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Characterization and Validation Studies of PowerPlex™ 2.1, a Nine-Locus Short Tandem Repeat (STR) Multiplex System and Penta D Monoplex*

ABSTRACT: In order to increase the power of discrimination for human identification purposes, a nine-locus short tandem repeat (STR) multiplex, the GenePrint® PowerPlex™ 2.1 system (PowerPlex™ 2.1) developed by Promega Corporation and a separate pentanucleotide-repeat locus, Penta D, were tested. This megaplex system includes the highly polymorphic loci FGA, TPOX, D8S1179, vWA, Penta E, D18S51, D21S11, TH01, and D3S1358 and may be used in combination with the eight-locus STR multiplex, the GenePrint® PowerPlex™ 1.1 system (PowerPlex™ 1.1) that has been previously developed. Three of the loci, TPOX, TH01 and vWA, have been included in both systems for quality control purposes. As with PowerPlex™ 1.1, PowerPlex™ 2.1 is also based on a two-color detection of fluorescent-labeled DNA products amplified by polymerase chain reaction (PCR) and provides a valuable tool for accurate and rapid allele determination. The primer sequences used in the PowerPlex™ 2.1/Penta D system are also presented in this report. To meet the "Quality Assurance Standards for Forensic DNA Testing Laboratories" (FBI), we tested the efficiency and reproducibility of the PowerPlex™ 2.1/Penta D system by several validation studies that were conducted as a joint project among seven laboratories. Validation tests included concordance studies, sensitivity, and species specificity determination, as well as performance in forensic and environmentally impacted samples. The results produced from these tests demonstrated the consistency and reliability of the PowerPlex™ 2.1/Penta D system.

KEYWORDS: forensic science, short tandem repeats, PowerPlex™ 1.1, PowerPlex™ 2.1, multiplex, polymerase chain reaction, allele microvariants, DNA typing, forensics, primer sequences, FGA, TPOX, D8S1179, vWA, Penta E, D18S51, D21S11, TH01, D3S1358, Penta D, CSF1PO, D16S539, D7S820, D13S317, D5S818

Short tandem repeat (STR) loci are small segments of repetitive DNA sequences three to seven base pairs in length which display highly polymorphic regions of the human genome (1,2). The small size of these loci facilitates DNA amplification by the polymerase chain reaction (PCR) (3,4). Development of simultaneous amplification of several such STR loci, known as multiplex PCR (5,6) allows for rapid human identification based on DNA polymorphisms. Detection and analysis of multiplexed PCR products may be conducted on platforms such as capillary (7,8) and flat bed gel electrophoresis (9) with concordant results. Since very small amounts of DNA are required even in a highly degraded form (10,11), this procedure has found many applications in forensic sciences, paternity testing, and other related fields where human identification is necessary (12–14).

Lins and colleagues (15) have previously reported the development of the highly discriminating eight-locus PowerPlex™ 1.1 STR multiplex system. In this study we describe an additional nine-locus STR multiplex, PowerPlex™ 2.1, and the pentanucleotide-repeat locus Penta D. The STR loci of these systems can be currently analyzed with three amplification reactions (one for each of the PowerPlex™ 1.1, PowerPlex™ 2.1, and Penta D monoplex, respectively) and quality control is monitored by confirmation of three locus profiles, TPOX, TH01, and vWA, that both multiplex systems include.

The PowerPlex™ 2.1 multiplex that is studied here utilizes the same fluorescent detection system as described for PowerPlex™ 1.1 (15) and includes eight tetranucleotide-repeat loci, FGA, TPOX, D8S1179, vWA, D18S51, D21S11, TH01, D3S1358, and one pentanucleotide-repeat locus, Penta E. When the PowerPlex™ 1.1 system is used, appearance of allele microvariants is rare with

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the exception of the TH01 9.3 allele. With the PowerPlex™ 2.1 system, however, microvariants are frequently observed especially at the FGA, D21S11, and D18S51 loci (16). Such loci frequently display microvariant alleles differing from the typical alleles by one to three base pairs (17,18). Optimal detection and resolution of microvariants is critical for interpretation of repeat sequences that are not part of the nominal motif. The pentanucleotide-repeat loci Penta E and Penta D are effective forensic genetic markers due to their high degree of polymorphism, low incidence of microvariants, and extremely low stutter. As a result, these loci are highly discriminating for both single source samples and complex mixtures thus aiding in allele interpretation of DNA profiles.

In order to meet the "Quality Assurance Standards for Forensic DNA Testing Laboratories" (19), a collaborative effort was initiated among seven laboratories to conduct validation studies the results of which are reported here. Furthermore, since the design and validation of individual STR primer sets is critical for successful use on casework evidence, primer sequence data for the PowerPlex™ 2.1/Penta D system are also presented.

Materials and Methods

The methods used by the reporting laboratories are basically as recommended in the PowerPlex™ 2.1 System Technical Manual (20) and are briefly described below. All the procedures were tested against the NIST Standard Reference Material (SRM 2391a).

DNA Sources

Single source samples included blood, saliva, urine, semen, and vaginal fluids/swabs. For tissue studies, incision scars, ear wax, fingernail, head and pubic hair, teeth and perspiration were also included. Other sources used were non-probative samples and samples from previously used proficiency tests. Samples that were obtained in dry form were kept temporarily at room temperature until analysis. Liquid samples were stored at 4°C until stains could be made. For long term storage, samples were kept at either −20°C or −40°C (as stains) or −80°C (extracted DNA).

Mixture studies included: a) preparations of a series of DNA:DNA ratios from already quantified samples by the methods described below, and b) mixtures of body fluids in known volumes prior to DNA extraction and quantification.

DNA Extraction and Concentration Determination

DNA was extracted using an organic method (21) followed by DNA purification and concentration through Microcon YM-100 filters (Amicon, Beverly, MA). Alternatively, the FTA Gene Guard System was used (Life Technologies, Gaithersburg, MD). For semen-containing samples, male fractions were separated by a differential method (22) followed by organic extraction and concentration with Microcon YM-100 filters.

DNA concentrations were determined using the Quantiblot kit (Perkin Elmer-Applied Biosystems, Foster City, CA), spectrophotometric assay, or agarose yield gels.

DNA Amplification

One ng of DNA template was used for amplification in a 25 μL reaction volume (or alternatively, 0.5 ng in 12.5 μL) unless otherwise mentioned, using the reagents provided in the GenePrint® PowerPlex™ 2.1 System kit including the Gold ST★R 10X reaction buffer (Promega, Madison, WI) and Ampli*Taq* Gold™ DNA polymerase (Perkin Elmer-Applied Biosystems, Foster City, CA).

Amplification conditions were performed as recommended in the PowerPlex™ 2.1 System Technical Manual (Promega, Madison, WI) for the 9600, 9700, or 480 Thermal Cyclers (Perkin Elmer-Applied Biosystems, Foster City, CA). More specifically the amplification conditions for the 480 cycler were as follows: 95°C for 11 min, then: 96°C for 2 min, then: 94°C for 1 min, 60°C for 1 min, 70°C for 1.5 min, for 10 cycles, then: 90°C for 1 min, 60°C for 1 min, 70°C for 1.5 min, for 20 cycles, then: 60°C for 30 min followed by 4°C soak. The program for the 9600 and 9700 cyclers was as described above with the exception that the duration of the 96°C denaturation step as well as each of the cycling steps (denaturation-annealing-extension) was reduced in half. With FTA-extracted samples, the last three amplification cycles were eliminated. Ramp times were as listed in the PowerPlex™ 2.1 System Technical Manual (Promega, Madison, WI) for all labs except for NCSBI, where the protocol for the 480 Thermal Cycler was followed (ramp times were not used).

Gel Electrophoresis and Detection of Amplified Products

Prior to electrophoresis, the amplification samples were combined with loading buffer and the Internal Lane Standard 600 (ILS) provided in the PowerPlex™ 2.1 System kit (Promega, Madison, WI), or with loading buffer alone depending on whether a three- or a two-color detection, respectively, was desired. The ILS 600 contains 22 fragments of 60–600 bases labeled with carboxy-X-rhodamine (CXR) for detection at 650 nm. Allelic ladder samples were also provided in the kit, containing 5′-labeled fragments with either carboxy-tetramethylrhodamine (TMR) for detection of FGA, TPOX, D8S1179, and vWA loci at 585 nm, or with fluorescein (FL) for detection of Penta E, D18S51, D21S11, TH01 and D3S1358 loci at 505 nm. When the Penta D monoplex was used, it was accompanied by its own allelic ladder for FL detection. Occasionally Penta D was detected at 585 nm when labeling was with 6-carboxy-4′, 5′-dichloro-2′, 7′-dimethoxyfluorescein (JOE).

