the behavior of a variety of STR loci derived from samples exposed to a diversity of environmental insults (16,19,22,37,39,40,49–51). These experiments were performed in order to: 1) identify potential PCR inhibitors from a variety of substrates, 2) determine the role artificial whitening agents found in a variety of detergents may have in interfering with the detection of fluorescently labeled DNA fragments, and 3) examine the robustness of the STR systems under various DNA degradation regimens. This series of experiments was carried out using 10 ng of input DNA with the triplex 1A and the ABI 373A DNA Sequencer.

Substrate Study—All bloodstains, regardless of the deposition substrate, generated STR-1A DNA profiles consistent with those of the control stains (data not shown). No signs of allele or locus dropout were observed for amelogenin, HumD21S11 and HumFGA. In addition, the yield of fluorescent amplified products was essentially the same for all substrates, including the dark-colored garments (black leather and blue denim). These materials have been known to cause difficulties when analyzed by PCR because of dyes or chemicals which inhibit or prevent optimal enzyme activity (19,39,52–54). In fact, the mean value for the yield of fluorescence for black leather and blue denim was calculated as 353.5 ± 30.7 FU (range 322 to 388 FU) for amelogenin, 586.3 ± 193.7 FU (range 416 to 801 FU) for HumD21S11 and 285.5 ± 32.3 FU (range 255 to 330 FU) for HumFGA. Values for the remaining substrates (100% cotton, 80% polyester/20% cotton, glass, linoleum floor tile, nylon rug and newspaper) were 198.9 ± 34.3 FU (range 144 to 254 FU) for amelogenin, 234 ± 72.6 FU (range 164 to 345 FU) for HumD21S11 and 158 ± 31.7 FU (range 121 to 205 FU) for HumFGA. No inhibitory effect on the yield of amplicons was noted from black leather or blue denim and the slight difference noted may be a consequence of quantitation or pipetting variation.

Substrate studies performed by van Oorschot et al. (39) during validation of the HumTHO1 STR locus, in which 37 different substrates were tested, revealed that no typeable amplification profiles for HumTHO1 (amplicon size range 155 to 178 bases) were obtained from leather substrates. Our studies indicate that amelogenin, as well as HumD21S11 and HumFGA, which represent a large range of amplicon sizes (106, 112, 204 to 244, 254–298 bases) can be reliably typed from blood deposited on leather. However, in our studies, the dried blood was swabbed off the substrate, whereas van Oorschot used the entire bloodstained surface during DNA extraction. Sparkes and colleagues (19) tested a variety of substrates using a broad range of amplicon sizes in their simulated case study for the validation of the second generation multiplex system (SGM). Although it is unclear which of the substrates failed to amplify, leather and denim were among the more challenging substrates examined. A study performed by Comey and Budowle (53), in which 34 different fabric types and surfaces were tested, indicated that DNA samples extracted from bloodstains on denim (white or blue) using four different extraction procedures, failed to amplify the HLA DQα locus (amplicon size of 239 or 242 basepairs (bp)). These authors reported successful HLA DQα typing from bloodstains prepared on leather using a modified organic extraction procedure or a nonorganic method (53). In a separate validation study, polymarker typing results (amplicon size range of 138 to 214 bp; control HLA DQα product of 239 or 242 bp) were generated from bloodstains made on denim but only half of the bloodstains prepared on leather produced polymarker genotypes (54). These observations reiterate the importance of conducting validation studies in different laboratories under different experimental conditions of DNA extraction, DNA amplification and detection. Although typing results for STR loci with amplicons in the same size range may be predictable for most substrates

FIG. 2—*Detection limits of STR-1B amplicons using the ABI Prism™ 377 DNA Sequencer. GM9948 control cell line DNA was serially diluted from 10 ng to 0.04 ng and amplified under conditions detailed in Materials and Methods, using 28 or 30 cycles of amplification. Typing results in the low range of target DNA are shown. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 350 ROX using the Genescan Analysis version 2.0.2 software. For each sample, 1.5 μL of the DNA/denaturing buffer mixture was loaded on the gel. (A) 0.32 ng of template DNA, 28 cycles, 1.5 μL of a 16 μL PCR used for analysis; (B) 0.32 ng, 28 cycles, 6 μL of a 16 μL PCR reduced to 1.5 μL using speed vacuum; (C) 0.32 ng, 30 cycles, 1.5 μL; (D) 0.32 ng, 30 cycles, 6 μL; (E) 0.16 ng, 28 cycles, 1.5 μL; (F) 0.16 ng, 28 cycles, 6 μL; (G) 0.16 ng, 30 cycles, 1.5 μL; (H) 0.16 ng, 30 cycles, 6 μL. HumD3S1358 primers were labeled with FAM (blue), HumD21S11 primers with FAM and HumFGA primers with JOE (green). Extraneous bands detected in blue or green are marked with a letter B or G, respectively.*

TABLE 4—*Temperature, moisture and substrate study using the multiplex STR-1A.*



(glass, paper, cotton, newspaper), different observations may be noted from different investigators using more challenging substrates such as dark-colored garments.

Temperature, Moisture and Substrate Study—All STR-1A profiles generated from DNA samples extracted from treated substrates were consistent with those determined for the untreated samples of the same origin (data not shown), and showed no signs of unequal amplification between two locus-specific alleles of an heterozygous profile (see Table 4). A full STR-1A profile was detected from the dry bloodstains prepared on cotton/polyester blend fabric, black leather, blue denim, newspaper, glass and floor tile subjected to 37°C for 30 min. Amelogenin, with its small size amplicons (106 and 112 bases), was the only system detected on the nylon rug following its incubation at 37°C for 30 min. Complete STR-1A profiles were observed from wet bloodstains prepared on black leather, newspaper and floor tile. No other wet bloodstains prepared at this temperature were available for our STR-1A validation studies.

The incubation at 56°C for 30 min supported amplification of full STR-1A profiles from the dry bloodstains prepared on the substrates tested with the exception of the nylon rug, which resulted only in the detection of the amelogenin locus. The typing results from the fresh (wet) bloodstains were significantly different. First, the overall yield of fluorescent amplified products was lower than that noted for the dry bloodstains (see Table 4). This was particularly true for the 100% cotton, floor tile and nylon rug at all three loci, and blue denim and glass at HumD21S11 and HumFGA. Signs of locus drop out were also noted for blue denim, glass and nylon rug. Using the threshold limit of 20 FU to score alleles, complete STR-1A profiles were generated from cotton, cotton/polyester blend fabric, black leather, newspaper and floor tile. Partial profiles (amelogenin only) were observed for blue denim and glass. No typing profile was detected for the nylon rug under these experimental conditions.

The incubation at 100°C for 30 min completely prohibited detection of HumFGA amplification products, regardless of the substrate and the nature of the bloodstain (dry or wet). HumD21S11 allele

profiles were generated from DNA extracted from dry bloodstains prepared on cotton, cotton/polyester blend fabric, black leather, blue denim, newspaper and floor tile, but all fluorescent signals were below 70 FU. The majority of the detection signals were close to the minimum threshold value of 20 FU. No profiles were detected from dry bloodstains prepared on the glass surface and the nylon rug. The HumD21S11 amplification was severely compromised in the wet bloodstains on the substrates. Samples from the cotton and cotton/polyester blend fabrics did yield STR profiles, but the signal intensity was very weak (in the 20 to 45 FU range). Locus dropout problems were noted for all remaining substrates tested at this temperature. Amelogenin was detected in most of the DNA extracts from dry and wet bloodstains, yielding fluorescent peak heights between 26 and 103 FU. Allele dropout was noted for the blue denim and the floor tile. No amelogenin signals were detected using the DNA extracted from the glass surface or the nylon rug.

Interestingly, DNA samples extracted from bloodstains applied to substrates incubated at 100°C for 30 min and subjected to agarose gel electrophoresis did not show any obvious degradation. Therefore, these STR typing results could be explained by the possible co-extraction of PCR inhibitors from the blood and/or the substrate, promoted by the 100°C incubation. This in turn could prevent efficient amplification.

None of the bloodstains, either dry or wet, prepared on the various substrates tested and incubated at 200°C for 30 min derived HumD21S11 and HumFGA STR results. Strong amelogenin signals (between 298 and 590 FU) were detected from floor tile (dry) and black leather (wet) (Table 4). Moderate signals (between 140 and 190 FU) were detected from glass (wet). Samples from cotton (wet), cotton/polyester blend (dry and wet), black leather (dry), newspaper (dry and wet) and glass (dry) generated weak signals (between 21 and 84 FU). The incubation at 200°C clearly compromised the DNA's suitability for amplification in all situations.

These STR-1A typing results corroborate the level of DNA degradation noted from yield gels run for this series of DNA samples (data not shown). As the samples degraded, loci with high molecular weight alleles such as HumFGA (amplicons between 254 and 298 bases), followed by HumD21S11 (amplicons between 204 and 244 bases), amplified less efficiently with the potential for leading to allele or locus dropout. Amelogenin, whose amplification generates the smallest size fragments (106 and 112 bases), was the last system affected by such degradation.

This study indicates that recovery of intact DNA suitable for PCR analysis is more problematic from bloodstains maintained at elevated temperatures, such as 100°C and 200°C, compared with samples maintained at 37°C and 56°C. Yield gels showed clear degradation of DNA extracted from 200°C substrates. However, no detectable sign of degradation was noted for genomic DNA extracted from substrates left at 100°C, 56°C or 37°C (data not shown). Partial STR-1A profiles obtained from bloodstains subjected to these temperatures may be explained by the presence of PCR inhibitors from blood and/or substrates which interfere with efficient amplification of STR loci in the size range of HumD21S11 and HumFGA, and favor amplification of the smaller amplicons such as amelogenin. Although dark-colored garments have been notorious for preventing optimal DNA amplification by PCR (19,39,52–54), our study indicates that substrate samples processed from black leather yielded intact DNA and excellent STR typing results. The worst substrate was the nylon rug, which yielded no results for both sets of bloodstains at all temperatures tested. Similar conclusions were drawn from other studies (55).

Stability of DNA Extracts Left at Room Temperature for Various Lengths of Time—The DNA from the individual selected and the two control cell lines GM9947A and GM9948 were amenable to PCR after four weeks at room temperature, and generated profiles for amelogenin, HumD21S11 and HumFGA that were consistent with those of the control DNAs left at 4°C for the same periods of time (data not shown).

Stability of DNA from Semen and Bloodstains Following Long- and Short-Term Exposures to Sunlight and Darkness—Bloodstains and semen stains maintained at room temperature in daylight or darkness for up to two years yielded the same amount of fluorescent STR-1A amplified products as the control stains from the same donor, and showed consistent allele size estimates (data not shown). In addition, the DNA recovered from the treated stains showed no signs of degradation, as revealed by yield gels or as corroborated by the intense fluorescent STR signals detected and the lack of allele dropout or spurious bands (data not shown).

These results complement studies performed by Lygo et al. (16), Sparkes et al. (40) and van Oorschot et al. (39) in which bloodstains and semen stains stored at room temperature for 11 or 15 years, 13 years, and 7 or 4 years, respectively, generated full profiles for the quadplex STR system (HumvWA, HumTHO1, HumF13A1, Humfes/fps), the SGM system (HumvWA, HumFGA, HumTHO1, HumD21S11, HumD18S51, HumD8S1179, amelogenin) and HumTHO1, respectively. These studies all demonstrate that blood and semen are very resistant to the effects of age. A similar conclusion was reached by other investigators using different PCR typing systems (53,54). In these studies, bloodstains and semen stains stored at room temperature for 16 or 20 weeks successfully produced HLA DQα and polymarker genotypes.

Detergent Study—The ten different detergents, fabric softeners and color brighteners used under normal washing conditions did not interfere in any way with the interpretation of STR-1A allele peaks (data not shown). No enhancement in the fluorescence background and no extraneous bands were noted. Regardless of the treatment, profiles for amelogenin, HumD21S11 and HumFGA showed the same fluorescence intensity, and no signs of unequal amplification or allele dropout, when compared with profiles obtained from control bloodstains prepared on untreated cloth. A previous study performed by Urquhart et al. (49), using a large number of fluorescent compounds including color brighteners, showed that although many compounds gave rise to identifiable peaks when run on the ABI 373A DNA Sequencer, standard phenol/chloroform extraction removed these fluorescent peaks and prevented interference with the interpretation of the quadplex STR allelic peaks. Our results, using North American detergents and softeners, complement their experiments and further support the PCR amplifiability of genetic material recovered from bloodstains and semen stains prepared on prewashed or pretreated cloth.

Other validation studies were published by Scheithauer and Weisser (56) and Andrews et al. (57). The first group of investigators processed a series of bloodstains prepared on linen pretreated with 20 different remedies used for cleaning and maintaining clothes to determine the effect on RFLP analysis. Three out of the 20 remedies tested caused disturbances, i.e., prevented optimal restriction endonuclease activity. Andrews and colleagues (57) examined the PCR amplifiability of genetic material recovered from washed blood, semen and saliva stains prepared on a variety of substrates. These authors reported that even tiny washed stains allowed successful PCR DNA typing.

Soiled DNA—The DNA recovered from our sample showed amplification of only the amelogenin locus. The HumD21S11 and HumFGA alleles were not detected. The five chemicals used together as a cocktail, i.e., gasoline, motor oil, dirt, bleach and sodium dodecyl sulfate, appeared to have a major impact on the STR typeability of the sample. Van Oorschot et al. (39) tested the same five chemicals separately and observed no detrimental effect on the capability of the HumTHO1 locus to be amplified (amplicon size 155 to 178 bases). Comey and Budowle (53) evaluated the effect of a variety of chemicals, including the five chemicals included in our study, and reported successful HLA DQα typing from all samples with the exception of those samples contaminated with soil. Failure to amplify the HLA DQα locus (amplicon size 239 or 242 bp) was explained by the fact that no DNA was recovered from these samples. These authors suggested that components of the soil may bind the DNA making it impossible to extract. Our result may be explained by the presence of chemical residues not totally removed during phenol-chloroform extraction, which interfered with efficient amplification of STR loci in the size range of HumD21S11 and HumFGA, and favored amplification of the smaller amplicons such as amelogenin. On the other hand, the extent of DNA degradation following exposure to the mixture of dirt and chemicals may be such that high molecular weight target sequences such as HumFGA and HumD21S11 are no longer amenable to amplification. Unfortunately, no agarose gel was run to verify this theory due to a very poor DNA yield.

Degradation Studies

DNA extracts were subjected to two different degradation regimens in order to examine the robustness of each STR locus comprising the multiplex 1A.

Boiling, Freezing, Thawing—As shown in Fig. 3, a full STR-1A profile identical to the control was obtained for DNA samples boiled for up to 10 min and subjected to 10 cycles of freezing (10 min at −70°C) and thawing (room temperature) (see Table 2 for actual step-by-step protocol). Strong fluorescent signals in the range of 300 to 600 FU were detected, with a lack of allele or locus dropout. No spurious bands were noted. However, the yield of amplified products for samples boiled for up to 10 min and subjected to 10 cycles of freeze/thaw was reduced by a factor of 1.5 to 2 compared with samples boiled for 30 s and subjected to only one cycle of freeze/thaw (compare panels 3B and 3C). Similar reduced yields were also observed for samples #9 to #12 (see Table 2 for details on samples and data not shown). These samples may contain a reduced number of target DNA molecules available for amplification. In fact, DNA degradation was noted from yield gels (data not shown). The degradation was detrimental to the RFLP analysis and prevented hybridization of the D2S44 probe (expected median size fragments of 2832 and 1837 basepairs for GM9948). In our study, the level of degradation was not significant enough to eliminate amplification signals from the STR loci HumD21S11 and HumFGA, but sufficient to reduce the yield of amplified products by a factor of approximately 2.

Sonication—A complete STR-1A profile was obtained for DNA samples sonicated for up to 2 min (Fig. 4). Balanced signals between the two locus-specific alleles of heterozygous profiles were observed. Our conditions of sonication did not interfere with successful amplification and detection of amelogenin, HumD21S11 and HumFGA alleles. Interestingly, none of our samples subjected to sonication for up to 2 min showed detectable degradation when run on agarose gels (data not shown). These observations are in contrast with those of Prinz and Schmitt (50), who noted severe DNA degradation (no high molecular weight DNA) after 30 s of exposure to ultrasonic waves using a different instrument. However, these authors also reported that prolonged exposure (150 min) to ultrasonic waves did not prevent detection of HumvWA profiles (longest allele 167 bases).

Nonhuman DNA Study

All three STR primer sets evaluated (HumD3S1358, HumD21S11 and HumFGA) failed to amplify products from DNAs of domestic and wild game animal bloods (see list in Materials and Methods/Validation Experiments). Nonspecific fragments were observed using 5 ng or 10 ng of DNA, but the fluorescent peak heights were always very close to the arbitrary 20 FU cutoff for the detection of true amplification products and/or fell outside the human allele size range distinct for each STR locus (Fig. 5). The nonspecific fragments were seldom seen below the smallest human allele detected in this study (i.e., 114 bases, HumD3S1358) or above the largest human allele (i.e., 300 bases, HumFGA). The use of 50 ng of DNA for amplification enhanced the production and/or detection of artefactual peaks (compare panels 5A and 5D, 5C and 5E). In most instances, although some of the nonspecific fluorescent peaks overlapped with human STR allele sizes, they were of a different color and would not interfere with the detection of a potential human STR profile (panel 5D). One exception was seen in sheep where two green bands sized at 275 and 279 bases (HumFGA allele size range) were detected (panel 5E). The use of 100 ng of DNA showed an inhibitory effect on the PCR as reflected by lower yields of amplified products and therefore, resulted in fewer nonspecific bands (panels 5F and 5G).

Only three of the 14 bacterial or yeast strains examined (i.e., *Bacteroides fragilis, Streptococcus agalactiae* and *Candida albicans*) consistently produced minor peaks (20 to 95 FU) using 10, 50 and 100 ng of DNA (Fig. 6). However, all peaks with one exception (see panel 6A) were detected in positions irrelevant to human allele sizes for the STRs surveyed. One blue peak in *Bacteroides fragilis* (5 ng target DNA), sized at 231 bases (66 FU), corresponds to a position within the allele size range for HumD21S11. However, this peak was not seen when larger amounts of DNA were used for amplification (panels 6D and 6G). These observations complement those of Sparkes et al. (40) who tested a different group of 28 bacterial strains with a heptaplex STR system which included HumD21S11 and HumFGA. Other microbial DNA challenge studies were also performed using different STR loci such as HumvWF, HumTHO1, HumF13A1, Humfes/fps (18), HumTPOX and HumCSF1PO (37). No amplified products were detected for any of the microorganisms examined in these studies.

Previous nonhuman DNA studies carried out on some tetranucleotide STR loci and amelogenin (18,38,40,49,58–60) indicated that human STR primers will anneal to genomic sequences of animals, in particular Higher Primates (chimpanzee, gorilla and orangutan), and promote the production of amplicons. Our study reaffirms these reports and presents an extensive survey of members belonging to the Higher Primate group or Old World and New World monkey groups.

Table 5 lists the fluorescent amplified products detected for all the individual animals examined. Figure 7 shows representative STR-1B profiles obtained for chimpanzees, gorillas and orangutans. All three STR loci tested were amplified and displayed some

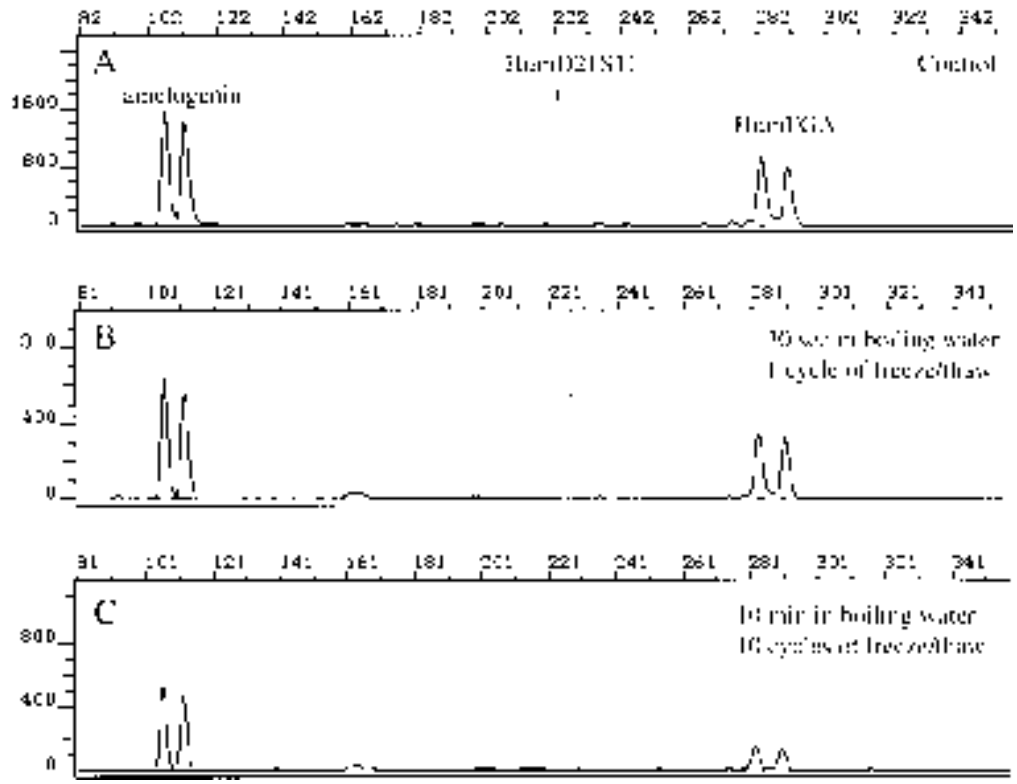


FIG. 3—*STR-1A typing analysis of degraded DNA following a boiling/freezing/thawing regimen. GM9948 control cell line DNA was subjected to repeated cycles of boiling (in water for 10 min), freezing (10 min at −70°C) and thawing (at room temperature) and then amplified under conditions detailed in Materials and Methods, using 28 cycles of amplification. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 2500 ROX run on the ABI Model 373A DNA Sequencer. (A) Untreated GM9948 control DNA; (B) Sample 1-1 (total of 30 s in boiling water, 1 cycle of freezing and thawing); (C) Sample 1-10 (total of 10 min in boiling water, 10 cycles of freezing and thawing; see Table 2 for details). Amelogenin primers were labeled with FAM, HumD21S11 primers with JOE and HumFGA primers with TAMRA.*

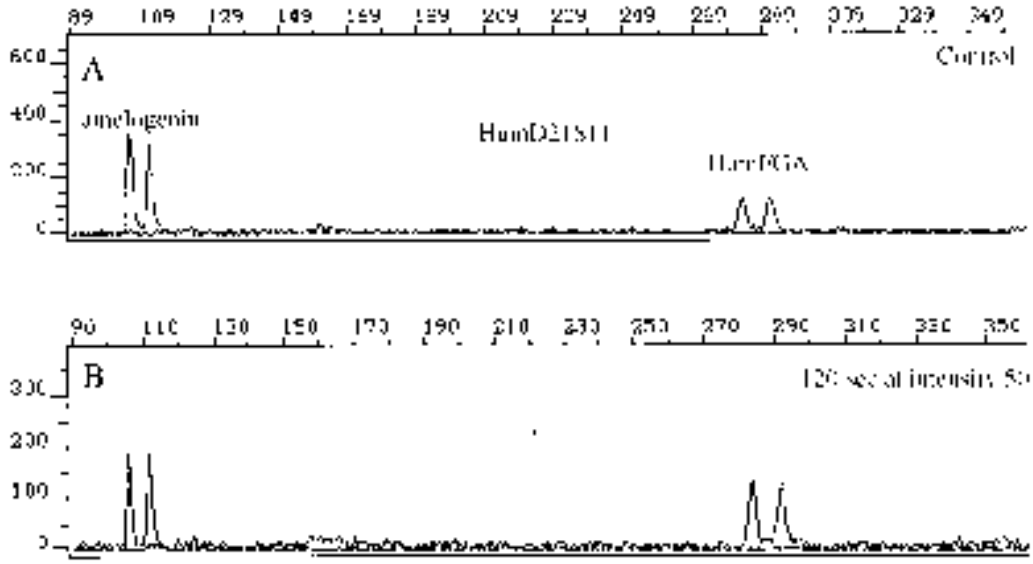


FIG. 4—*Effect of sonication on the STR-1A typing analysis of GM9948 DNA. GM9948 DNA was subjected to ultrasounds at intensity 50 for different time spans (2, 4, 8, 10, 20, 30, 40, 50, 60, 90 and 120 s) and processed for PCR, using 28 cycles of amplification as detailed in Materials and Methods. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 2500 ROX run on the ABI Model 373A DNA Sequencer. (A) Untreated GM9948 control DNA; (B) 10 ng DNA subjected to 120 s of sonication. Amelogenin primers were labeled with FAM, HumD21S11 primers with JOE and HumFGA primers with TAMRA.*

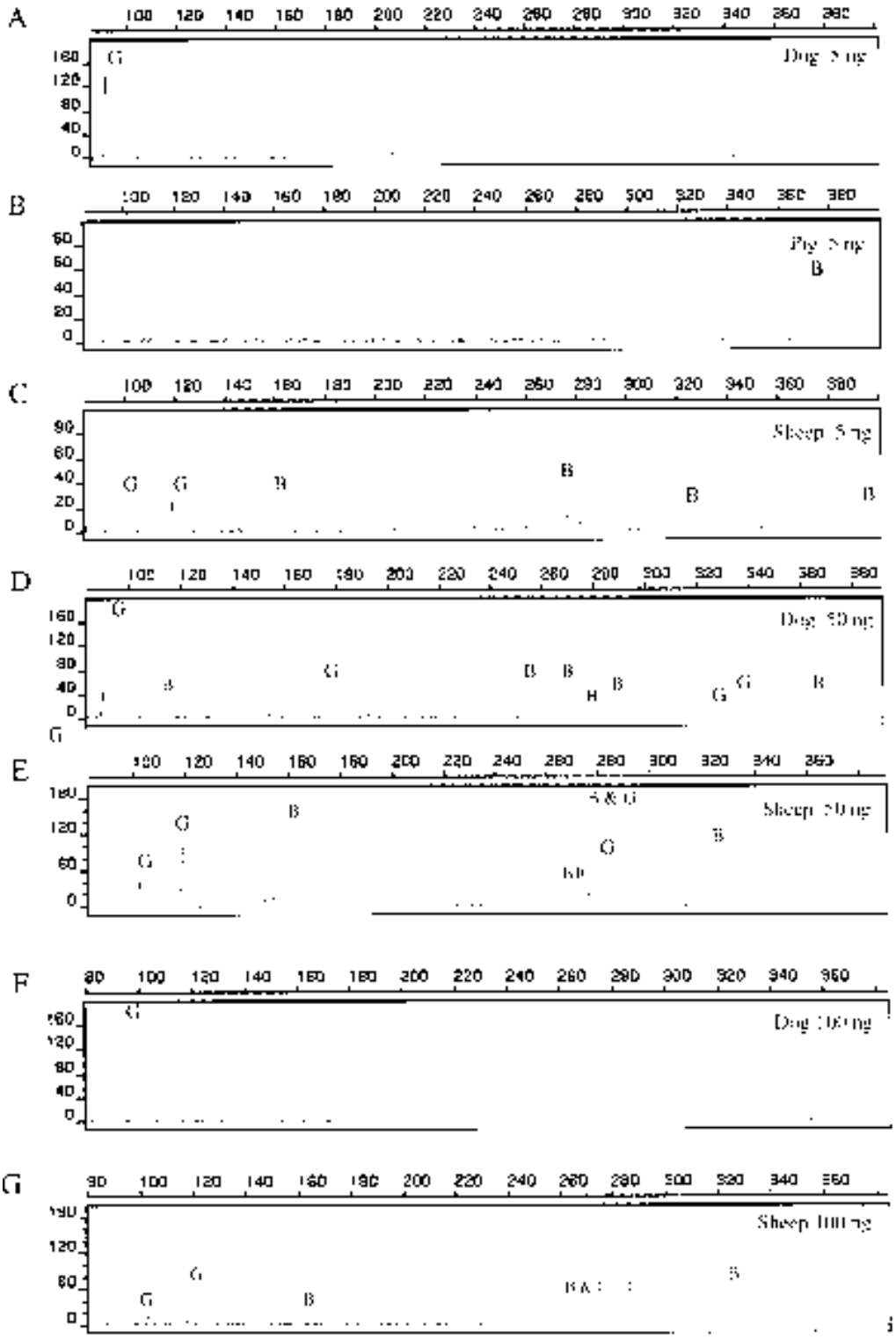


FIG. 5—*Species specificity of HumD3S1358, HumD21S11 and HumFGA primers using nonprimate target DNAs. The STR-1B system was tested on DNA from 24 different nonprimate species and analysed on the ABI Prism™ 377 DNA Sequencer. Examples of amplicons obtained from domestic animals using different amounts of DNA (5, 50 and 100 ng) are shown. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 350 ROX run on the ABI Prism™ 377 DNA Sequencer. (A), (D), (F): 5, 50 and 100 ng DNA, respectively, from dog; (B) 5 ng DNA from pig; (C), (E), (G): 5, 50 and 100 ng DNA, respectively, from sheep. HumD3S1358 primers were labeled with FAM, HumD21S11 primers with FAM and HumFGA primers with JOE. G refers to a green-colored peak and B, to a blue-colored peak. The size in bases of the bands detected and their respective fluorescence intensities in FU are the following: (A) 88.69 (169); (B) 375.00 (53); (C) 100.60 (22), 118.27 (29), 160.53 (28), 275.23 (37), 321.70 (26) and 392.00 (21); (D) 88.58 (2570), 111.68 (44), 179.93 (47), 254.37 (43), 266.45 (45), 278.34 (22), 285.40 (30), 325.98 (22), 337.22 (28) and 365.89 (23); (E) 101.24 (83), 118.57 (138), 160.61 (143), 267.26 (28), 272.20 (30), 275.43 (141 in blue, 67 in green), 279.61 (82) and 321.85 (82); (F) 88.75 (550); (G) 101.11 (34), 118.42 (117), 160.50 (50), 275.10 (62 in blue, 46 in green), 279.34 (76) and 322.17 (84).*