After addition of the loading buffer, the samples were denatured for 2 min at 95°C and the amplification products were separated through a 5% Long Ranger™ denaturing polyacrylamide gel (FMC BioProducts, Rockland, ME), or a 6% Page-Plus for PBSO (Amresco, Solon, OH), containing 6M urea and 1X TBE. Allelic ladders were present on every gel for allelic calls. Electrophoresis was performed at 60 watts for 1 h and 30 min or at 45–50 W for 1 h and 45 min, using the 43 cm long gels (BRL, Bethesda, MD). The optimized electrophoresis conditions for PBSO were at 60 watts for 1 h and 55 min using the 44.3 cm long gels (BRL, Bethesda, MD). The gels were pre-run for 10–20 min at the above conditions in order to achieve a surface temperature of approximately 50°C. Following electrophoresis, fluorescent images were detected with the FMBIO® II Fluorescent Scanner and analyzed by the FMBIO® Analysis software (1-D gel analysis) and StarCall™ software (MiraiBio Inc., Alameda, CA). As part of the concordance study (at Charlotte/Mecklenburg Police Department, NC) and for allele confirmation (at PSP and TXDPS), samples were also amplified using the ABI AmpFℓSTR™ Profiler Plus™/Cofiler™ kits and were analyzed by the ABI PRISM® 310 Genetic Analyzer using the Genotyper 2.0 software (Perkin Elmer-Applied Biosystems, Foster City, CA)

Stutter Cutoff Determination

Stutter cutoff values were determined by the VDFS laboratory as follows: 88 samples were analyzed on a total of 5 gels and were examined for the presence of stutter bands. The total number of alle-

les was as follows: FGA: 166; TPOX: 158; D8S1179: 148; vWA: 149; Penta E: 161; D18S51: 165; D21S11: 174; TH01: 146 and D3S1358: 150. For heterozygous samples, the analyst looked for alleles that were at least 8 bp apart so that the stutter of the higher molecular weight allele would not inflate the OD value of the lower molecular weight allele. The following numbers of stutter bands were found for each locus: FGA: 81; TPOX: 23; D8S1179: 72; vWA: 93; Penta E: 0; D18S51: 24; D21S11: 50; TH01: 23 and D3S1358: 30. An average optical density (OD) with background was calculated for the stutter bands and their preceding alleles observed in each locus. Then the percentage of the stutter OD average: allele OD average was calculated. The standard deviation (σ) of this percentage was determined and a three standard deviation value (3σ) was obtained. The stutter cutoff values are the percentages of stutter OD average: allele OD average at the 3σ value, with a 99% confidence interval.

Sensitivity Studies

The ranges of DNA quantities used for amplification in a standard volume of 25 μL reaction were as follows:

- Virginia Division of Forensic Science (VDFS): 0.0625 ng, 0.125 ng, 0.25 ng, 0.5 ng, 0.75 ng, 1 ng and 2 ng for five donors that donated blood and buccal samples each.
- Promega Corporation/Bode Technology Group (PC/BT): 0.1 ng, 0.2 ng, 0.5 ng, 1 ng, 2 ng, 5 ng, 10 ng and 25 ng for the K562, CCRF-SB, RAJI, KG-1 and IM9 human cell lines. Two other cell lines, GM 9947A and GM 9948, and one human genomic DNA sample were tested in the range of 0.1–2.5 ng.
- Pennsylvania State Police (PSP): 0.03125 ng, 0.0625 ng, 0.125 ng, 0.25 ng, 0.5 ng and 1 ng for control K562 DNA and for three individuals that donated blood/urine, blood/semen, and blood/saliva, respectively.
- North Carolina State Bureau of Investigation (NCSBI): 0.1 ng, 0.2 ng, 0.3 ng, 0.4 ng, 0.5 ng, 0.75 ng, 1 ng, 2.5 ng, 5 ng, and 10 ng for the K562 human cell line.
- Palm Beach County Sheriff's Office (PBSO): 0.08 ng, 0.15 ng, 0.3 ng, 0.6 ng, 1.25 ng, 2.5 ng, 5 ng, and 10 ng for the K562 cell line.

Species Specificity

To determine species specificity, various DNA quantities from the following animal and microbial species were amplified in a standard reaction volume of 25 μL as follows:

- NCSBI: 1 and 10 ng template DNA were used for amplification for cat, horse, partridge, broiler chicken, rabbit, dog, chicken, deer, bushbaby, African green monkey, Fascicularis monkey, mouse, gorilla, crested cockatoo, mallard duck, wild turkey, sheep, pig, cow, lemur, Siwatu Prosimian bushbaby, rhesus monkey, stumptail monkey, rat, Himalayan brown bear, *Bacillus (cereus, megaterium* and *subtilis), Micrococcus luteus, Staphylococcus (epidermidis, capitis, aureus* and *hominus), Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas fluorenscens, Serratia marcescens, Chlamydia trachomatis, Neisseria gonnoherra, Enterococcus faecalis, Salmonella choleraesuis, Candida albicans, B. marcescens, Aspergillus niger, Streptococcus somguis* and *beta-hemolytic Strep. (*Group G*).*
- PC/BT: 0.5 and 5 ng template DNA for rat, mouse, rabbit, chicken, dog, cow, monkey, orangutan, gorilla, chimpanzee, *Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* Hepatitis B virus, Human papilloma virus and *Candida albicans.*
- PBSO: 1 ng template DNA was used for amplification for bovine, dog, mouse, rabbit, rat, gorilla, chimpanzee, orangutan, monkey, cow, frog, shark, sea lion, damsel fish, pig and chicken.
- VDFS: 1 ng template DNA was used for amplification for armadillo, bear, bovine, cat, chimpanzee, crane, deer, dog, gibbon, gorilla, hawk, horse, orangutan, pig, rat and viper.
- PSP: 0.5 ng template DNA was used for amplification for rabbit, dog, cat, ferret and baboon.

Environmental Impact Study

Environmental impact on samples was tested by three different procedures:

- NCSBI: Various substances were used to soil individual white, clean cloth pieces of 3 × 4 in. in size which were subsequently stained with blood and allowed to dry before DNA extraction. DNA was extracted with the organic method described above. Liquid substances were left to dry before blood staining. The substances tested by this method were the following: silver, black and magnetic fingerprint powder, red clay, brown dirt, bleach, detergent, perspiration, motor oil, urine and gasoline. Two known donors provided blood samples.
- VDFS: Twenty-four small pieces of cotton were each soiled with the following substances: vigorol hairdressing, two different types of lubricant lotion, feminine deodorant spray, vaginex, static guard, hand cream, isopropyl alcohol, household cleaner, ammonia, feminine wash, baby oil, mold, heat, moisture, heat and moisture, super glue, soap, motor oil, luminol, blackpowder, ninhydrin, redwop fingerprint powder and bleach. Blood or seminal fluid was then applied on the soiled cotton. DNA was extracted by the organic method described above. Six known donors provided body fluids.
- PSP: Blood samples from a known donor were deposited on several substrates and were left to air-dry overnight prior to DNA extraction. The following substrates were used: 5 pieces of broken glass 8 × 1 mm each, 1 cm^2 of oily rag, 1 cm^2 of dirty tire, 2 cm^2 of green leaf, 1 cm^2 of leather shoe, 7 pieces of wood 8 × 1 mm each, 1 cm^2 of denim, 1 cm^2 of tennis shoe and 5 pieces of rusty metal 8 × 1 mm each.

Mixture Studies

For DNA mixture studies, DNA quantities from two sources were mixed at various ratios. For fluid mixtures, body fluids from two sources were mixed at different ratios and stains of the mixtures were subsequently made to be processed for DNA extraction. For both types of mixtures a total of 1 ng DNA was used in a 25 μL reaction volume for amplification. The ratios were as follows:

- VDFS: DNA ratios of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10 were tested for two DNA mixtures derived either from blood or buccal samples donated by two individuals. Also seventeen vaginal plus semen fluid mixtures were prepared (vaginal swabs were spotted with dilutions of seminal fluid) at approximate ratios of 1:1, 10:1 and 20:1 and compared with seventeen individual standards.