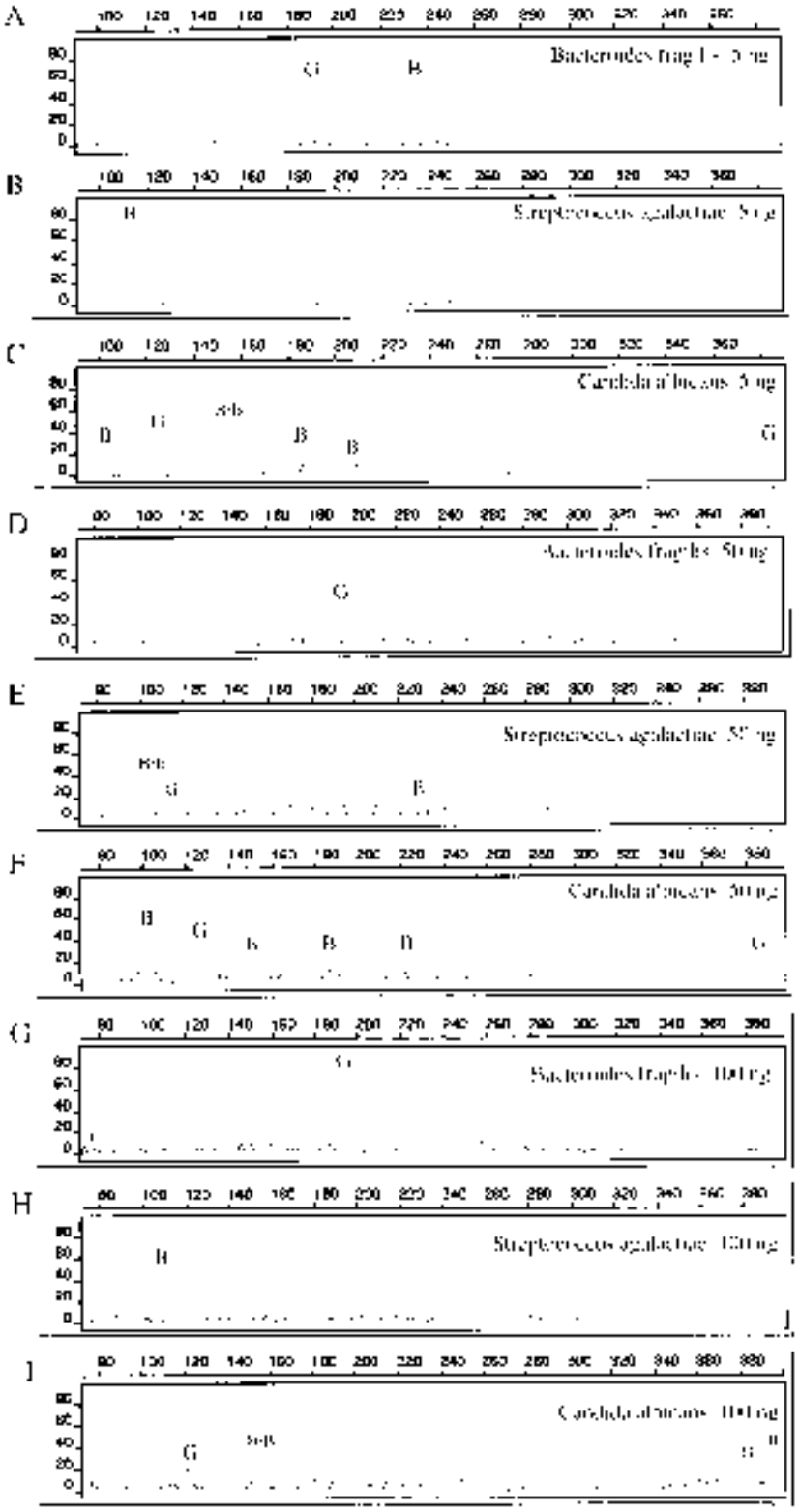


FIG. 6—*Microbial DNA challenge study using STR-1B primers and 14 bacterial and yeast strains. A total of 5, 50 and 100 ng of DNA was used with 30 cycles of amplification. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 350 ROX run on the ABI Prism™ 377 DNA Sequencer. (A), (D), (G): 5, 50 and 100 ng DNA, respectively, from* Bacteroides fragilis; *(B), (E), (H): 5, 50 and 100 ng DNA respectively, from* Streptococcus agalactiae; *(C), (F), (I): 5, 50 and 100 ng DNA, respectively, from* Candida albicans. *HumD3S1358 primers were labeled with FAM, HumD21S11 primers with FAM and HumFGA primers with JOE. G refers to a green-colored peak and B, to a blue-colored peak. The size in bases of the bands detected and their respective fluorescence intensities in FU are the following: (A) 190.69 (60), 231.57 (66); (B) 107.50 (95); (C) 99.16 (21), 121.54 (33), 150.75 (39), 151.71 (37), 185.87 (16), 208.19 (13) and 388.00 (27); (D) 190.76 (43); (E) 107.58 (39), 108.82 (29), 109.04 (19 in green) and 226.19 (19); (F) 99.29 (54), 121.59 (44), 151.45 (28), 186.10 (24), 222.19 (20) and 388.00 (25); (G) 190.70 (75); (H) 107.61 (51); (I) 121.63 (23), 150.77 (27), 151.65 (32), 388.00 (27) and 406 (32).*

TABLE 5—*Species specificity study using the multiplex STR-1A and STR-1B primers with primate DNAs.*

DNA sample	Number of alleles on the ABI PRISM DNA Sequencer	Number of alleles on the ABI Prism 377 DNA sequencer	Number of complete DNA profiles for STR-1A typing analysis only	Number of complete DNA profiles for STR-1B typing analysis only	Amelogenin on the ABI PRISM DNA Sequencers (Note: could not be scored)	Hum vWA 31/35 on Perkin-Elmer 377 DNA Sequencer (size estimate in bases)	Hum FES/FPS on the ABI PRISM DNA Sequencers (Size estimate in bases)	Hum TPOX on ABI PRISM DNA Sequencer (size estimate in bases)	Hum D8S1179 on Perkin-Elmer 377 DNA Sequencer (size estimate in bases)
Orang	2	2	8, 10	9, 10	106/112	N/D	137 bp	296/298	ND
					104/112	N/D	173 bp	296/298	ND
					108/109	151/157	151 bp	ND	301/308, 311/318
					108/112	129/129	135 bp, 138 bp	ND	299/301, 311/317
					108/108	129/129	134 bp, 141 bp	ND	307/307, 317/318
					106/112	157/157	174 bp, 174 bp	ND	299/301, 311/317
					ND	134/158	135 bp, 148 bp	ND	299/312, 313/313
					ND	130/130, 134/134	133 bp, 148 bp	ND	299/306, 313/313
Orangutan	4	2	9, 10	9, 10	106/112	150/150	129 bp, 135 bp	290/294, 300/304	301/307, 317/320
					106/112	138/141, 150/157	133 bp, 148 bp	290/294, 312/315	299/305, 313/322
					108/110	138/154	130 bp, 148 bp	294/294, 312/320	299/307, 313/320
					108/108	ND	127/141, 129/141	294/294, 312/312	ND
					108/108	ND	129/129, 138/138	ND	299/299, 313/320
					106/112	ND	129/133, 146/146	ND	308/311, 322/322
					106/108	ND	129/129, 146/146	ND	299/299, 322/322
					ND	137/141, 150/150	130/133, 146/146	ND	299/313, 322/322
Chimpanzee	2	2	8, 10	9, 10	106/112	174/174, 176/176	188/190, 190/194	264/270, 268/270	299/313, 317/329
					106/106, 112/112	172/172, 176/176	188/194, 206/212	264/264, 268/270	299/313, 317/317
					108/108, 112/112	172/172, 174/176	200/206, 211/211	272/272	311/311
					108/108, 112/112	176/176	206/212	268/270, 270/270	307/307, 317/317
					106/106, 112/112	172/172, 176/176	188/194, 198/212	264/270, 270/270	299/313, 313/317

TABLE 5—*Continued*.

[illegible] Monkeys	7	5	III	1 × 50	16,28	ND			ND
					66 *1	ND			ND
					85 8	162 16 *(167) 259 *93 (262)	139 (197) (197)	ND	212 23 (262) 264 *72 (292) 295 17 (327)
					105 *8	162 23 (167) 259 *95 (262)	139,18 (221)	ND	212 22 (276) 264 *77 (315) 294 *3 (331)
					ND	162 23 (167) 259 *95 (262)	139,19 (172)	ND	212 69 (276) 264 *77 (315) 294 43 (347)
					ND	162 23 (167) 259 *95 (262)	139,37 (161)	ND	212 64 (276) 264 *74 (302) 294 *7 (347)
					ND	162 23 (167) 259 *91 (262)	139,18 (221)	ND	212 56 (276) 264 *74 (302) 294,33 (338)
					ND	162 *11 (167) 264 *91 (311)	139 (37) (117)	ND	212 61 (276) 264 18 (302) 294 (331) (338)
African Green Monkey	2	2	ID	46, 50	105 87 (108) 111 *90 (276)	ND		ND	164 56 (176) 254 61 (302)
					105 87 (108) 111 *70 (94)	ND		ND	164 56 (166) 254 71 (348)
Rhesus Monkey	9	1	III	46 × 50	106 15	162 23 (166) 259 *95 (262)	139,6 (181)	ND	208 *7 (276) 264,25 (275)
					105 *7	162 23 (183) 259 (95) (96)	139,72 (142)	ND	208 *7 (276) 264,25 (302)
					105 87 (108) 111 *201 (210)	162 23 (166) 259 (96) (262)	139 (2 (181)	ND	208 (2 (276) 264,25 (302)
					105 *8	162 23 (262) 259 *92 (266)	139 21 (271)	ND	208 (9 (276) 264 (28) 300) 294 *7 (335)
Marmoset	1	1	ID	4 × 50	101 12				
Mouse-ear	1	1	ID	4 × 50	109 10				

* N, IUPAC primers (nucleotides 1[n=1]251–3[n=1]284 of IL10 GAT of NHE IB primers (i.e., 1[n=1]251–3[n=1]284, GenBank GAT no. M65291) and 2[n=1]251–3[n=1]284, GenBank GAT no. M65291) designed on these GAT data.

† ND, not detected.

‡ PCR products from 10 cycles of PCR of 75 attoMol of detected white 50 ng of DNA were used to amplify at 60°. Numbers in parentheses represent the peak height in RFU of the fragment detected.

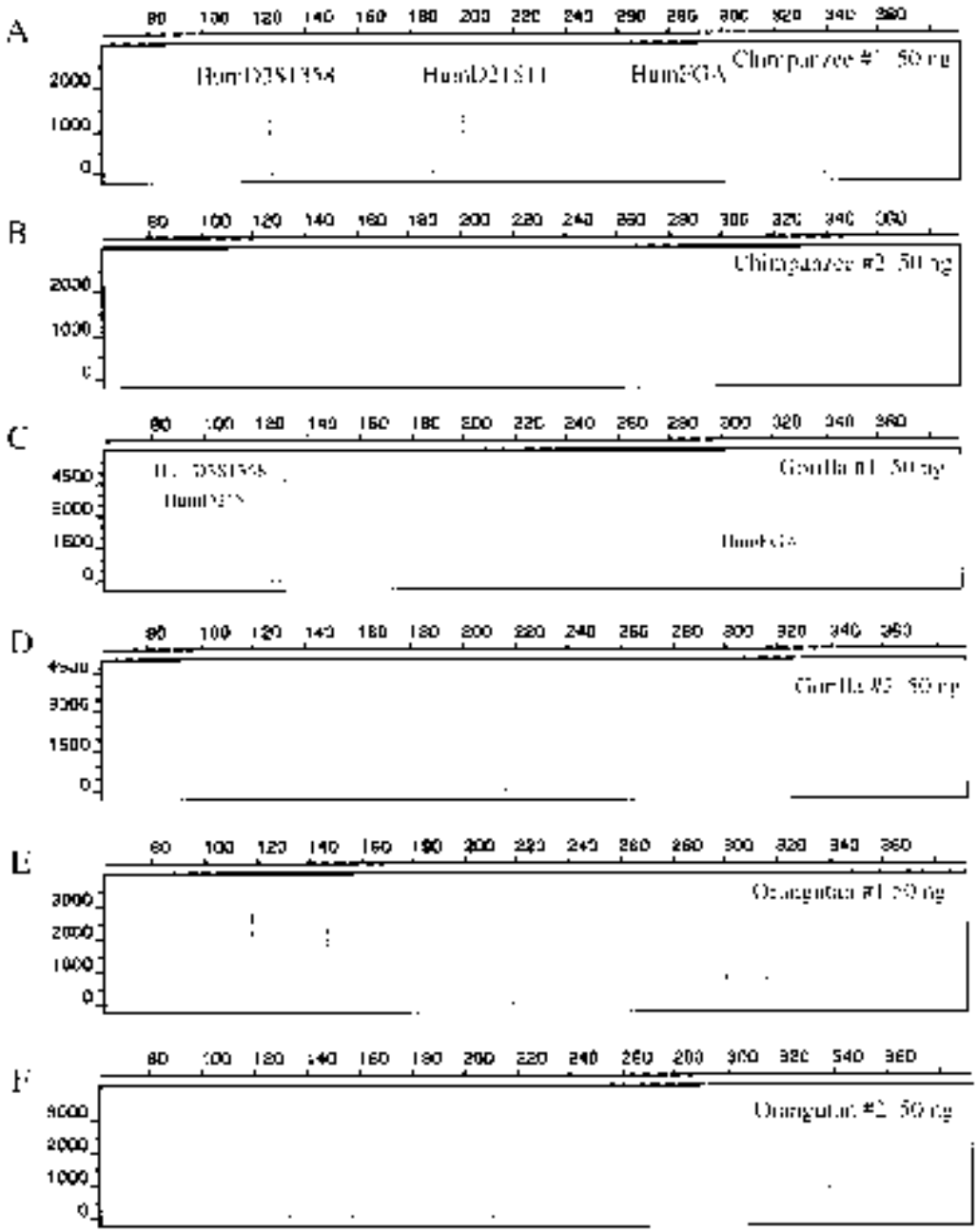


FIG. 7—*Species specificity of STR-1B primer sequences in Higher Primates. The STR-1B system was tested on DNA (5 and 50 ng) from 8 gorillas, 8 orangutans and 5 chimpanzees and analyzed on the ABI Prism™ 377 DNA Sequencer. Examples of amplicons obtained for each species are shown. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 350 ROX run in each lane. (A), (B): chimpanzees, 50 ng DNA; (C), (D): gorillas, 50 ng DNA; (E), (F): orangutans, 50 ng DNA. HumD3S1358 primers were labeled with FAM, HumD21S11 primers with FAM and HumFGA primers with JOE.*

degree of polymorphism in all three species. In addition, profiles showed balanced signals across the three STR loci in STR-1B. This was especially true for the chimpanzees, whereas the high molecular weight HumFGA alleles in gorillas and orangutans were less efficiently amplified. The HumD3S1358 alleles all fell within the allele size range noted for humans (i.e., 114 to 142 bases, Ref 46); gorilla allele sizes were between 126 and 134 bases, orangutan allele sizes between 118 and 134 bases, and chimpanzee allele sizes between 122 and 130 bases. The HumD21S11 alleles were much smaller in size than those of humans; gorilla and orangutan allele sizes were between 126 and 148 bases and chimpanzee allele sizes were between 184 and 212 bases, which are much closer in size to the human D21S11 alleles (202 to 250 bases, Ref 61). Meyer et al. (60) reported similar observations using different individuals from the Higher Primate group. In contrast, other investigators (40,49) observed HumD21S11 alleles for gorillas and orangutans that were larger or in the top range of the human allele sizes using the same set of primers in a different multiplex STR system. In the same studies (40,49), chimpanzees showed alleles smaller than those of humans.

The HumFGA alleles observed in the gorillas and orangutans tested were all in the top range of the human allele sizes reported (238 to 372 bases, 373A instrument, Ref 61), and were between 294 and 344 bases (on the 373A instrument) or between 296 and 346 bases (on the 377 instrument). Allele sizes for chimpanzees were between 260 and 272 bases (on the 373A instrument) which correspond to a size range where frequent HumFGA alleles were reported in humans (61). Similar observations were made by other groups (40,49).

Other animals tested included members of the Lower Primates, Old World and New World monkey groups. No polymorphism was observed at any of the STR loci examined. Rather, monomorphic bands or nonspecific fragments of the same size were detected in all animals (Table 5 and Fig. 8) and only sequencing experiments could differentiate between these two possibilities. The 139 base fragment amplified using the HumD21S11 primers lies outside the human D21S11 allele size range, but the 255 base and 295 base fragments generated using the HumFGA primer set fall within the human FGA allele size range. The use of 50 ng of DNA for amplification promoted mispriming events and generated a number of nonspecific bands of different sizes with fluorescence intensities between 59 and 440 FU (see Table 5). The majority of these bands fell outside the HumD3S1358, HumD21S11 and HumFGA allele size range determined for humans, with the exception of a 288 base fragment which corresponds to a HumFGA allele. Detection of these bands in forensic situations is unlikely and to our knowledge, monkey DNA has never been encountered or reported by any international forensic laboratories.

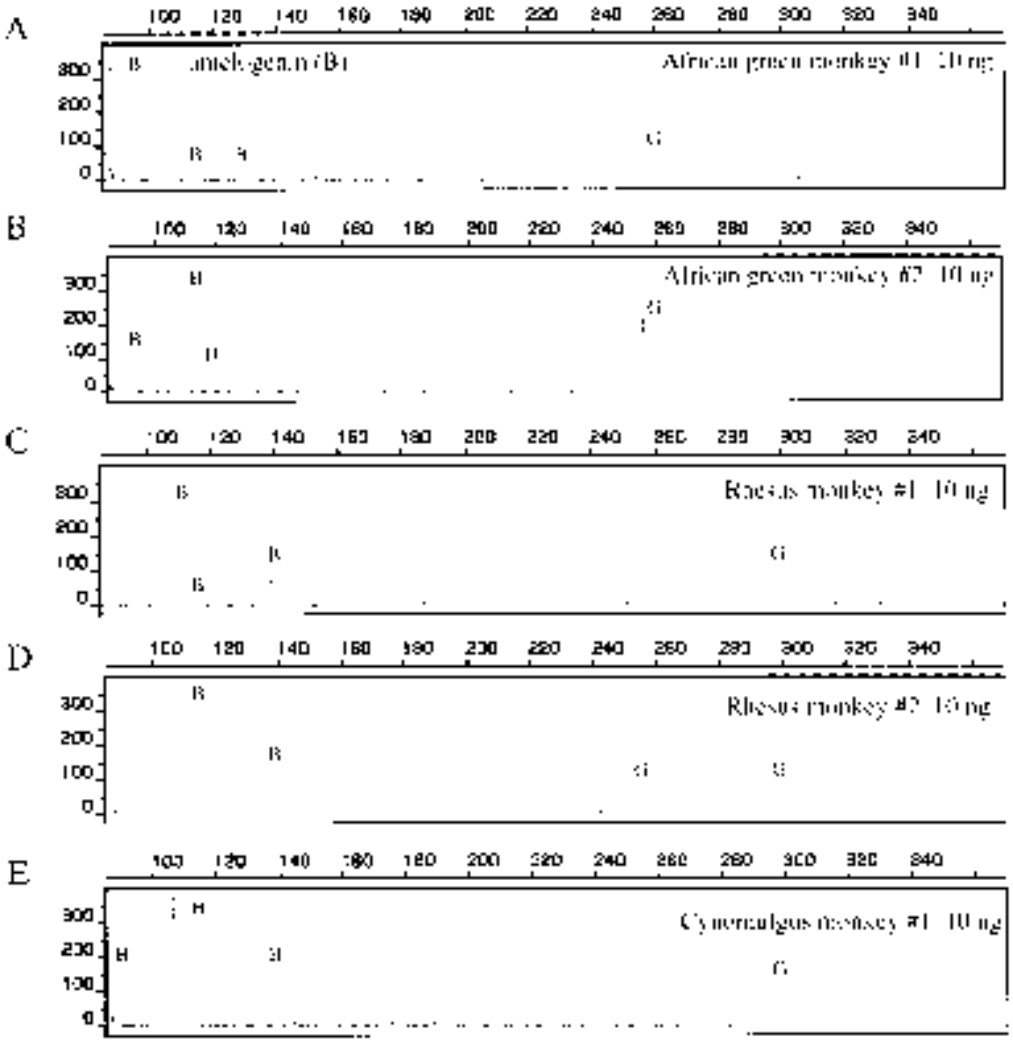
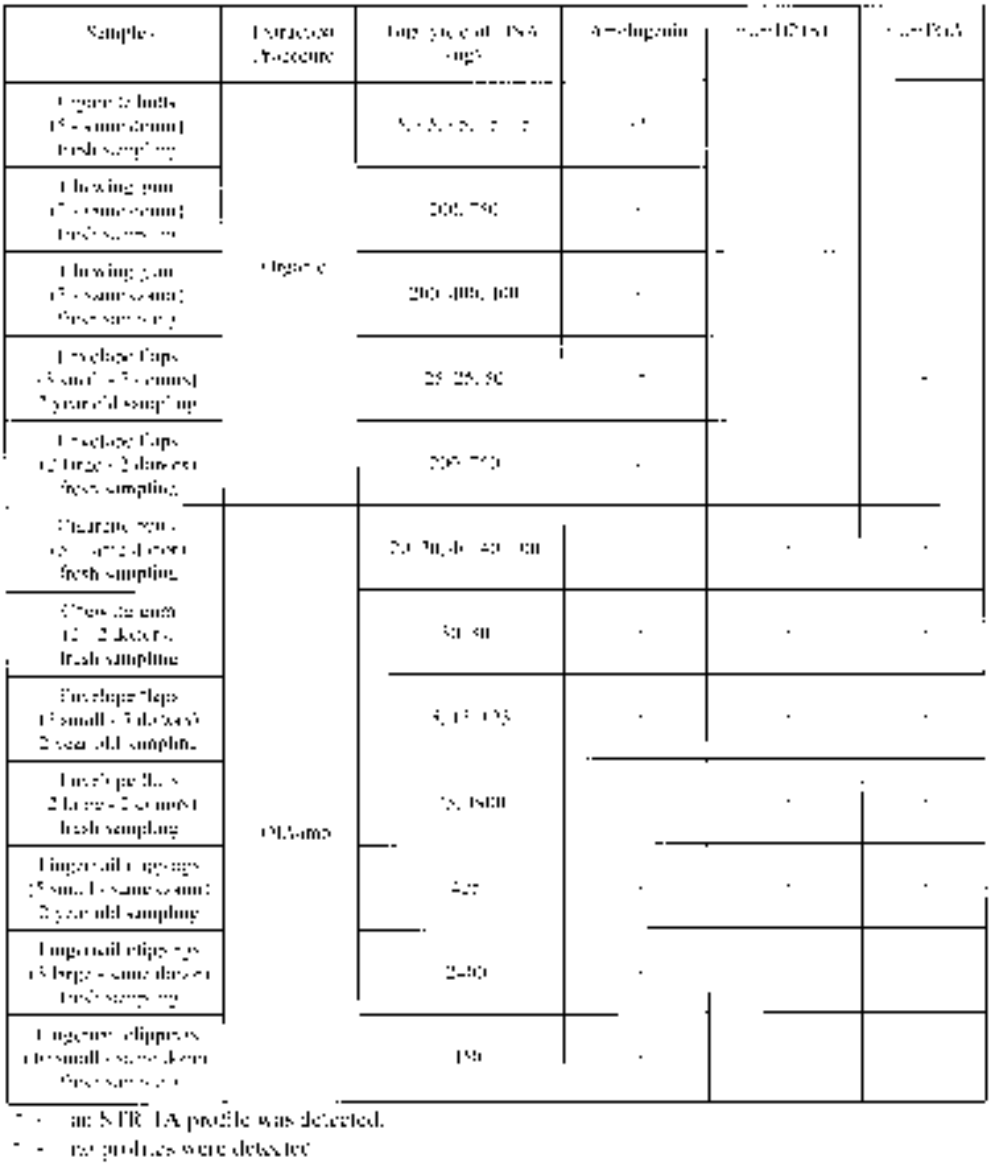


FIG. 8—*Species specificity of STR-1A primer sequences in Old World Monkeys. The STR-1A system was tested on DNA from 8 Cynomulgus monkeys, 2 African green monkeys and 4 Rhesus monkeys and analyzed on the ABI Prism™ 377 DNA Sequencer. Examples of amplicons obtained for each species are shown. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 350 ROX run in each lane. (A), (B): African green monkeys, 10 ng DNA; (C), (D): Rhesus monkeys, 10 ng DNA; (E) Cynomulgus monkeys, 10 ng DNA. Amelogenin primers were labeled with FAM, HumD21S11 primers with FAM and HumFGA primers with JOE. B refers to a blue-colored peak and G, to a green-colored peak. The size in bases of the bands detected and their respective fluorescence intensities in FU are the following: (A) 88.26 (407), 105.87 (407), 111.90 (25), 127.84 (27) and 254.68 (86); (B) 90.36 (132), 105.87 (1000), 111.79 (94) and 254.67 (226); (C) 105.87 (305), 112.01 (21), 139.72 (84) and 294.25 (89); (D) 105.87 (1227), 139.72 (130), 253.73 (63) and 294.27 (80); (E) 88.36 (174), 105.87 (310), 139.60 (160) and 294.17 (123).*

This series of results suggest that the STR loci under study have been conserved throughout evolution, with profiles in chimpanzees showing a tendency towards smaller STR alleles for HumD3S1358 (122 to 130 bases versus 114 to 142 bases), HumD21S11 (184 to 212 bases versus 202 to 250 bases) and HumFGA (260 to 272 bases versus 238 to 372). Rubinsztein et al. (62,63) showed that microsatellites started with a shorter ancestral state and evolved more rapidly in humans than in chimpanzees. They also presented models to explain the propensity for humans to expand the repeats. The high degree of conservatism between humans and chimpanzees had previously been noted using dinucleotide and trinucleotide repeats (62–65). Our study, using a large survey of animals belonging to the Higher Primate group, shows that this high degree of conservatism also extends to tetranucleotide repeats. This further suggests that the HumD3S1358, HumD21S11 and HumFGA loci predated the divergence between humans and chimpanzees. A similar observation was made by other investigators concerning other STR loci (59,65).