- PC/BT: DNA mixture ratios of 100:0, 99:1, 97.5:2.5, 95:5, 90:10, 80:20, 50:50, 20:80, 10:90, 5:95, 2.5:97.5, 1:99 and 0:100, were used in the following human cell line mixtures: K562+CCRF-SB, GM 9947A+RAJI, and IM9+KG-1.
- NCSBI: DNA ratios of 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18 and 1:19, were tested for a DNA mixture of two donors.
- PBSO: NIST samples #2 and #3 were used as a DNA mixture with the following DNA ratios: 100:0, 99:1, 97.5:2.5, 95:5, 90:10, 80:20, 50:50, 20:80, 10:90, 5:95, 2.5:97.5, 1:99 and 0:100. Also, one body fluid mixture of blood plus blood was tested with volume ratios of 100:0, 95:5, 90:10, 80:20, 50:50, 20:80, 10:90, 5:95, and 0:100.
- PSP: four individuals provided one specimen each for the preparation of a total of two fluid mixtures, vaginal plus semen and blood plus saliva, using fluid ratios of 1:20, 1:10, 1:5, 1:3, 1:2, 1:1, 2:1, 3:1, 5:1, 10:1 and 20:1.

Non-Probative and Proficiency Testing

PSP examined the Cellmark 9902, 9903, 9904, Collaborative 99-512 and CAP FID-B 1999 proficiency tests and 30 mixed source specimens (8 vaginal plus semen, 2 semen plus blood and 20 semen plus saliva) where previous PowerPlex™ 1.1 data were available or analysis had been performed with Perkin Elmer 310 or 377 Genetic Analyzers. NCSBI tested nine adjudicated cases where RFLP or CTT results had been previously obtained, and analyzed six proficiency tests that included CAP FID-A and B 1998, CAP-FID-A 1999 and CAP PI-A, B and C 1998, which had been tested before with PowerPlex™ 1.1. VDFS tested thirteen non-probative cases previously analyzed with PowerPlex™ 1.1. PBSO tested eight cases where CTT and/ or PowerPlex™ 1.1 data were previously available. PC/BT tested two cases provided by PBSO.

Concordance Studies

One hundred blood stain samples from convicted offenders provided by the NCSBI were analyzed by NCSBI, VDFS and PSP. Twenty-five of these samples were also analyzed by PC/BT and PBSO/Charlotte/Mecklenburg Police Department (NC) laboratories. All 25 samples tested in Charlotte/Mecklenburg laboratory were analyzed using the ABI amplification kits and instruments as described above in the *Gel electrophoresis and detection of amplified products.* Also, 80 separate concordant samples were processed between the PSP regional laboratories in Greensburg and Bethlehem, PA.

Family Studies

Families were studied by PBSO/TXDPS and VDFS as follows:

- PBSO/TXDPS: Blood or buccal swabs were obtained from members of three different families consisting of: thirty-five individuals (Family A/PBSO), twenty-two individuals (Family B/ TXDPS) and eighteen individuals (Family C/PBSO). All members of these families were tested with both ABI and Promega STR multiplex systems.
- VDFS: Buccal samples were collected from the members of four different families, consisting of five, seven, eight and seventeen members, respectively.

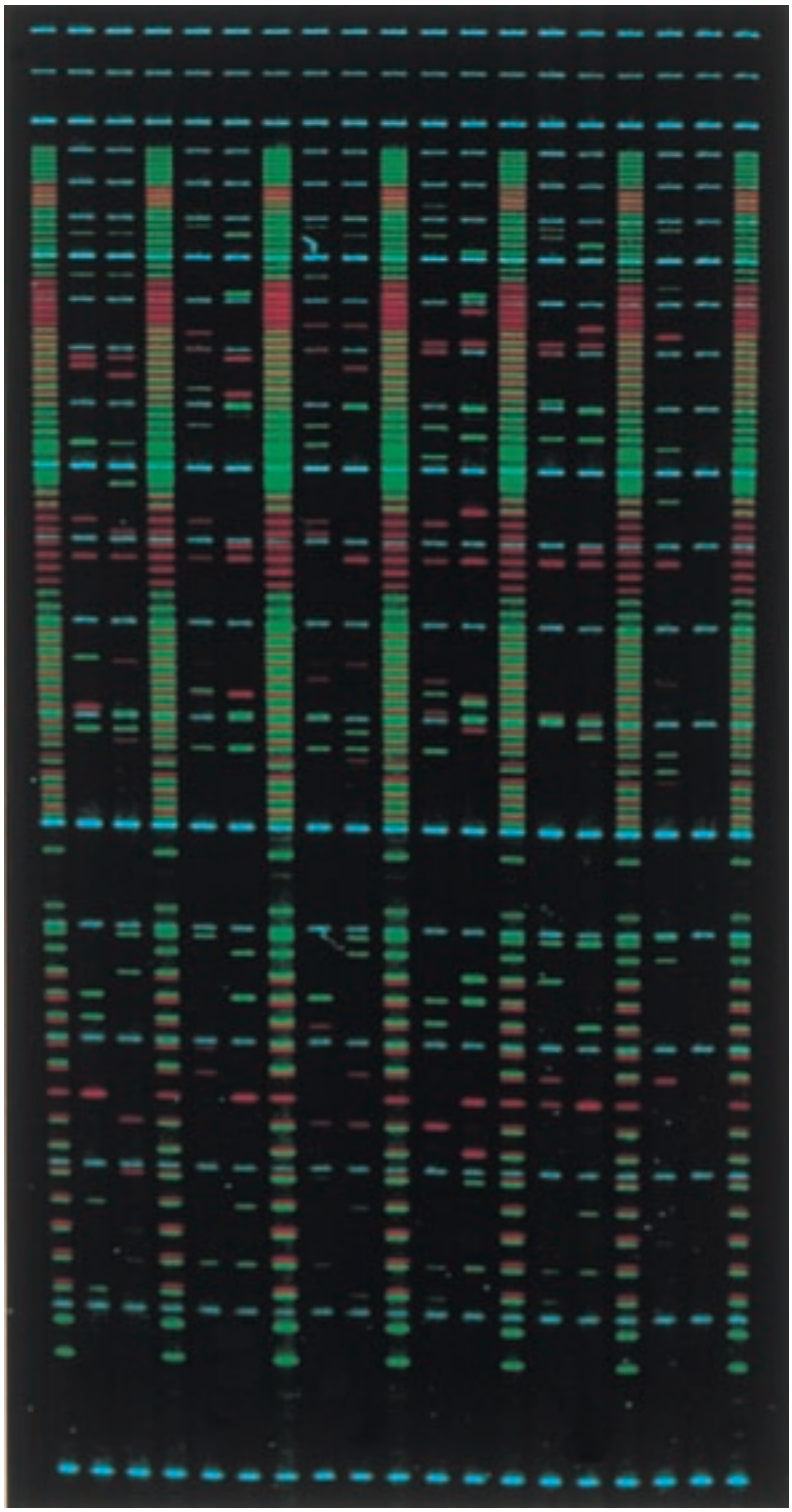
Results and Discussion

PowerPlex™ 2.1 and Penta D Monoplex Characterization

Using PCR technology, forensic DNA typing exploits the polymorphic nature of STR sequences in the human genome thus allowing for fast, reproducible results even when only small amounts or poor quality of DNA samples are available (10,11). The PowerPlex™ 2.1 and Penta D loci have been selected based on their high degree of polymorphism and efficient amplification with minimal artifacts. The multiplex loci used in these studies have been extensively investigated and comply with the "Quality Assurance Standards for Forensic DNA Testing Laboratories," effective October 1998 (19), as well as with international standards (23). Analysis of 13 core STR loci is required prior to inclusion in the National DNA Index System (NDIS) for searching the U.S. National Database of convicted offender profiles. Lins et al. (15) have previously reported the development of PowerPlex™ 1.1 which included eight of these loci, CSF1PO, TPOX, TH01, vWA, D16S539, D7S820, D13S317 and D5S818. In this report we describe the validation of PowerPlex™ 2.1 which includes the remaining five tetranucleotide-repeat loci that compose the 13 loci core, FGA, D8S1179, D18S51, D21S11, D3S1358, and an additional pentanucleotide-repeat locus, Penta E. Furthermore, we have tested another pentanucleotide-repeat locus, Penta D which can be used separately as a monoplex.