Sex typing results also revealed that gender determination in domestic and wild game animals, as well as in orangutan and Old World monkeys, was not possible using the human amelogenin primer set selected for this study. Indeed, only one band sized at 102 to 103 bases (for domestic animals [dog, cat, pig, cow, horse, sheep, mouse, hamster] and members of the Cervidae group ([deer, moose, bison, goat]) or 106 bases (for orangutan) was observed regardless of the gender of the animals surveyed (data not shown). As was suggested by other investigators (66), this may result, in some species, from the lack of sex differences between the X and Y homologues of the amelogenin gene; a situation that prevails for bovines (67). Alternatively, and as reported for the mouse (68),

TABLE 6—*STR-1A typing using DNA from miscellaneous samples extracted using the organic extraction method or the QIAamp extraction protocol.*



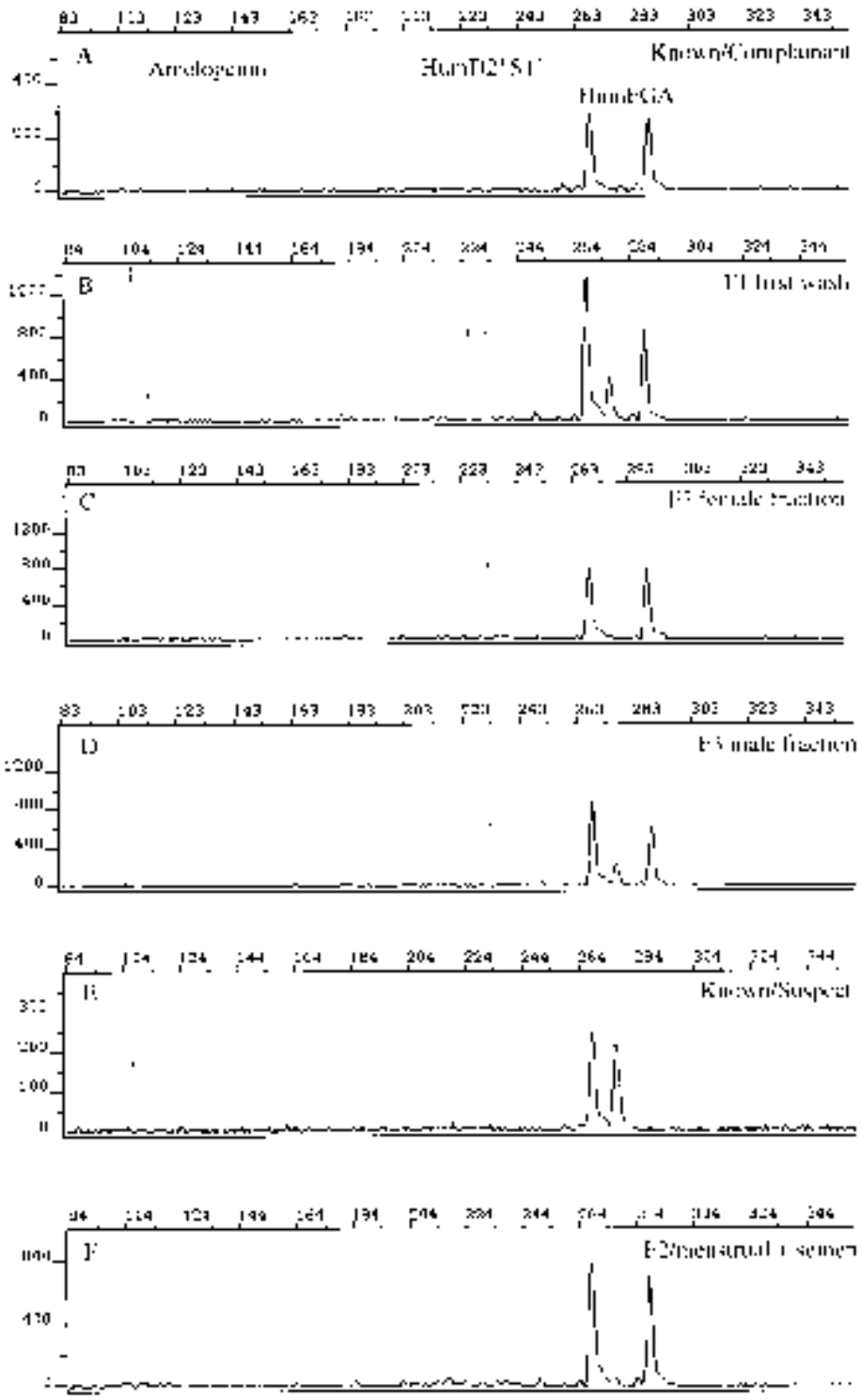


FIG. 9—*STR-1A typing analysis of simulated sexual assault cases (Case #1). Mock sexual assault cases were prepared from vaginal swabs (with and without menstrual blood) obtained from two female donors and semen from three different male donors chosen at random among the laboratory personnel. A total of 10 ng DNA was amplified under conditions detailed in Materials and Methods using 28 cycles of amplification. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 2500 ROX run on the ABI Model 373A DNA Sequencer. (A) Control from complainant, vaginal swab; (B) first wash of vaginal/semen swab (F1 fraction); (C) female epithelial cell fraction (F2 fraction); (D) male sperm cell fraction (F3 fraction); (E) control from suspect, semen; (F) female epithelial cell fraction from menstrual/semen swab (F2 fraction). Amelogenin primers were labeled with JOE, HumD21S11 primers with JOE and HumFGA primers with TAMRA.*

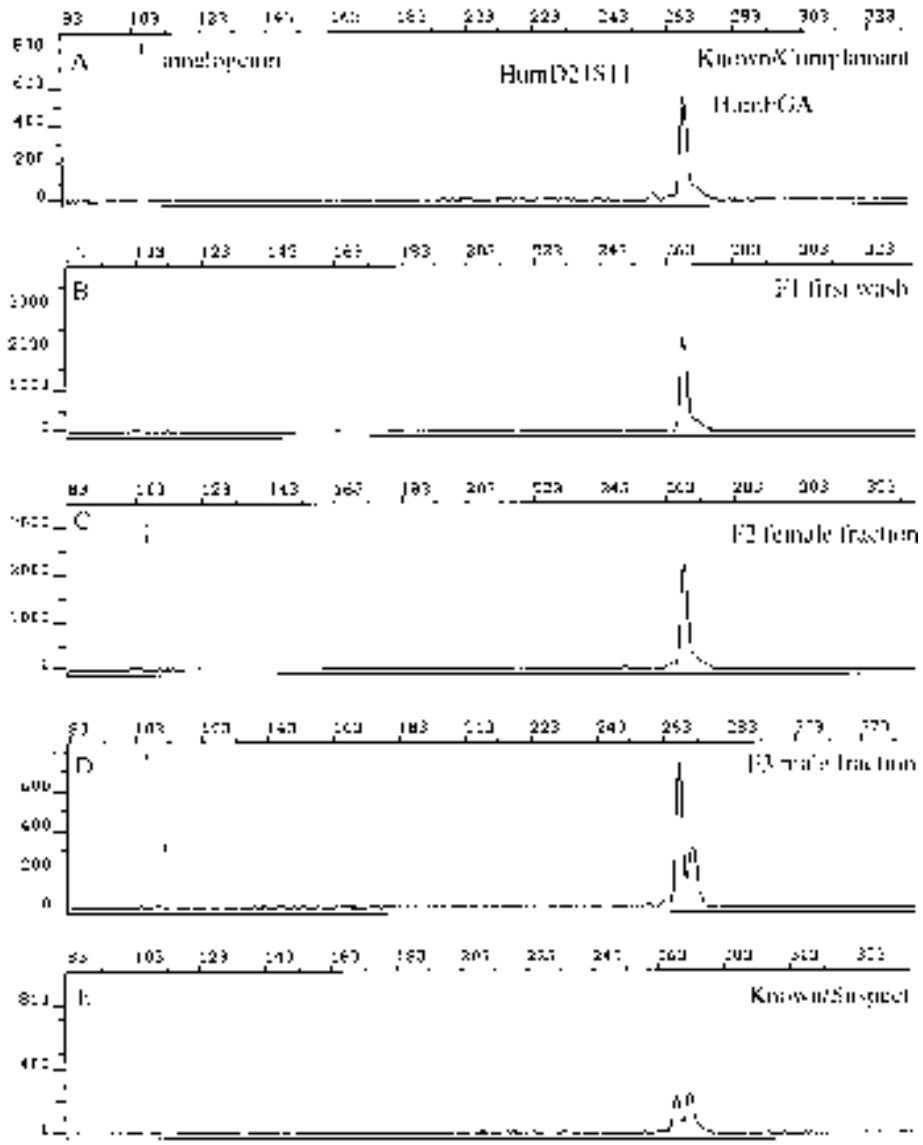


FIG. 10—*STR-1A typing analysis of simulated sexual assault cases (Case #2). Mock sexual assault cases were prepared from vaginal swabs (with and without menstrual blood) obtained from two female donors and semen from three different male donors chosen at random among the laboratory personnel. A total of 10 ng DNA was amplified under conditions detailed in Materials and Methods using 28 cycles of amplification. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 2500 ROX run on the ABI Model 373A DNA Sequencer. (A) Control from complainant, vaginal swab; (B) first wash of vaginal/semen swab (F1 fraction); (C) female epithelial cell fraction (F2 fraction); (D) male sperm cell fraction (F3 fraction); (E) control from suspect, semen. Amelogenin primers were labeled with JOE, HumD21S11 primers with JOE and HumFGA primers with TAMRA.*

amelogenin sequences in some species may be present exclusively on the X chromosome. Studies performed by Bailey et al. (69) revealed a remarkable degree of conservation between amelogenin X-Y homologues among the Great Apes and Old World monkeys. Using their primer set, gender identity was possible for chimpanzees, gorillas, orangutans, rhesus monkeys and crab-eating macaques. Failure to detect a Y product in orangutan using our primers (which map to a different position in the first intron of the gene) may reflect sequence divergence in the primer site on the Y homologue. Alternatively, in these animals, our primers may flank a region of the gene which shows a high degree of conservation between the X and Y homologues and lacks the 6 base pair difference targeted for gender identity. Sequence experiments could provide more information regarding these two possibilities.

Although two bands, sized at 106 and 112 bases, were detected in green monkeys and rhesus monkeys, a strong differential was noted between the peak heights of the two bands (112/106 ratios ranged from 6% to 9%); Fig. 8, panels A-C. Such a differential could potentially prevent the detection of the 112 band in situations of reduced number of target DNA. In view of the studies performed by Bailey et al. (69), failure to detect a balanced X/Y product ratio in Old World monkeys using our amelogenin primers possibly reflect sequence divergence in the primer site on the Y homologue.

Miscellaneous Samples

As indicated in Table 6, both extraction procedures provided amplifiable DNA from cigarette butts, chewing gum and fingernail

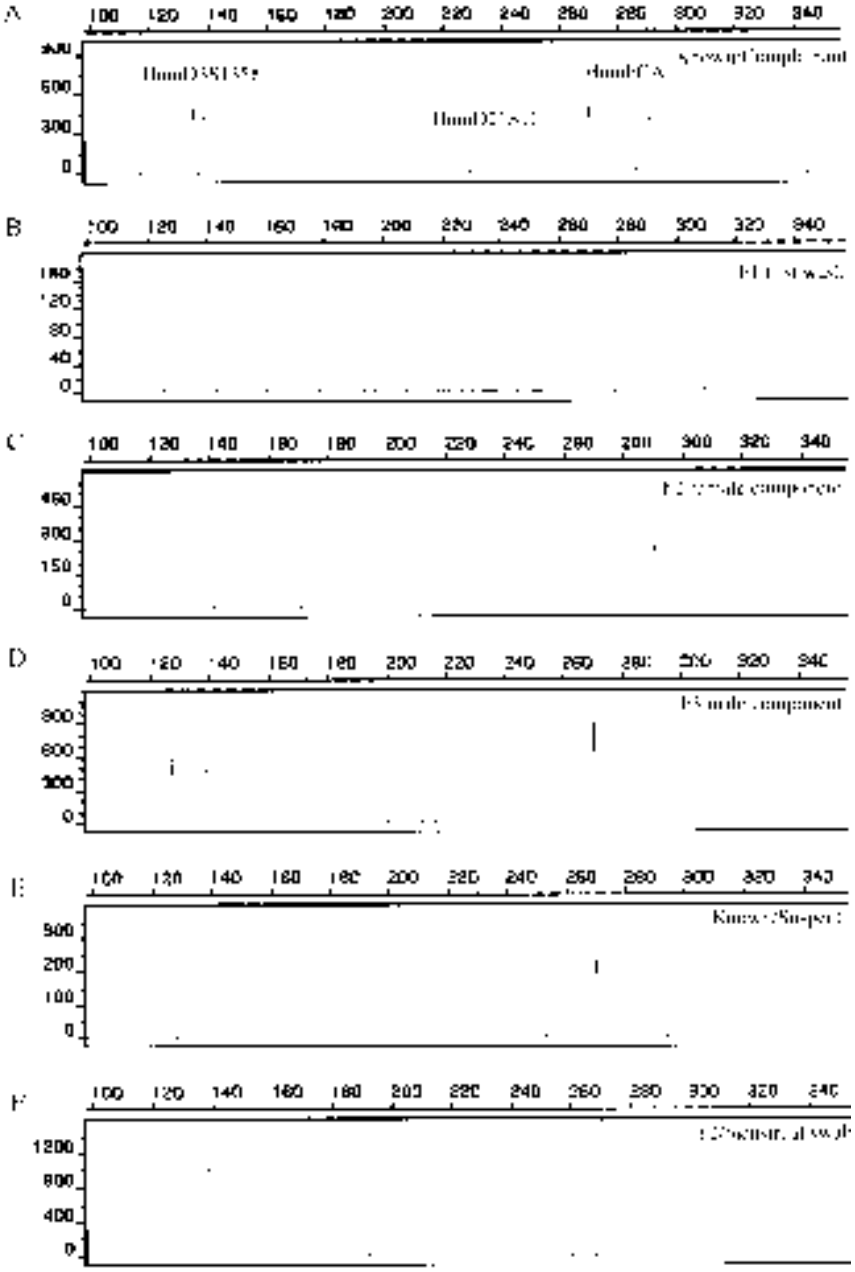


FIG. 11—*STR-1B typing analysis of simulated sexual assault cases. Mock sexual assault cases were prepared from vaginal swabs (with and without menstrual blood) obtained from two female donors and semen from three different male donors chosen at random among the laboratory personnel. A total of 5 ng DNA was amplified under conditions detailed in Materials and Methods using 30 cycles of amplification. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 350 ROX run on the ABI Prism™ 377 DNA Sequencer. (A) Control from complainant, vaginal swab; (B) first wash of vaginal/semen swab (F1 fraction); (C) female epithelial cell fraction (F2 fraction); (D) male sperm cell fraction (F3 fraction); (E) control from suspect, semen; (F) female epithelial cell fraction from menstrual/semen swab (F2 fraction); HumD3S1358 primers were labeled with FAM, HumD21S11 primers with FAM and HumFGA primers with JOE.*

clippings, even the fingernail clippings stored at room temperature for two years. All STR profiles generated were consistent with those of the control samples from the donors. However, the yield of DNA recovered from the specimens varied greatly between extraction procedures. The amount of DNA obtained from the ten cigarette butts received from the same individual was significantly lower (by a factor of 4 to 20) using the organic extraction procedure. The QIAamp protocol appeared better suited for recovering DNA from cigarette butts. Although not compared to the organic procedure, excellent yields were also obtained for fingernail clippings using the QIAamp kit. Interestingly, greater yields of DNA were recovered from the chewing gum samples using the organic extraction procedure (4 to 15 times for donor A and 3 to 5 times for donor B) compared to the QIAamp protocol. In addition, the fast and easy QIAamp extraction protocol appeared better suited to recover amplifiable DNA from envelope flaps. Indeed, although DNA from envelope flaps was obtained using the organic procedure, no STR profiles were generated. STR profiles produced from the DNA extracted using the QIAamp kit were consistent with those of the control samples from the donors.

Simulated Sexual Assault Cases

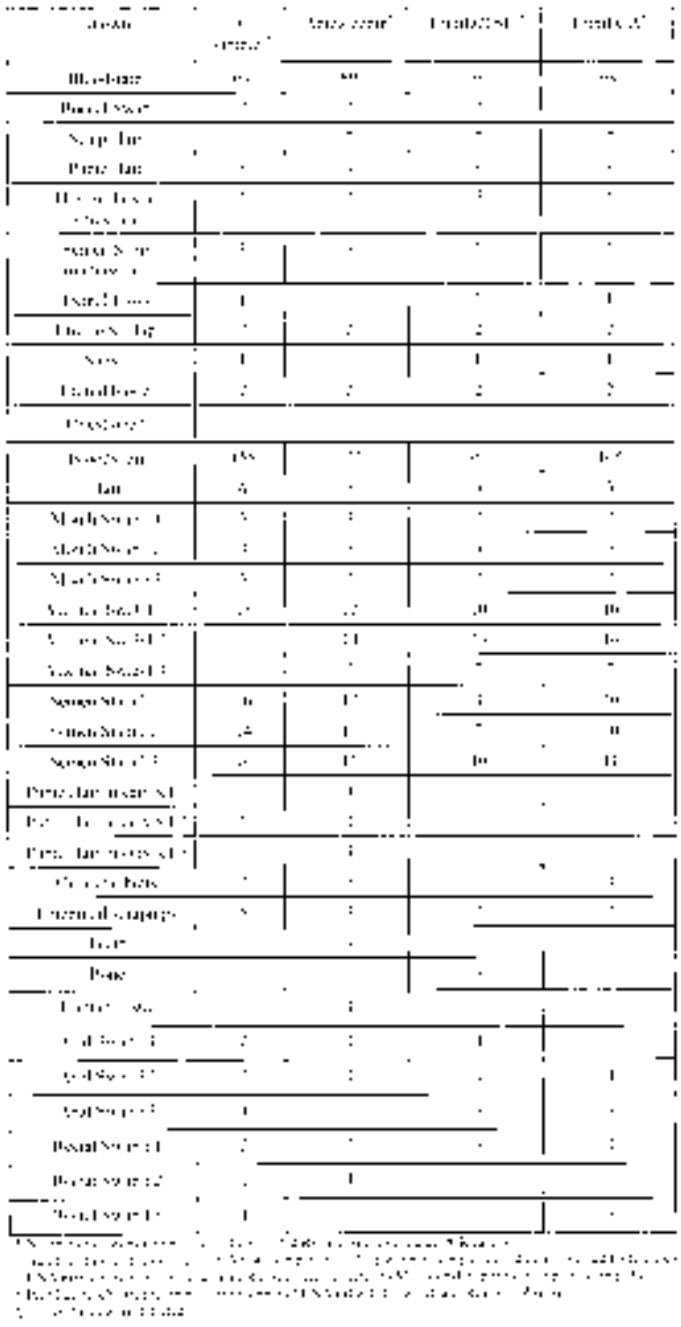
Mock cases from the nine different sexual assault situations simulated using two females (menstrual and nonmenstrual swabs) and three semen donors are presented in Figs. 9–11; Figs. 9 and 10 represent STR-1A typing results generated on an ABI Model 373A Sequencer and Fig. 11, STR-1B typing results as analyzed on the ABI Prism™ 377 DNA Sequencer. The differential extractions were successful in most instances and allowed female and male STR profiles to be clearly distinguished. Indeed, the F2 fractions (female component) from the six swabs without menstrual blood consistently generated STR profiles corresponding to the female donors only (Figs. 9–11, panel C). Two of the F2 fractions from the three swabs with menstrual blood however, showed mixed profiles with a minor contribution from the male component (Figs. 9 and 11, panel F). All F3 fractions (male component) appeared to contain some carryover of female cell components as evident by the detection, in these samples, of both female and male contributor's STR profiles (Figs. 9–11, panel D).

The use of F1 fractions in STR profiling, which represent the first wash of the swabs and often contain cellular debris, was examined in this study. The F1 fractions provided valuable information on the male contributor's profile in 3 out of 9 (33%) instances due to premature lysis of the sperm cells (Fig. 9, panel B). In 8 out of 9 samples (89%), the F1 fractions confirmed the female contributor's profile (Figs. 9 and 10, panel B). Only one F1 fraction showed no STR results (Fig. 11, panel B). The data strongly suggest that the F1 fractions are important samples to consider when processing mixed swabs or stains by PCR STR analysis. As more challenging samples are received for PCR analysis, and likely those with a high degree of cell damage, F1 fractions containing these damaged cells may become a major source of information about the male contributor of the semen on a swab. Already, this situation has been encountered in sexual assault cases in a number of laboratories including our own.

Casework Samples

As summarized in Table 7, all known samples (a total of 97), with the exception of the human muscle DNA, produced complete STR-1A profiles. Approximately 64% of the questioned DNA

TABLE 7—*STR-1A typing analysis using casework samples.*



extracts (a total of 277) generated full profiles that were consistent with those of the corresponding known samples. Another 17% of the questioned specimens failed to amplify, whereas 8% showed results for amelogenin only. Partial profiles consisting of amelogenin and HumFGA were noted in 9% of exhibits examined. Only 2% of specimens showed partial profiles composed of amelogenin and HumD21S11. Examples of exhibits that failed to produce a complete STR-1A profile are shown in Fig. 12, and included bloodstains (on nylon pants, rope, paper towels, black running shoes, black leather jacket, black or blue T-shirts, black, navy blue or gray pants, brown carpet, floormat of a truck, blue vinyl seat, kitchen knife, glove, $10 bill), cigarette butts, fingernails, scalp hair, anal swabs, rectal swabs, swabs from door handles and human

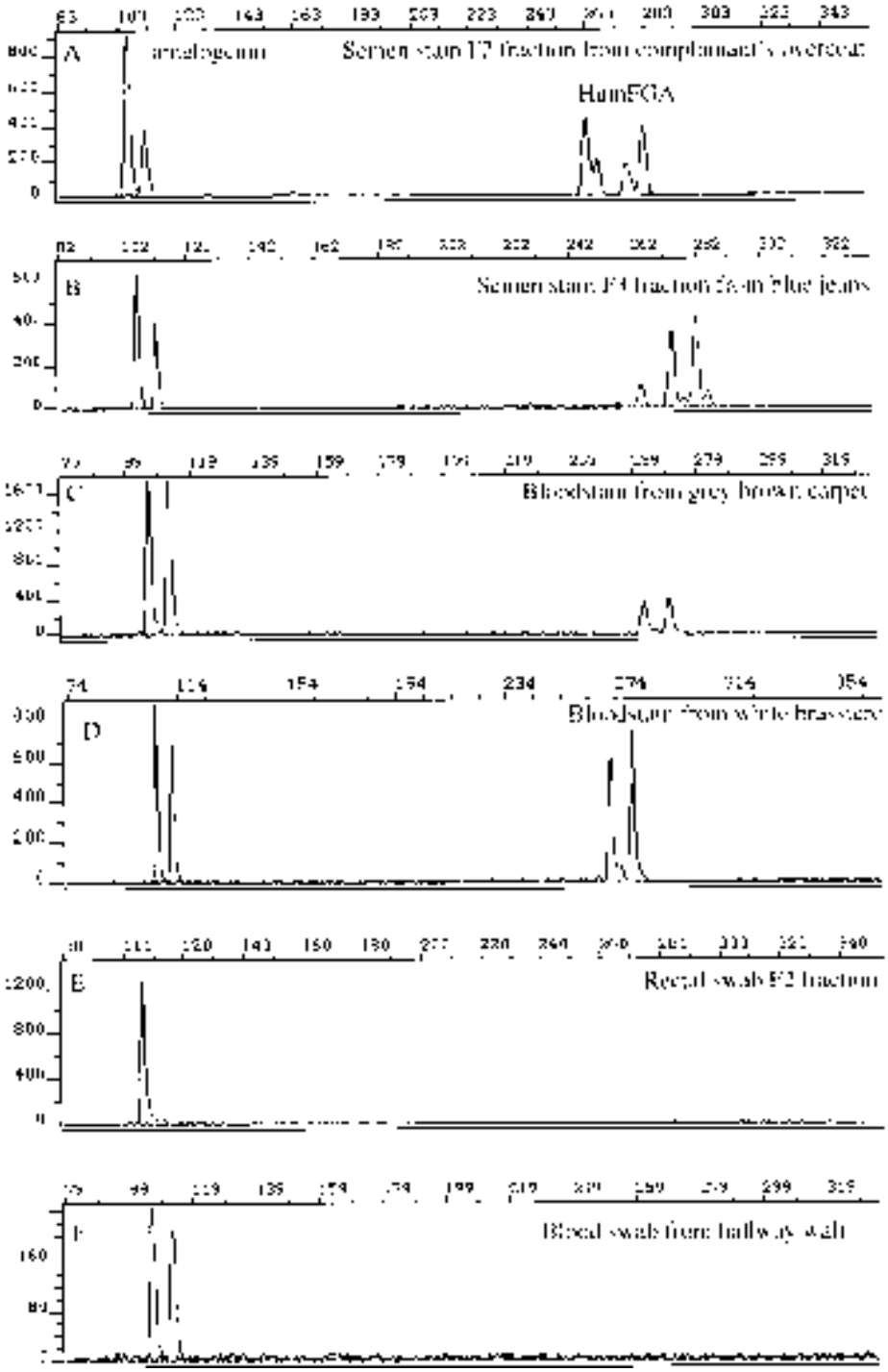


FIG. 12—*Partial STR-1A profiles observed with casework samples. A total of 8 to 9.5 ng of DNA was used with 28 cycles of amplification. Amplicons were analyzed on the ABI 373A DNA Sequencer. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 2500 ROX run in each lane. (A) Semen stain F2 fraction from complainant's overcoat; (B) semen stain F3 fraction from blue jeans; (C) bloodstain from gray brown carpet; (D) bloodstain from white brassiere; (E) rectal swab F2 fraction; (F) blood swab from hallway wall. Amelogenin primers were labeled with FAM, HumD21S11 primers with JOE and HumFGA primers with TAMRA.*

muscle. In these instances, DNA degradation and limited quantities of DNA recovered from the specimens, along with potential PCR inhibitors, could be possible explanations for the lack or weak amplification results.

Interestingly, of the 277 questioned samples tested, 219 (79%) provided a result for amelogenin versus 189 (68%) for HumFGA and 165 (60%) for HumD21S11. Most of the samples that produced partial STR-1A profiles lacking the HumD21S11 alleles were bloodstains. Other samples that showed the HumD21S11 locus dropout phenomenon included semen stains F1, F2 and F3 fractions and cigarette butts. Thus, it appears that HumD21S11 may be less efficiently amplified when subjected to certain insults. Selective degradation of the HumD21S11 locus (chromosome 21) over the HumFGA locus (chromosome 4) is unlikely since further purification of the DNA extracts remaining from RFLP cases, using Microcon-100 size-exclusion columns, promoted successful amplification of HumD21S11 in most of these samples (see next section).

Table 8 reveals that 63% of the exhibits examined, which had generated a result following RFLP analysis, showed STR-1A profiles. Another 23% of casework specimens which gave reportable STR-1A results had not been processed using RFLP single locus probes because of DNA degradation or insufficient DNA. Yet another 12% of samples, not processed for RFLP, remained refractory to amplification and provided no STR-1A results. This may be due to high levels of DNA degradation, very low amounts of DNA or the presence of PCR inhibitors in the DNA extracts. Only 2% of the items tested, which had failed to produce RFLP results, generated STR profiles. More importantly, the case conclusions derived from the STR-1A typing analysis corroborated those of the RFLP analysis.

Typing results for the triplex STR-1B system are summarized in Table 9. Thirty-seven cases (23 completed RFLP cases and 14 actual PCR cases) representing 156 samples were processed using this STR system. All known samples (a total of 62) produced a full profile with the exception of two exhibits. One pair of blue underwear from a suspect (indicative of epithelial cells, unidentified biological material) generated a partial HumD3S1358/HumFGA profile (Fig. 13, panel A). A mouth swab from a suspect showed no STR results. Approximately 85% (80 out of 94) of the questioned DNA extracts generated STR-1B profiles which were consistent with those of the corresponding known samples. Another 8% of the questioned exhibits yielded no results. Amplification failure was usually associated with the extent of degradation of the DNA, and/or the minute amounts of DNA recovered from the exhibits (scalp hair from clothing, saliva stains on a black nylon stocking, one F1 fraction from a vaginal swab with semen, one F2 fraction from an anal swab, one bloodstain from a facial tissue paper and one bloodstain from a brown carpet). Partial profiles were also noted in 7% of the questioned specimens examined. The partial profile HumD3S1358/HumFGA was observed more frequently (5%) than the profiles consisting of HumD21S11 alone (1%) or HumFGA alone (1%). Partial profiles were generated from two vaginal swabs (F2 fractions), a bloodstained jacket, two bloodstains on the interior door of a car and two bloodstains on brown suede shoes (Fig. 13).

As noted before with casework items processed using the triplex

TABLE 8—*Success rate comparison between RFLP and STR-1A typing analysis.*

Typing results	# of samples
RFLP and STR-1A:	86/142[a]
[illegible]	41
[illegible]	3
[illegible]	2
RFLP (partial digest) and STR-1A[b]	6/142
[illegible]	1
RFLP but no STR-1A[c]	2/142
STR-1A but no RFLP[d]:	7/142
[illegible]	5
[illegible]	1
STR-1A but no RFLP[e]	35/142
[illegible]	15
[illegible]	9
[illegible]	9
No triplex STR and no RFLP[f]	17/142

[a] 142 cases/total number of samples examined.

[b] RFLP analysis was performed on two separate vaginal swabs: one F2 fraction from a vaginal swab and one F2 fraction from an anal swab.

[c] No STR-1A profiles were obtained for samples processed for RFLP: 2/11 fractions, 1 F2 fraction with [illegible] degraded DNA, 3/13 bloodstains with [illegible] degraded DNA, 2 bloodstains with [illegible] degraded DNA, 2 bloodstains with sufficient DNA but did not proceed to RFLP analysis.

TABLE 9—*STR-1B typing analysis using casework samples.*

[illegible]	[illegible] samples*	[illegible]†	[illegible]‡	[illegible]§
Bloodstain	45	35	38	34
Dried Blood	2	2	2	2
Saliva	4	4	4	4
Semen	1	1	1	
Scalp Hair	3	3	3	3
Pubic Hair	1	1	1	
[illegible]	2	2	1	1
Questioned				
Bloodstain	17	15	15	17
Hair/Scalp¶	1	1	1	1
Vaginal Swab E1	8	7	7	
Vaginal Swab E2	12	11	11	11
Vaginal Swab E3	5	5	5	5
Semen Stain E1	5	4	4	4
Semen Stain E2	2	2	2	2
Semen Stain E3	8	8	8	8
Anal Swab E2	2	1	1	
Anal swab E3	2	2	2	2
Sperm [illegible]	3	3	3	3
Cigarette butt	1	1		1
Unidentified substance	2	2	2	2
Human Tissue	1	1	1	1
[illegible]	2	2	2	2

* Number of casework samples tested: 87 (37 from completed RFLP cases, 11 questioned, 39 [illegible] cases).
† Number of exhibits examined: 62 casework samples, 5 [illegible] samples (only HumD3 and HumTH01 DNA extracts from the 37 RFLP cases were subjected to Microcon-100 purification prior to amplification).
‡ Profiling results are presented as the number of DNA extracts for which a profile was observed.
§ [illegible] alleles were detected.