The PowerPlex™ 2.1 system permits amplification of nine loci in a single reaction that is analyzed through gel electrophoresis as shown in Fig. 1 for 10 NIST SRM2391a DNA samples. Detection is performed using the Hitachi FMBIO® II fluorescent scanner and is based on the differential labeling of the loci primers. The loci FGA, TPOX, D8S1179 and vWA are displayed in red, and Penta E, D18S51, D21S11, TH01 and D3S1358 are displayed in green (Fig. 1*A*). Identically labeled allelic ladders (shown flanking every set of two DNA samples in Fig. 1*A*) are loaded on every gel for sizing the most common alleles for each locus, both visually and by StarCall™ software analysis of the digitized images. The internal lane standard (ILS) is displayed in blue (Fig. 1*A*). The ILS allows precise allele position determination by migrating with each individual sample through various gel electrophoresis conditions (i.e., gel "smiling" effect, bubbles, etc). Color-separated images can be generated from this multicolor image as shown in black and white in Fig. 1*B,* where for every sample each locus is clearly distinguished. The image produced using the monoplex Penta D for 11 population database samples is illustrated in Fig. 2.

The characterization of each of the STR loci of the PowerPlex™ 2.1 and Penta D locus including chromosome location, locus definition, repeat sequence and sequence of primers used for amplification is displayed in Table 1. In addition, allelic ladder characteristics such as size range and number of repeats for each ladder component are shown. The primer sequences have been selected based on the need to manage *Taq* DNA polymerase associated artifacts. Common examples of such artifacts include terminal extra nucleotide addition (mostly adenine) of PCR-generated fragments (24,25) and loss of one repeat unit or repeat slippage (26,27) thus generating a band of lower intensity described as "stutter." The uniformity of the terminal nucleotide addition has been managed by primer design and addition of a 30 min final extension step of 60°C to the amplification program (20). Interference of stutter can be regulated by determination of stutter cutoff values as percentages of true allele values for each locus and incorporation of the obtained percentages in the StarCall™ software. Therefore, any value that falls below the stutter cutoff percentage for each locus (shown in Table 1 as determined by the VDFS and Promega laboratories) is automatically considered stutter. Penta D is presented as a locus with very low stutter (<1%, Table 1) followed by Penta E (1–2%), TPOX (1.8%), and TH01 (2.8%). D8S1179 and FGA follow (5.0% and 5.3%, respectively) with the rest of the loci exhibiting higher



A

FIG. 1—*Fluorescent imaging of amplified DNA products using PowerPlex™ 2.1*

One ng from each of 10 genomic DNA samples (SRM2391a samples #1–10, from left to right, respectively), a positive amplification control (CCRF-SB, lane 17) and a negative amplification control (lane 18), were amplified using the PowerPlex™ 2.1 multiplex and the amplified products were analyzed by electrophoresis through a 5% Long Ranger polyacrylamide gel. Detection was performed using the Hitachi FMBIO® II fluorescent scanner. Every two DNA samples are shown flanked by allelic ladders the component sizes of which are displayed in Table 1. For each DNA sample, all nine loci were derived from a single amplification reaction and are represented within one lane. A: Color image displaying: FGA, TPOX, D8S1179 and vWA loci segments labeled with TMR for detection at 585 nm (red); Penta E, D18S51, D21S11, TH01 and D3S1358 loci segments labeled with FL for detection at 505 nm (green); the internal lane standard labeled with CXR for detection at 650 nm (blue) with sizes of 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, and 600 bases. B: The color image of panel A was separated into two individual black and white images for TMR- (left panel) and FL-labeled (right) products.

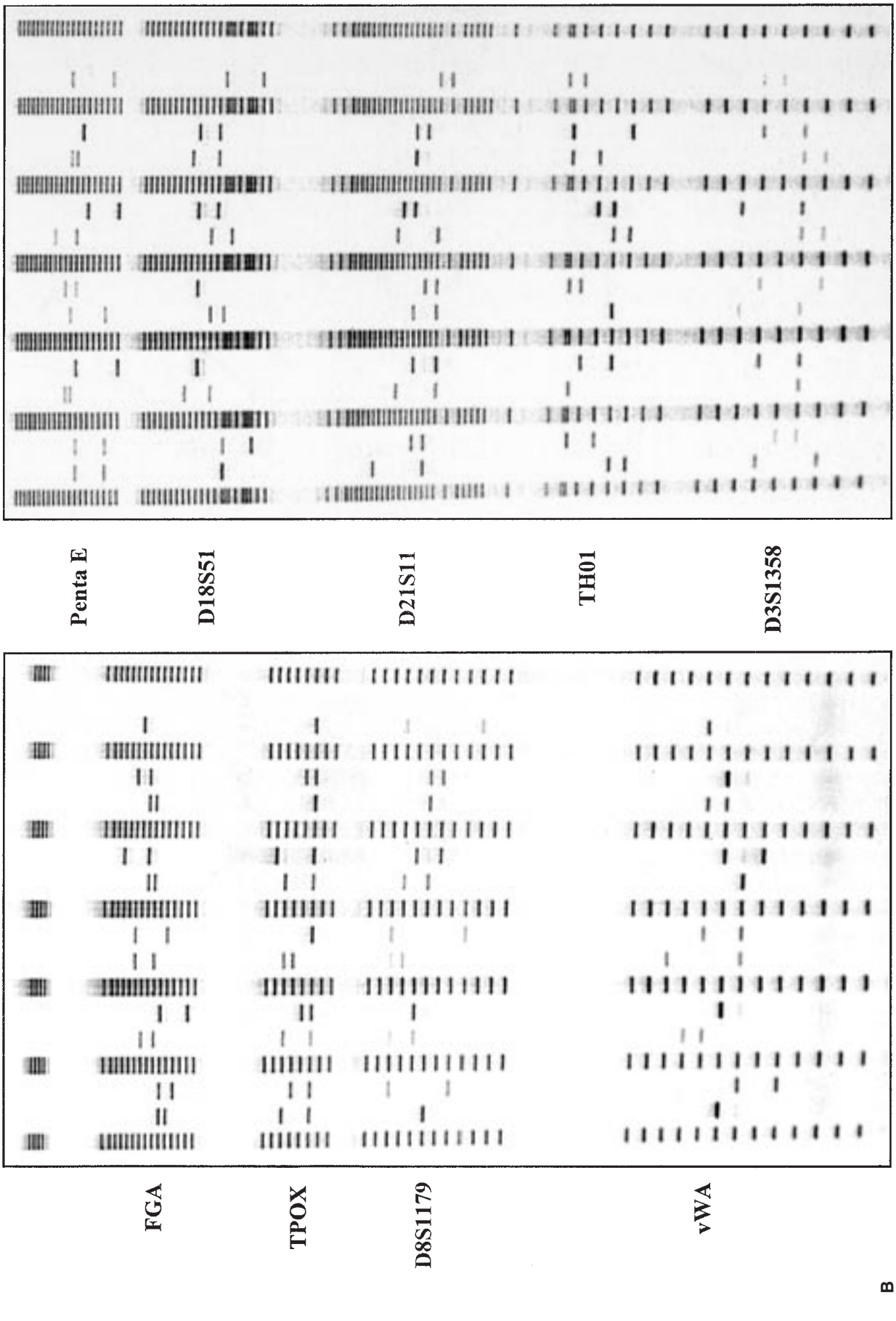


FIG. 1—*(continued)*

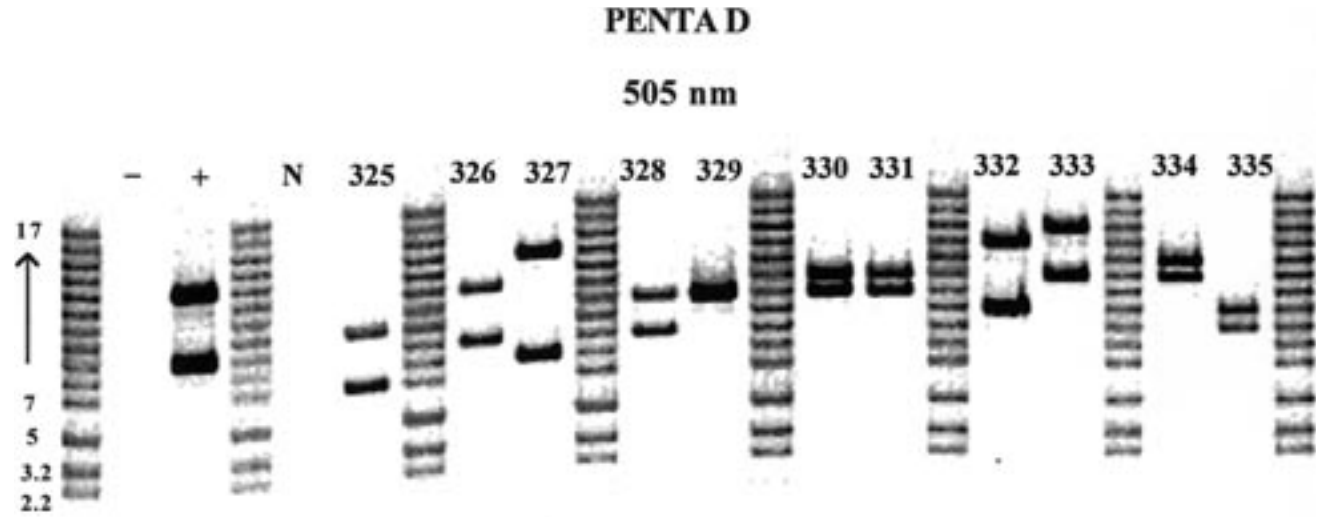


FIG. 2—*DNA profiling using the Penta D monoplex*

Genomic DNA from eleven population database samples (325–335) was amplified using the Penta D monoplex and the amplified products were analyzed by electrophoresis through a 5% Long Ranger polyacrylamide gel. Detection was performed using the Hitachi FMBIO® II fluorescent scanner at 505 nm for FL-labeled products. Repeat sizes of the Penta D allelic ladder are shown at the first lane from left (2.2, 3.2, 5, 7–17); (−): negative amplification control (amplification reagents without DNA), (+): positive amplification control (DNA from the K562 cell line) and N: negative DNA extraction control (extraction reagents without blood-stained sample).