STR-1A, but to a lesser extent, the HumD21S11 locus appeared to be less efficiently amplified than HumFGA. Of 94 questioned samples, 85 (90%) provided a result for HumD3S1358 versus 81 (86%) for HumD21S11 and 86 (91%) for HumFGA. The reason why no parallel decrease in HumD21S11 detection was noted using the STR-1B system was that most samples (remaining from RFLP completed cases), which showed partial STR-1A profiles lacking HumD21S11 alleles, were consumed during the validation of the triplex STR-1A system. Since these samples were not included in the validation of the STR-1B system, the percentage of samples showing full profiles was much higher for STR-1B.

Comparison of the STR-1B typing results with those of the RFLP analysis performed on the same casework items revealed that 85% of exhibits examined generated both RFLP and STR profiles. Another 11% of casework items which yielded reportable STR-1B results had not been processed using RFLP single locus probes because of degradation or insufficient DNA. Only 1% of specimens which failed to produce RFLP results, generated STR-1B results. In addition, 3% of exhibits which showed RFLP profiles were not successfully typed using the STR-1B system due to an insufficient amount of DNA remaining after RFLP analysis. In these instances, STR analysis was attempted in spite of the fact that no detectable DNA was noted.

Microcon-100 Column Testing

Initial results obtained from casework items indicated that detectable levels of DNA were often extracted from stained areas or swabs but amplification products were not always obtained. The use of a size-exclusion spin column (Microcon-100, Amicon) was found to be successful in removing PCR inhibitors from most casework DNA extracts received from completed RFLP cases. As shown in Table 10, this additional step increased the chances of PCR amplification on most challenging samples such as a soiled kitchen knife, a soiled $10 bill, bloodstained black running shoes, a black leather jacket, a blue vinyl seat, navy blue pants, brown carpet and anal and rectal swabs. Of the specimens that showed partial profiles (77%) or no profiles (23%) prior to Microcon-100 treatment, 62% were converted to full profiles following the treatment, 23% remained partial and 15% still did not generate any STR-1A results.

Furthermore, the use of the Microcon-100 column was crucial

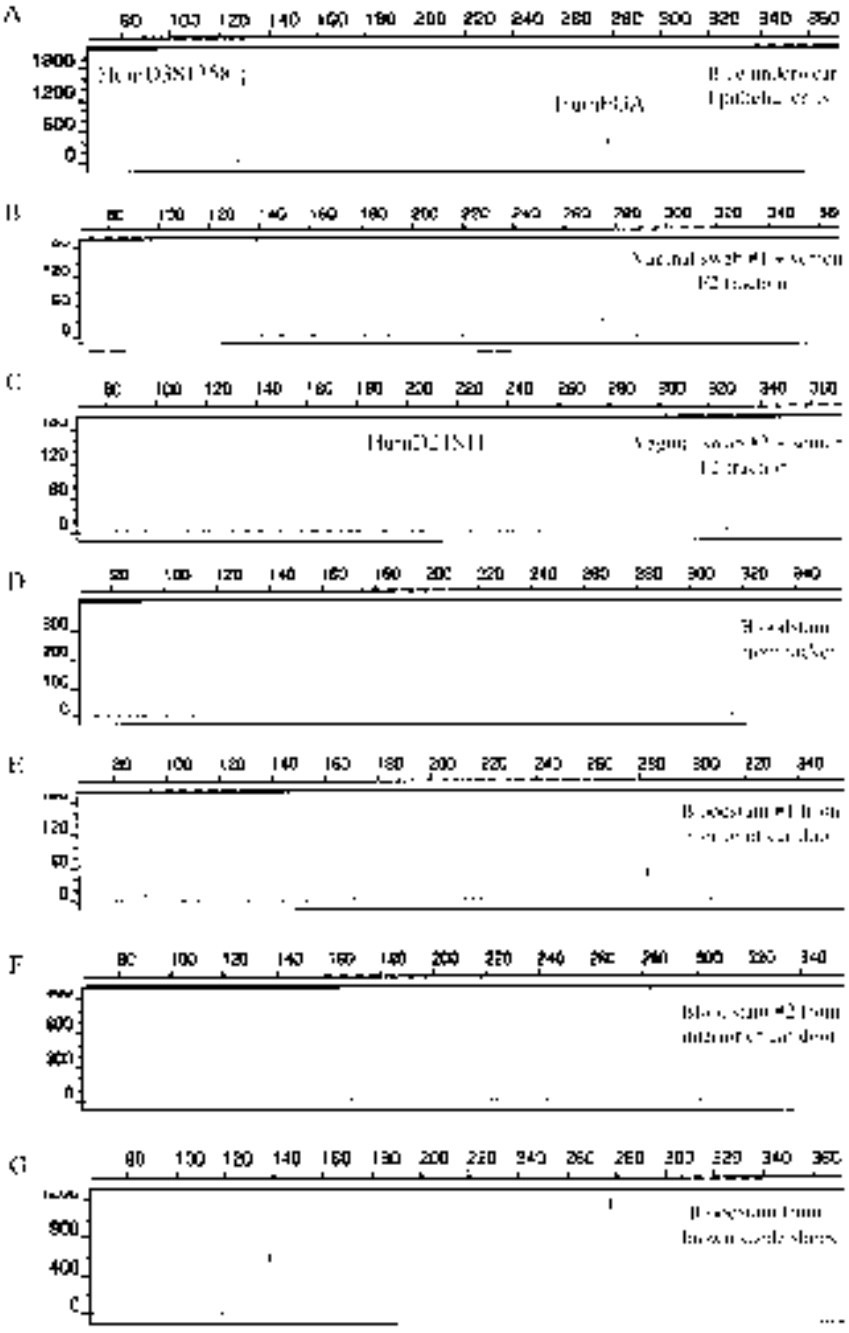


FIG. 13—*Partial STR-1B profiles observed with casework samples. A total of 5 ng of DNA was used with 30 cycles of amplification. Amplicons were analyzed on the ABI Prism™ 377 DNA Sequencer. Each panel depicts the fluorescence intensity (FU, Y-axis) and the size estimate in bases (X-axis) derived from the internal standard Genescan 350 ROX run in each lane. (A) Blue underwear from suspect, indicative of epithelial cells; (B) vaginal swab #1 with semen (F2 fraction); (C) vaginal swab #2 with semen (F2 fraction); (D) bloodstain from jacket; (E) bloodstain #1 from interior of car door; (F) bloodstain #2 from interior of car door; (G) bloodstain from brown suede shoes. HumD3S1358 primers were labeled with FAM, HumD21S11 primers with FAM and HumFGA primers with JOE.*

TABLE 10—*Improved STR-1A typing results following Microcon-100 size-exclusion spin column purification.*

Typing Result			Prior to Microcon-100 purification	Following Microcon-100 purification
			# samples (total = 260)*	# samples (total = 260)*
Complete STR-1A profiles:			8	16 (62%)
Amelogenin	HumD21S11	HumFGA		
†			8	16
Partial STR-1A profiles			28 (11%)	6 (2.3%)
			9	3
			7	0
			4	1
			0	1
No STR-1A profiles			6 (2.3%)	4 (1.5%)
			6	4

* DNA extracts from 2 to 5 year-old case exhibits.

† = alleles were detected.

‡ = no alleles were detected.

in eliminating or reducing the differential amplification observed with the X and Y homologues of the amelogenin gene. Indeed, as shown in Table 11, a number of casework samples (possible mixtures), as well as database samples (nonmixture samples), demonstrated a significant differential amplification of the X and Y homologues of the amelogenin gene (range between 0.1 to 55 times more of the X product over the Y product). This phenomenon could be potentially misleading in the interpretation of mixtures (i.e, assigning the major component of the postulated mixture as female when, in fact, the sample originated from a single male donor). After further purification using the Microcon-100 columns, the fluorescent peak height ratio of the X and Y homologues for most samples were all very close to 1.0 (highest differential was 6, observed only once). Although ratios of 1.0 are indicative of single sources for database samples, the possibility exists for casework samples that a minor female component is present and hidden under the X of the major male component. Some samples such as a bloodstain on carpet and blood swabs were incorporated in the list to show that the Microcon-100 treatment does not have any effect on the ratio of the X and Y homologues of the amelogenin gene when this ratio is close to 1.0 prior to purification on Microcon-100 columns. In addition, the strength of the fluorescent signals never interfered with the generation of ratios of 1.0. Amplifications producing peak heights in the 300 FU range as well as the 1000, 2000, 6000 FU ranges all resulted in ratios of 1.0 between the X and Y homologues of the amelogenin gene.

The use of the Microcon-100 size-exclusion spin columns facilitated the interpretation of multiplex STR-1A profiles and also significantly improved the yield of HumD21S11 and HumFGA amplified products, as noted by the strong fluorescent signals obtained following Microcon-100 treatment. The multiple advantages of the Microcon-100 treatment prompted its use in routine procedures for our laboratory system. Similar observations were reported by others (18,23).

Conclusion

Validation studies described in this paper demonstrate that the multiplex STR systems consisting of amelogenin or HumD3S1358, and HumD21S11 and HumFGA are both robust and reliable, and allow typing of forensic samples that were not suitable for the traditional RFLP procedure due to insufficient and/or degraded DNA. Forensic exhibits from which genetic material was obtained generated STR-1A and STR-1B profiles always consistent with those of control or reference samples. At no time were false positive or false negative results produced. The evolution in fluorescence-based detection technology and related instrumentation dictated the modification of the amplification conditions and design of the triplex STR-1A defined for the model 373A GeneSequencer in order to optimize the detection and interpretation of the profiles on the model 377 GeneSequencer. Both STR-1A and STR-1B systems have been fully validated on the ABI models 373A and 377 DNA Sequencers and provided a rich source of data for the comprehensive development of casework interpretation guidelines. STR mixture interpretation is one of the most challenging situations encountered in our validation studies, and a detailed review with suggested interpretation guidelines will be the subject of a separate publication. Further support of the reliability and validity of these typing systems has been demonstrated repeatedly by STR analysis of actual casework samples in the RCMP Forensic Laboratory Directorate.

Acknowledgments

The authors would like to thank members of the RCMP Central Forensic Laboratory in Ottawa who provided extracted DNA samples from completed RFLP cases for this validation study. We also thank Dr. Benoît Leclair for his critical evaluation of this manuscript.

TABLE 11—*Elimination or reduction of the differential amplification of the X and Y amelogenin bands following Microcon-100 column purification.*

Sample	Amelogenin allele					
	Before Microcon-100 purification			After Microcon-100 purification		
	X allele peak height (RFU)	Y allele peak height (RFU)	X:Y peak height ratio	Y allele peak height (RFU)	Y allele peak height (RFU)	X:Y peak height ratio
Database 010	1755	760	1.8	1622	535	1:3
Database 046	1078	470	2.2	1322	655	1:1
Database 048	1673	1450	1:1	2301	2256	1:1
Database 052	749	315	1:7	951	758	1:2
Database 056	1342	801	1:2	3311	2707	1:2
Database 060	1215	642	1:9	2111	1754	1:7
Database 010	1416	459	1:6	1118	938	1:1
Database 013	567	344	2:3	905	855	1:2
Database 017	707	196	4	1004	1734	1:1
Database 018	761	317	2:1	3459	2754	1:1
Database 019	427	219	2:0	1231	2564	1:3
Database 022	881	408	8	2012	1699	1:2
Database 024	428	210	2:6	780	1495	1:1
Database 17	4033	1225	8	11	15	1:3
Database 18	3441	2139	7	270	257	1:1
Database 19	3013	1333	9	72	143	1:2
DataBase 021	2152	1209	8	370	253	1:2
Bone Sample 33 Section	3385	253	13:1	211	1:1	4:0
Semen Swab 01 Section	3815	1934	2:0	3695	0:242	0
Saliva Swab 1 Section	4508	1:5	45	2385	078	1:0
Vaginal Swab 11 Section	5003	1654	3:2	3762	5102	1
STORM Model with Blood	861	81	6:2	222	1784	1:2
Bloodstain on carpet	1542	1552	1:0	6750	6819	1:0
Blood Stain on cut	755	519	1:2	6:15	5783	1:7
Semen swab 02 freezer	29	1758	1:7	4521	2109	1:8
Blood swab	1216	987	2:1	5812	5241	1:0

[illegible] Concentration too low

References

1. Sweet DJ, Sweet CHW. DNA analysis of dental pulp to link incinerated remains of homicide victim to crime scene. J Forensic Sci 1995;40:310–4.
2. Gill P, Ivanov PL, Kimpton C, Piercy R, Benson N, Tully G, et al. Identification of the remains of the Romanov family by DNA analysis. Nature Genet 1994;6:130–5.
3. Hochmeister MN, Budowle B, Borer UV, Eggmann U, Comey CT, Dirnhofer R. Typing of deoxyribonucleic acid (DNA) extracted from compacted bone from human remains. J Forensic Sci 1991; 36:1649–61.
4. Morris JW, Sanda AI, Glassberg J. Biostatistical evaluation of evidence from continuous allele frequency distribution of deoxyribonucleic acid (DNA) probes in reference to disputed paternity and identity. J Forensic Sci 1989;34:1311–7.
5. Mulhare P, McQuillen E, Collins C, Heintz N, Howard P. An unusual case using DNA polymorphisms to determine parentage of human remains. Am J Forensic Med Pathol 1991;12:157–60.
6. Corach D, Sala A, Penacino G, Sotelo A. Mass disasters: Rapid molecular screening of human remains by means of short tandem repeats typing. Electrophoresis 1995;16:1617–23.
7. Whitaker JP, Clayton TM, Urquhart AJ, Millican ES, Downes TJ, Kimpton CP, et al. Short tandem repeat typing of bodies from a mass disaster: high success rate and characteristic amplification patterns in highly degraded samples. BioTechniques 1995;18: 670–7.
8. Sajantila A, Ström M, Budowle B, Karhunen PJ, Peltonen L. Identification of fire victims by using DNA amplification (PCR). In: Rittner C, Schneider PM, editors. Advances in forensic haemogenetics. Proceedings of the 14th Congress of the International Society for Forensic Haemogenetics, 18–21 Sept. 1991, Oslo. Norway: Springer-Verlag, 1992;102–5.
9. Clayton TM, Whitaker JP, Maguire CN. Identification of bodies from the scene of a mass disaster using DNA amplification of short tandem repeat (STR) loci. Forensic Sci Int 1995;76:7–15.
10. Mannucci A, Casarino L, Bruni G, Lomi A, De Stefano F. Individual identification of flood victims by DNA polymorphisms and autopsy findings. Int J Leg Med 1995;107:213–5.
11. O'Briain DS, Sheils O, McElwaine S, McCann SR, Lawler M. Sorting out mix-ups: the provenance of tissue sections may be confirmed by PCR using microsatellite markers. Am J Clin Pathol 1996;106:758–64.
12. Hagelberg E, Gray IC, Jeffreys AJ. Identification of the skeletal