TABLE 1. Definition and Characterization of Loci

* signifies a complex repeat sequence

Locus	Chromosome Location	GenBank® Locus or Locus Definition	Allelic ladder size range (bases)	Repeat number of allelic ladder components	Repeat Sequence (5'---3')	Primer sequences (5'---3')	Average % Stutter
Penta E	15q	N/A	379-474	5-24	AAAGA	Forward (OH): ATTACCAACATGAAAGGGTACCAATA Reverse (FL): TGGGTTATTAATTGAGAAAACTCCTTACAATTT	1-2.0
D18S51	18q21.3	HUMUT574	290-366	8-10,10.2, 11-13, 13.2, 14-27	AGAA	Forward (FL): TTCTTGAGCCCAGAAGGTTA Reverse (OH): ATTCTACCAGCAACAACACAAATAAAC	8.3
D21S11	21q11-21q21	HUMD21LOC	203-259	24, 24.2,25, 25.2, 26-28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36-38	TCTA*	Forward (OH): ATATGTGAGTCAATTCCCCAAG Reverse FL): TGTATTAGTCAATGTTCTCCAGAGAC	9.2
TH01	11p15.5	HUMTH01, Human Tyrosine Hydroxylase gene	156-195	4-9, 9.3, 10-11, 13.3	AATG	Forward (FL): GTGATTCCCATTGGCCTGTTC Reverse (OH): ATTCCTGTGGGCTGAAAAGCTC	2.8
D3S1358	3p	N/A	115-147	12-20	TCTA*	Forward (OH): ACTGCAGTCCAATCTGGGT Reverse (FL): ATGAAATCAACAGAGGCTTGC	9.7
FGA	4q28	HUMFIBRA, Human fibrinogen alpha chain gene	326-444	17-30, 31.2, 43.2, 44.2, 45.2, 46.2	TTTC*	Forward (TMR): GGCTGCAGGGCATAACATTA Reverse (OH): ATTCTATGACTTTGCGCTTCAGGA	5.3
TPOX	2p23-2pter	HUMTPOX, Human thyroid peroxidase gene	262-290	6-13	AATG	Forward (OH): GCACAGAACAGGCACTTAGG Reverse (TMR): CGCTCAAACGTGAGGTTG	1.8
D8S1179	8	N/A	203-247	7-18	TCTA*	Forward (OH): ATTGCAACTTATATGTATTTTTGTATTTCATG Reverse (TMR): ACCAAATTGTGTTCATGAGTATAGTTTC	5
vWA	12p12-pter	HUMVWFA31, Human von Willebrand factor gene	123-171	10-22	TCTA*	Forward (OH): GCCCTAGTGGATGATAAGAATAATCAGTATGTG Reverse (TMR): GGACAGATGATAAATACATAGGATGGATGG	7.9
Penta D	21q	N/A	376-441	2.2, 3.2, 5, 7-17	AAAGA	Forward (JOE): GAAGGTCGAAGCTGAAGTG Reverse (OH): ATTAGAATTCTTTAATCTGGACACAAG	<1.0

Penta E and Penta D values were provided by Promega.

values such as vWA (7.9%), D18S51 (8.3%), D21S11 (9.2%) and D3S1358 (9.7%). Similar data were obtained previously as reported in Promega Technical Manual (20). Appearance of stutter bands becomes an important issue when mixtures of samples are being analyzed that contain bands of variable intensities. In such cases, interpretation may rather be based on analyst's discretion and experience by visual examination of the profiles than on stutter values incorporated in the StarCall™ software. Along these terms, the importance of highly polymorphic loci with low stutter, such as Penta D and E, becomes apparent in resolving controversial situations.

Another feature characteristic for some loci of PowerPlex™ 2.1 such as FGA, D21S11 and D18S51, is the frequent presence of microvariant alleles or alleles with sizes greater than the typical allele size by one, two or three bases (17,18). To achieve accurate allele determination in such cases, the allelic ladders for these loci have been supplemented with bands of one half repeat size. The ILS was also utilized so that allele calls may be based on both sizing systems. Appropriate adjustments may also be necessary when using the StarCall™ software to allow for precise size evaluation of such microvariants. The user may vary the allele call window for each locus as he desires, so that when in a particular locus microvariants are frequently flagged "out of range" and the used window is, for example, 1 bp (−1.00 to +1.00), a tighter window may be chosen for that locus to allow for the appropriate allele call (such as a −0.50 to +0.49 bp range) which will reduce the "out of range" calls.

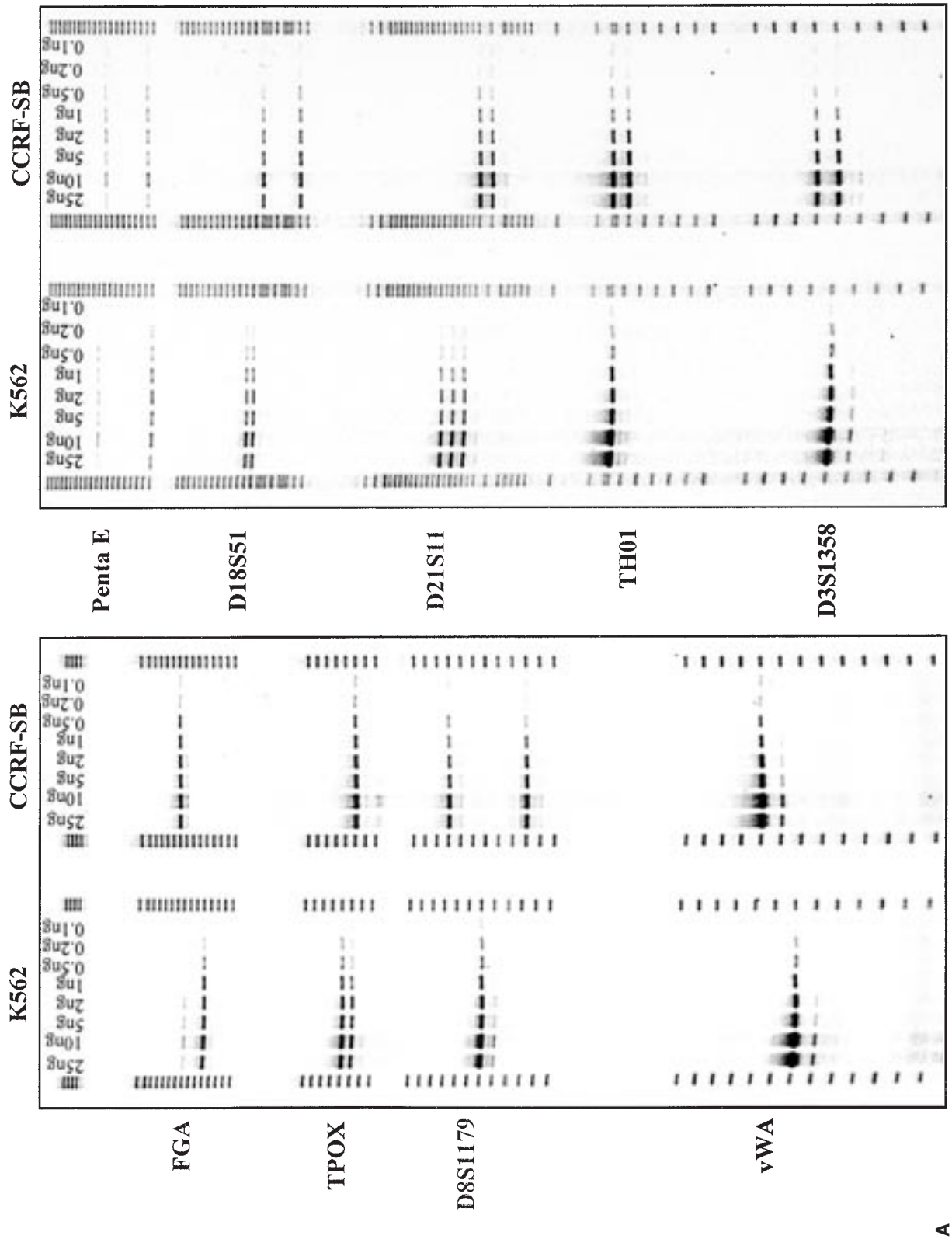


FIG. 3—*Sensitivity of PowerPlex™ 2.1/Penta D*
Various amounts of DNA templates were used for amplification, the amplified products were separated in a denaturing 5% Long Ranger polyacrylamide gel and analyzed by the Hitachi FMBIO® II fluorescent scanner. A: The DNA profiles are derived from K562 and CCRF-SB cell lines using for amplification 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 25.0 ng of genomic DNA from each cell line. B: K562 DNA was tested for Penta D in quantities of 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, 2.5, 5.0 and 10.0 ng. (−): negative amplification control.

Sensitivity

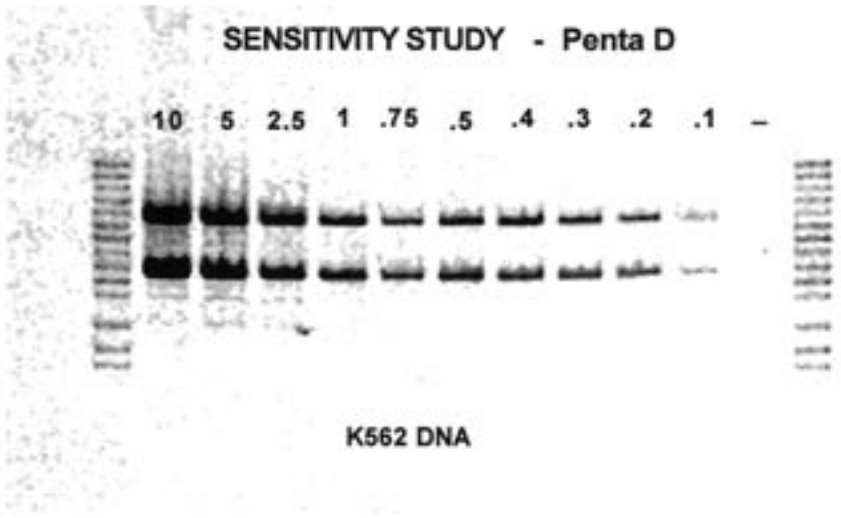
Sensitivity of the system was tested by amplification of various DNA quantities ranging from 0.03125 ng to 25 ng (per 25 μL reaction). All samples tested at PC showed complete profiles at DNA template quantities as low as 1 ng. Also, amplification of most loci was noted in almost all samples at PC even at quantities of 0.2 and 0.1 ng. For the reference sample K562, as shown in Fig. 3*A* (data provided by PC), at 0.5 ng of K562 DNA, one of the FGA alleles (upper band, allele 24) has dropped out. At 0.1 ng K562 DNA other loci have started to drop out (Penta E, D18S51, and D21S11). In contrast, the CCRF-SB cell line gave detectable results in all loci even at the lowest DNA quantity tested (0.1 ng). NCSBI and PBSO results were in agreement with the results obtained by PC for the K562 DNA (Table 2). PSP reported allelic dropouts mostly at Penta E, FGA and D18S51, at DNA quantities as low as 0.0625 ng, 0.125 ng and 0.5 ng depending on the sample examined. Tests performed at VDFS showed allelic dropout at all quantities below 0.5 ng. Amplification for Penta D locus was observed even at 0.1 ng of DNA template (Fig. 3*B,* NCSBI data). It is noteworthy that in those situations that some loci failed to amplify, there was still a substantial amount of information that could be obtained from the remaining loci. It also appears that sensitivity may be template dependent, as for example, in the K562 cell line where the apparent difference of intensity between the two FGA alleles resulted in a switch of the sensitivity threshold from 0.1 to 0.5 ng DNA. The allelic FGA imbalance in the reference sample K562 provides for a useful positive control of amplification of the weaker bands since it is possible that samples in question to exhibit a similar locus imbalance. A summary of the sensitivity results is illustrated in Table 2 which indicates that allelic dropouts may be observed below 0.5–0.6 ng of DNA template. Taking into account all the results derived from the participant laboratories an optimal range of 0.75 to 2 ng DNA was determined to be used for amplification. Nevertheless, this does not imply that complete profiles cannot be successfully produced at very low DNA amounts as it was occasionally noticed. In such situations, one must have confidence in the obtained results especially when full profiles are generated. This observation is particularly useful in forensic casework where frequently only limited evidence material is available and there is no option of using the optimal DNA amount. We have also noticed that when DNA quantities of 5 ng or greater are amplified, background level as well as stutter may increase and an imbalanced profile among loci may be observed as a result of better amplification of smaller loci as compared to the larger ones.

Consistency Among Tissues

To determine that the allelic profile remains the same from tissue to tissue obtained from a single individual, two studies were performed where samples from various tissues such as blood, saliva, vaginal fluid, semen, head hair, fingernail, ear wax, urine and incision scar (VDFS, five donors), or blood, saliva, vaginal fluid/semen, perspiration, teeth and head and pubic hair (PSP, four donors) were tested. All tissues obtained from the same donor gave identical profiles (data not shown). These results demonstrate that regardless of the tissue tested, each individual is characterized by a single allele profile for all the loci of PowerPlex™ 2.1.

Species Specificity

In order to determine if bacterial, fungal, or animal DNA could yield amplified products from human-derived STR primers, extracted DNA from a panel of microbial and animal species was tested. Penta D locus showed no amplification products in any microbial and animal species tested at the NCSBI. For all the microbial species studied at the NCSBI and PC/BT, amplification of PowerPlex™ 2.1 STR alleles was negative as well. Negative amplification results were also obtained for all the non-primate samples tested by all the reporting laboratories. For the higher primates a DNA banding pattern was observed with the majority of alleles migrating off ladder or between ladder bands. The data for the primates are summarized in Table 3 which indicates the loci where amplification is likely to be observed for each species. It should be noted, that use of monoplexes may identify the locus that each allele is assigned to. More specifically: For monkey, amplification was noted at Penta E, FGA and TPOX (NCSBI: Stumptail and Fascicularis monkey), or only at Penta E and FGA (PC/BT). For orangutan, bands were observed at Penta E, TH01 and FGA (VDFS); at Penta E, TH01, D3S1358 and FGA (PC/BT); and at every locus except TPOX, D8S1179 and vWA (PBSO). For gorilla, bands were seen at all loci (PBSO, PC/BT), except at TH01, TPOX



B

FIG. 3—*(continued)*

TABLE 2. Sensitivity Summary

DNA (ng)	VDFS	PC/BT	PSP	NCSBI	PBSO
0.03125			+		
0.0625	+		+		
0.08					+
0.1		+		+	
0.125	+		+		
0.15					+
0.2		+		+	
0.25	+		+		
0.3				+	+
0.4				+	
0.5	+	+	+	+	
0.6					+
0.75	++			++	
1.0	++	++	++	++	
1.25					++
2.0	++	++			
2.5				++	++
5.0		++		++	++
10		++		++	++
25		++			

(+): DNA quantity tested (allele dropouts were observed)
(++): Complete profiles recorded

TABLE 3. PowerPlex™ 2.1 loci amplification in primates

SPECIES	Penta E	D18S51	D21S11	TH01	D3S1358	FGA	TPOX	D8S1179	vWA
monkey	+	-	-	-	-	+	+	-	-
orangutan	+	+	+	+	+	+	-	-	-
gorilla	+	+	+	+	+	+	+	+	+
chimp	+	+	+	+	+	+	+	+	+
baboon	+	-	-	-	-	+	+	-	-
gibbon	-	-	-	+	-	+	+	-	-

(+): loci where amplification is likely to be noted
(-): loci where negative amplification results were obtained

TABLE 4. PowerPlex™ 2.1 allele dropout under environmental insults

SUBSTANCES	FGA	TPOX	D8S1179	vWA	Penta E	D18S51	D21S11	TH01	D3S1358
hand cream	_	_	_	+	_	_	_	+	_
mold	_	_	_	_	_	_	_	_	_
heat + moisture	_	_	_	_	_	_	_	_	_
moisture	+	+	+	+	_	+	+	+	+
super glue	+	_	+	+	_	+	+	+	+
motor oil	+	_	+	+	_	+	+	+	+
bleach	+	+	+	+	_	+	+	+	+
red clay	+	+	+	+	_	+	+	+	+
perspiration	+	+	+	+	_	+	+	+	+

(+): loci that amplified
(-): loci where amplification failed

and D8S1179 (NCSBI), or D21S11 and D8S1179 (VDFS). For chimpanzee amplification was also seen at all loci (PBSO), except at Penta E (PC/BT), or D3S1358 and D8S1179 (VDFS). For baboon, amplification was noticed at Penta E, FGA and TPOX (PSP), and for gibbon at TH01, FGA and TPOX (VDFS).