remains of a murder victim by DNA analysis. Nature 1991;352: 427–9.
13. Kurosaki K, Matsushita T, Ueda S. Individual DNA identification from ancient human remains. Am J Hum Genet 1993;53:638–43.
14. Sajantila A, Ström M, Budowle B, Karhunen PJ, Peltonen L. The polymerase chain reaction and post-mortem forensic identity testing: application of amplified D1S80 and HLA-DQ loci to the identification of fire victims. Forensic Sci Int 1991;51:23–34.
15. Weber JL, May PE. Abundant class of human polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 1989;44:388–96.
16. Lygo JE, Johnson PE, Holdaway DJ, Woodroffe S, Whitaker JP, Clayton TM, et al. The validation of short tandem repeat (STR) loci for use in forensic casework. Int J Leg Med 1994;107:77–89.
17. Frégeau CJ, Fourney RM. DNA typing with fluorescently tagged short tandem repeats: a sensitive and accurate approach to human identification. BioTechniques 1993;15:100–19.
18. Andersen JF, Greenhalgh MJ, Butler HR, Kilpatrick SR, Piercy RC, Way KA, et al. Further validation of a multiplex STR system for use in routine forensic identity testing. Forensic Sci Int 1996; 78:47–64.
19. Sparkes R, Kimpton C, Gilbard S, Carne P, Andersen J, Oldroyd N, et al. The validation of a 7-locus multiplex STR test for use in forensic casework. II. Artefacts, casework studies and success rates. Int J Leg Med 1996;109:195–204.
20. De Stefano F, Bruni G, Casarino L, Costa MG, Mannucci A. Multiplexed DNA markers from cigarette butts in a forensic casework. In: Carracedo A, Brinkmann B, Bär W, editors. Advances in forensic haemogenetics. Proceedings of the 16th Congress of the International Society for Forensic Haemogenetics, 12–16 Sept. 1995, Santiago de Compostela. Spain: Springer-Verlag, 1996;252–4.
21. Mevåg B, Jacobsen S, Olaisen B. Three intriguing identification cases. In: Carracedo A, Brinkmann B, Bär W, editors. Advances in forensic haemogenetics. Proceedings of the 16th Congress of the International Society for Forensic Haemogenetics, 12–16 Sept. 1995, Santiago de Compostela. Spain: Springer-Verlag, 1996; 310–2.
22. Thomson J, Phillips C, Beckett D, Summerfield O, Lincoln P. Analysis of STR loci in old blood stains using automated and manual genotyping systems. In: Carracedo A, Brinkmann B, Bär W, editors. Advances in forensic haemogenetics. Proceedings of the 16th Congress of the International Society for Forensic Haemogenetics, 12–16 Sept. 1995, Santiago de Compostela. Spain: Springer-Verlag, 1996;328–30.
23. Robertson JM, Sgueglia JB, Badger CA, Juston AC, Ballantyne J. Forensic applications of a rapid, sensitive, and precise multiplex analysis of the four short tandem repeat loci HUMVWF31/A, HUMTHO1, HUMF13A1, and HUMFES/FPS. Electrophoresis 1995;16:1568–76.
24. Frégeau CJ, Bowen KL, Elliott JC, Robertson JM, Fourney RM. PCR-based DNA identification: a transition in forensic science. In: Proceedings of the 4th International Symposium on Human Identification, Sept. 1993, Scottsdale, AZ. Madison, WI: Promega Corporation, 1993;107–18.
25. Leclair B, Frégeau CJ, Aye MT, Fourney RM. DNA typing for bone marrow engraftment follow-up after allogeneic transplant: a comparative study of current technologies. Bone Marrow Transplant 1995;16:43–55.
26. Sharma V, Litt M. Tetranucleotide repeat polymorphism at the D21S11 locus. Hum Molec Genet 1992;1:67.
27. Mills KA, Even D, Murray JC. Tetranucleotide repeat polymorphism at the human alpha fibrinogen locus (FGA). Hum Molec Genet 1992;1:779.
28. Nakahori Y, Takenaka O, Nakagome Y. A human X-Y homologous region encodes ''Amelogenin''. Genomics 1991;9:264–9.
29. Sullivan KM, Mannucci A, Kimpton CP, Gill P. A rapid and quantitative DNA sex test: fluorescence-based PCR analysis of X-Y homologous gene amelogenin. BioTechniques 1993;15:636–41.
30. Li H, Schmidt L, Wei M-H, Hustad T, Lerman MI, Zbar B, et al. Three tetranucleotide polymorphisms for loci: D3S1352, D3S1358, D3S1359. Hum Molec Genet 1993;2:1327.
31. Adams DE, Presley LA, Baumstark AL, Hensley KW, Hill AL, Anoe KS, et al. Deoxyribonucleic acid (DNA) analysis by restriction fragment length polymorphisms of blood and other body fluid stains subjected to contamination and environmental insults. J Forensic Sci 1991;36:1284–98.
32. McNally L, Shaler RC, Baird M, Balazs I, De Forest P, Kobilinsky L. Evaluation of deoxyribonucleic acid (DNA) isolated from human bloodstains exposed to ultraviolet light, heat, humidity, and soil contamination. J Forensic Sci 1989;34:1059–69.
33. McNally L, Shaler RC, Baird M, Balazs I, Kobilinsky L, De Forest P. The effects of environment and substrata on deoxyribonucleic acid (DNA): the use of casework samples from New York City. J Forensic Sci 1989;34:1070–7.
34. Laber TL, Giese SA, Iverson JT, Liberty JA. Validation studies on the forensic analysis of restriction fragment length polymorphism (RFLP) on LE agarose gels without ethidium bromide: effects of contaminants, sunlight, and the electrophoresis of varying quantities of deoxyribonucleic acid (DNA). J Forensic Sci 1994;39: 707–30.
35. Cosso S, Reynolds R. Validation of the AmpliFLP™ D1S80 PCR amplification kit for forensic casework analysis according to TWGDAM guidelines. J Forensic Sci 1995;40:424–34.
36. Lienert K, Fowler JCS. Analysis of mixed human/microbial DNA samples: a validation study of two PCR AmpFLP typing methods. BioTechniques 1992;13:276–80.
37. Fernández-Rodríguez A, Alonso A, Albarrán C, Martín P, Iturralde MJ, Montesino M, et al. Microbial DNA challenge studies of PCR-based systems used in forensic genetics. In: Carracedo A, Brinkmann B, Bär W, editors. Advances in forensic haemogenetics. Proceedings of the 16th Congress of the International Society for Forensic Haemogenetics, 12–16 Sept. 1995, Santiago de Compostela. Spain: Springer-Verlag, 1996;177–9.
38. Van Oorschot RAH, Gutowski SJ, Robinson SL. HumTHO1: amplification, species specificity, population genetics and forensic applications. Int J Leg Med 1994;107:121–6.
39. Van Oorschot RAH, Gutowski SJ, Robinson SL, Hedley JA, Andrew IR. HumTHO1 validation studies: effect of substrate, environment, and mixtures. J Forensic Sci 1996;41:142–5.
40. Sparkes R, Kimpton C, Watson S, Oldroyd N, Clayton T, Barnett L, et al. The validation of a 7-locus multiplex STR test for use in forensic casework. I. Mixtures, ageing, degradation and species studies. Int J Leg Med 1996;109:186–94.
41. Frégeau CJ, Aubin RA, Elliott JC, Gill SS, Fourney RM. Characterization of human lymphoid cell lines GM9947 and GM9948 as intra- and interlaboratory reference standards for DNA typing. Genomics 1995;28:184–97.
42. Waye JS, Presley LA, Budowle B, Shutler GG, Fourney RM. A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts. BioTechniques 1989;7:852–5.
43. Möller A, Meyer E, Brinkmann B. Different types of structural variation in STRs:HumFES/FPS, HumVWA and HumD21S11. Int J Leg Med 1994;106:319–23.
44. Urquhart A, Kimpton CP, Downes TJ, Gill P. Variation in short tandem repeat sequences—a survey of twelve microsatellite loci for use as forensic identification markers. Int J Leg Med 1994;107: 13–20.
45. Barber MD, McKeown BJ, Parkin BH. Structural variation in the alleles of a short tandem repeat system at the human alpha fibrinogen locus. Int J Leg Med 1996;108:180–5.
46. Lazaruk K, Walsh PS, Oaks F, Gilbert D, Rosenblum BB, Menchen S, et al. Genotyping of forensic short tandem repeat (STR) systems based on sizing precision in a capillary electrophoresis instrument. Electrophoresis 1998;19:86–93.
47. Kimpton C, Fisher D, Watson S, Adams M, Urquhart A, Lygo J, et al. Evaluation of an automated DNA profiling system employing multiplex amplification of four tetrameric STR loci. Int J Leg Med 1994;106:302–11.
48. Kimpton CP, Oldroyd NJ, Watson SK, Frazier RRE, Johnson PE, Millican ES, et al. Validation of highly discriminating multiplex short tandem repeat amplification systems for individual identification. Electrophoresis 1996;17:1283–93.
49. Urquhart A, Chiu CT, Clayton T, Downes T, Frazier R, Jones S, et al. Multiplex STR systems with fluorescent detection as human identification markers. In: Proceedings of the 5th International Symposium on Human Identification, 9–11 Oct. 1994, Scottsdale, AZ. Madison, WI: Promega Corporation, 1995;73–83.
50. Prinz M, Schmitt C. Effect of degradation on PCR based DNA typing. In: Bär W, Fiori A, Rossi U, editors. Advances in forensic haemogenetics. Proceedings of the 15th Congress of the International Society for Forensic Haemogenetics, 13–15 Oct. 1993, Venezia. Italy: Springer-Verlag, 1994;375–8.

51. Alvarez García A, Muñoz I, Pestoni C, Lareu MV, Rodriguez-Calvo MS, Carracedo A. Effect of environmental factors on PCR-DNA analysis from dental pulp. Int J Leg Med 1996;109:125–9.
52. Keyser C, Montagnon D, Ludes B, Crubezy E, Cardon D, Walton Rogers P, et al. Influence of medieval clothes colour pigments on DNA extraction and amplification. In: Carracedo A, Brinkmann B, Bär W, editors. Advances in forensic haemogenetics. Proceedings of the 16th Congress of the International Society for Forensic Haemogenetics, 12–16 Sept. 1995, Santiago de Compostela. Spain: Springer-Verlag, 1996;292–4.
53. Comey CT, Budowle B. Validation studies on the analysis of the HLA DQα locus using the polymerase chain reaction. J Forensic Sci 1991;36:1633–48.
54. Budowle B, Lindsey JA, DeCou JA, Koons BW, Giusti AM, Comey CT. Validation and population studies of the loci LDLR, GYPA, HBGG, D7S8, and Gc (PM loci), and HLA-DQα using a multiplex amplification and typing procedure. J Forensic Sci 1995; 40:45–54.
55. Lotterle J, Hantschel M. DNA profiling on carpeting—methods of purification, restriction and detection. In: Bar W, Fiori A, Rossi U, editors. Advances in forensic haemogenetics. Proceedings of the 15th Congress of the International Society for Forensic Haemogenetics, 13–15 Oct. 1993, Venezia. Italy: Springer-Verlag, 1994; 164–6.
56. Scheithauer R, Weisser HJ. DNA profiling of bloodstains on linen pretreated with remedies used for cleaning and maintaining clothes. Int J Leg Med 1991;104:273–5.
57. Andrews C, Coquoz R. PCR DNA typing of washed stains. In: Bär W, Fiori A, Rossi U, editors. Advances in forensic haemogenetics. Proceedings of the 15th Congress of the International Society for Forensic Haemogenetics, 13–15 Oct. 1993, Venezia. Italy: Springer-Verlag, 1994;343–5.
58. Crouse CA, Schumm J. Investigation of species specificity using nine PCR-based human STR systems. J Forensic Sci 1995;40: 952–6.
59. Meyer E, Wiegand P, Rand SP, Kuhlmann D, Brack M, Brinkmann B. Microsatellite polymorphisms reveal phylogenetic relationships in primates. J Mol Evol 1995;41:10–4.
60. Meyer E, Rand S, Wiegand P. STR polymorphisms in non-human primates. In: Bär W, Fiori A, Rossi U, editors. Advances in forensic haemogenetics. Proceedings of the 15th Congress of the International Society for Forensic Haemogenetics, 13–15 Oct. 1993, Venezia. Italy: Springer-Verlag, 1994;418–20.
61. Frégeau CJ, Tan-Siew WF, Yap KH, Carmody GR, Chow ST, Fourney RM. Population genetic characteristics of the short tandem repeat HumD21S11 and HumFGA loci in eight diverse human populations. Hum Biol 1998;70:813–44.
62. Rubinsztein DC, Amos W, Leggo J, Goodburn S, Jain S, Li S-H, et al. Microsatellite evolution—evidence for directionality and variation in rate between species. Nature Genet 1995;10: 337–43.
63. Rubinsztein DC, Leggo J, Amos W. Microsatellites evolve more rapidly in humans than in chimpanzees. Genomics 1995;30: 610–2.
64. Deka R, Shriver MD, Yu LM, Jin L, Aston CE, Chakraborty R, et al. Conservation of human chromosome 13 polymorphic microsatellite $(CA)_n$ repeats in chimpanzees. Genomics 1994;22: 226–30.
65. Crouau-Roy B, Service S, Slatkin M, Freimer N. A fine-scale comparison of the human and chimpanzee genomes: linkage, linkage disequilibrium and sequence analysis. Hum Molec Genet 1996;5: 1131–7.
66. Buel E, Wang G, Schwartz M. PCR amplification of animal DNA with human X-Y amelogenin primers used in gender determination. J Forensic Sci 1995;40:641–4.
67. Gibson CW, Golub EE, Abrams WR, Shen G, Ding W, Rosenbloom J. Bovine amelogenin message heterogeneity: alternative splicing and Y-chromosomal gene transcription. Biochemistry 1992;31:8384–8.
68. Lau EC, Mohandas TK, Shapiro LJ, Slavkin HC, Snead ML. Human and mouse amelogenin gene loci are on the sex chromosomes. Genomics 1989;4:162–8.
69. Bailey DMD, Affara NA, Ferguson-Smith MA. The X-Y homologous gene amelogenin maps to the short arms of both the X and Y chromosomes and is highly conserved in primates. Genomics 1992;14:203–5.

Additional information and reprint requests:
Ron M. Fourney, Ph.D.
Royal Canadian Mounted Police
Central Forensic Laboratory
DNA Methods and Data Base
1200 Vanier Parkway
Ottawa, Ontario
Canada K1G 3M8