In conclusion, amplification products were not obtained in microbial and non-primate animal species. From the primates tested, the majority exhibited alleles migrating between ladder bands and off ladder and in several occasions more than two bands per locus were noticed. It should also be mentioned that no individual primate showed allele bands with the appropriate number of alleles or sequence lengths for all 10 loci, in a manner characteristic for human species.

Environmental Impact Study

These studies were designed to determine the efficiency and accuracy of the PowerPlex™ 2.1/Penta D system when biological samples have been exposed to different types of environmental insults. Therefore, a variety of such substances, both liquid and solid, were tested by contaminating samples of donors with known profiles using different experimental approaches. It was observed that regardless of the DNA source, the contaminant, or the method used (substrate directly stained with blood, or substance-soiled cloth and subsequently blood-stained), the loci that successfully amplified produced profiles that matched their respective standards. PSP reported amplification in all loci for all the substrates tested. For the contaminants tested by NCSBI and VDFS, amplification was noticed for all loci with the exceptions of allele dropouts for the contaminants shown in Table 4. For mold, or heat plus moisture amplification failed in all loci (VDFS). Also samples contaminated with hand cream amplified only in vWA and TH01 loci (VDFS). Penta E dropouts were also noticed when moisture, super glue, motor oil and bleach (data from VDFS), or red clay and perspiration were tested (NCSBI data). From the VDFS studies it was noticed

that TPOX was also lost when super glue and motor oil were tested. Amplification in the Penta D locus remained unaffected by all the substances tested by the NCSBI laboratory (data not shown). From these studies we concluded that DNA typing may be successfully produced using the PowerPlex™ 2.1 and Penta D loci even under circumstances that might be considered at first as inhibitory, such as the in presence of detergents, bleach, gasoline, alcohol, soap, ammonia or household cleaners. More importantly, other than the observation that a few loci failed to amplify in the presence of some of the substances, the obtained DNA profile remained unaltered. These results confirm previous studies on the effect of various environmental insults on DNA typing by either RFLP (28), or PCR (29).

Mixture Studies

Mixture studies were conducted to determine the efficiency of DNA amplification from two sources at different ratios. The importance of testing the sensitivity of the PowerPlex™ 2.1/Penta D system in mixtures lies in the fact that often a single forensic sample may contain DNA from two or more individuals at unpredictable ratios and the profiles need to be correctly identified. In these situations, information from as many loci as possible is desirable. DNA mixtures and body fluid mixtures were used at various ratios of the mixture components. For each mixture, a cutoff ratio was determined below which partial or no profile was detected from the source with lower DNA concentration.

DNA mixtures: The PC/BT laboratory reported a cutoff ratio of 20:80 for the mixtures of K562+CCRF-SB (Fig. 4*A*), GM 9947A+RAJI and IM9+KG-1 (data not shown). Nevertheless, as shown in Fig. 4*A* for the mixture of K562+CCRF-SB some loci of the sample with lower DNA concentration showed amplification in lower ratios tested, such as FGA, D8S1179, vWA and D21S11 for the K562 sample. The cutoff ratios reported for the DNA mixtures tested by VDFS were 20:80 and 30:70. NCSBI reported a cutoff ratio of 1:10 whereas several loci of the low DNA concentration sample showed amplification even at the lowest ratio tested (1:19), as for example Penta D (Fig. 4*B*). PBSO tested a DNA mixture that showed a cutoff ratio of 20:80.

Body fluid mixtures: VDFS typed correctly the profiles of the vaginal plus semen mixtures tested as compared with their respective standards, when the ratio of 1:1 was used. When greater dilutions of seminal fluid used at VDFS (10 or 20 times more dilute), partial or complete dropout of the sperm fraction profile was observed. PSP tested two mixtures of vaginal plus semen and blood plus saliva and whereas in the vaginal plus semen mixture all loci of the sperm fraction were detected at all ratios examined (1:20 to 20:1), in the blood plus saliva mixture the cutoff ratio was 1:2. Among all ratios tested at PBSO (100:0 to 0:100) for a blood plus blood mixture, complete profiles of both sources were reported at a ratio of 50:50.

In summary (Table 5), DNA mixtures with ratios between 1:10 and 30:70 gave *complete* profiles of both donors at all loci tested, whereas body fluid mixtures exhibited efficient ratios between 1:2 and 1:1. The difference in the ratios observed among the mixtures could partly be attributed to the fact that each mixture is unique in terms of biochemical composition and size of its allele components. Consequently, amplification rates may vary depending on the combination of the alleles that are present in each mixture. In addition, body fluid ratios generated by volume to volume mixing of different body fluids, may approximate the actual ratios of their DNA contributors. In forensic mixture samples, one doesn't know either the relative concentrations of the DNA donors, or the volume ratios of body fluids. For that reason, the mixture experiments served as a guide to determine the theoretical amplification expectations of the multiplex, since they only mimic actual forensic mixtures. As it was demonstrated from these studies, the multiplex successfully identified potential donors in mixtures similar to the ones seen in forensic samples.

Intralaboratory Consistency

To confirm the reliability of the PowerPlex™ 2.1 System within each individual laboratory, specific samples were re-analyzed. The results of the PowerPlex™ 2.1 system testing were compared to previous results obtained from testing by other analysts or other approaches, such as RFLP, CTT, PowerPlex™ 1.1, etc, or by using different instrumentation (Hitachi FMBIO system versus ABI technology). The samples examined were from non-probative cases, old proficiency tests, and body fluid mixtures as described in Materials and Methods section. In every laboratory, the allele typing based on PowerPlex™ 2.1 system was consistent regardless of the analyst or instrumentation and confirmed interpretations made on previously analyzed non-probative cases.

Interlaboratory Consistency

The consistency of the PowerPlex™ 2.1 multiplex was validated among three laboratories (NCSBI, VDFS, and PSP) through a concordance study of 100 convicted offender samples obtained from the NCSBI DNA database (data not shown). The PC/BT and PBSO/Charlotte/Mecklenburg laboratories analyzed the first 25 of these samples. All the samples were typed correctly and in agreement among the laboratories with the following exceptions: a 22.3 FGA allele which was typed as 22.2 or 23, a 21.2 D18S51 allele which was typed as 22 and one FGA allele that four laboratories reported it as a 22.2 and one laboratory reported it as a 22.3. After reanalysis of these samples, all laboratories reported the same results with the exception of the last FGA allele that remained a non-resolved discrepancy (22.2 or 22.3). It should be noted that all three samples involved microvariant calls and the discrepancies were no more than one to two base pairs apart. Such allele calls that approach the resolution and detection limits of the system may be subjective and reflect slight experimental variations among laboratories. Such factors include length of electrophoresis, gel migration artifacts, sample quantity loaded on the gel, use or not of ILS, and sizing window settings in the StarCall™ software to allow for the microvariant definition. Minor discrepancies of this kind should not affect CODIS searches since CODIS matching algorithms allow for a high stringency match when there is a 1 bp difference between two alleles. Also, CODIS searching parameters allow for moderate stringency searches that can return for example, a 12 locus match when two 13 loci samples differ in one locus. Such a match will draw the analyst's attention for further examination and analysis of these samples.

To test the compatibility of the PowerPlex™ 2.1 system with the ABI technology, STR profiles of the 25 samples analyzed at the Charlotte/Mecklenburg laboratory, were obtained using the ABI amplification and analysis systems. The results were in concordance with the PowerPlex™ 2.1 data returned from the other laboratories.

Family Studies

PBSO and TXDPS analyzed three families consisting of several generations to determine if there were parent/child STR allele

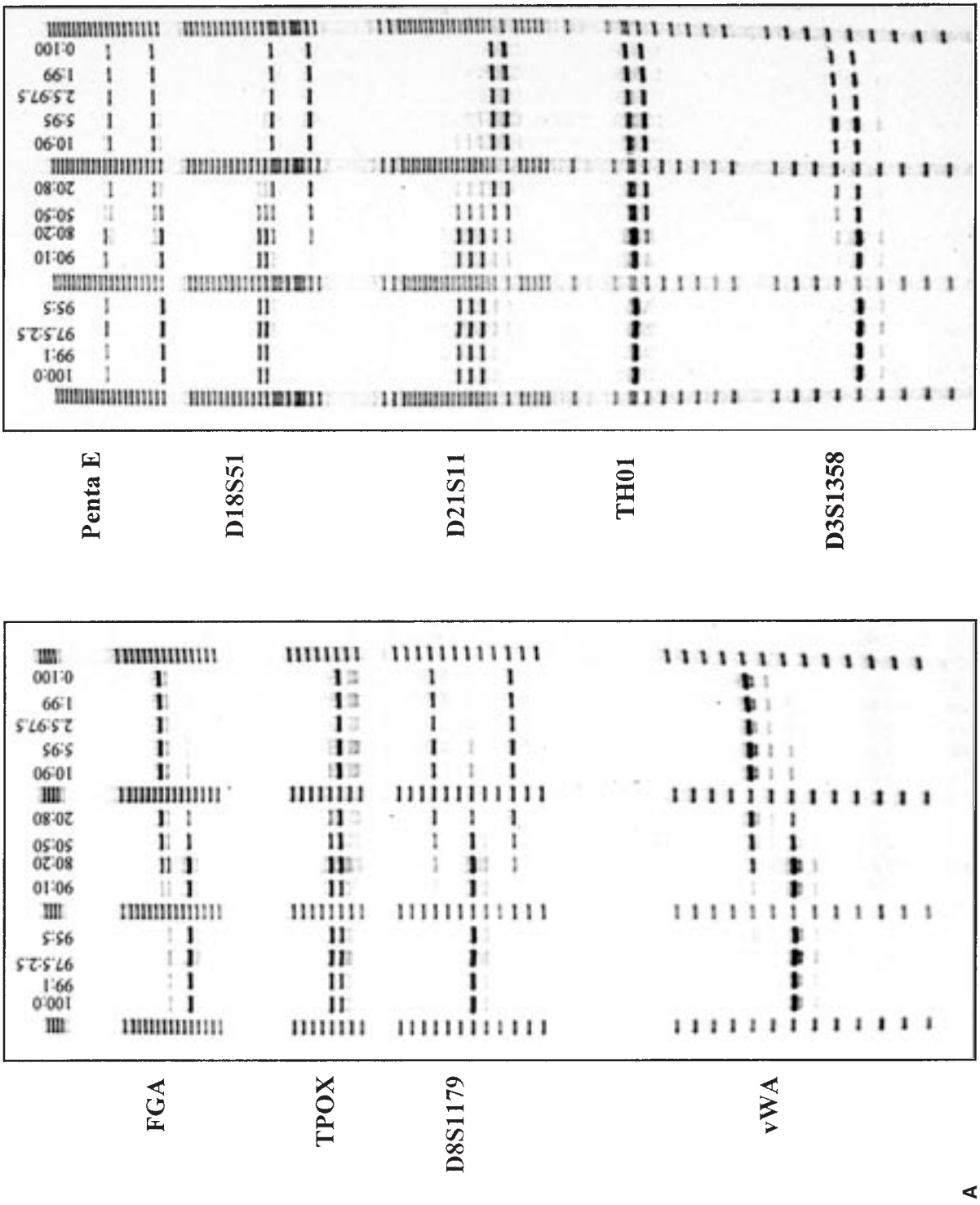
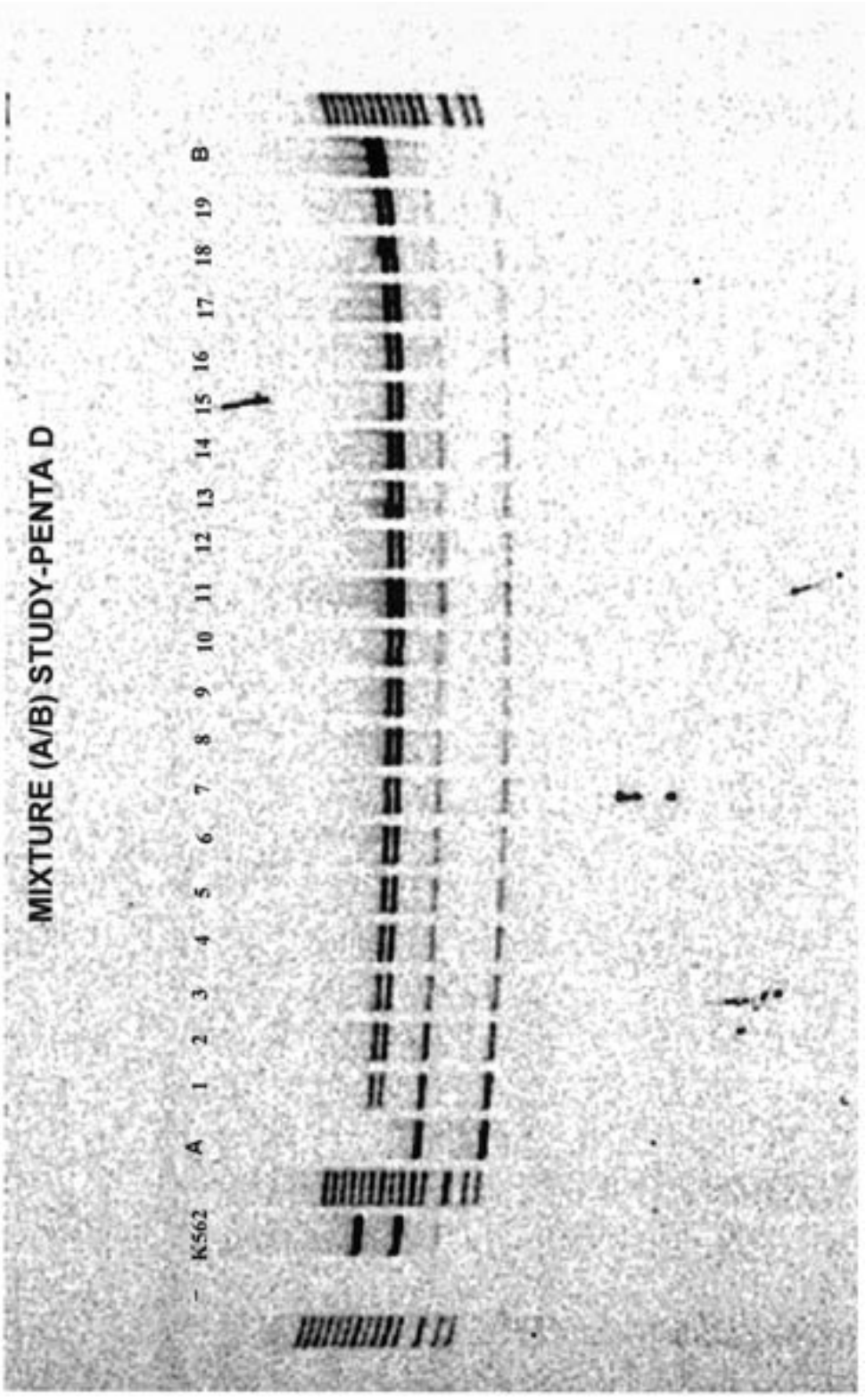


FIG. 4—*Performance of PowerPlex® 2.1/Penta D in DNA mixture studies*

DNA samples mixed in various ratios were used for amplification, the amplified products were separated in a denaturing 5% Long Ranger polyacrylamide gel and analyzed by the Hitachi FMBIO® II fluorescent scanner. A total of 1 ng of DNA was used for amplification. A. DNA mixture of K562 and CCRF-SB cell lines with ratios: 100:0, 99:1, 97.5:2.5, 95:5, 90:10, 80:20, 50:50, 20:80, 10:90, 5:95, 2.5:97.5, 1:99 and 0:100. B. DNA samples from two donors (A and B) were mixed at ratios: 1:1 (1), 1:2 (2), 1:3 (3), etc. to 1:19 (19), and the mixtures were analyzed for Penta D. (−): negative amplification control. (K562): positive amplification control.



B

FIG. 4—*(continued)*

TABLE 5—*Cutoff ratios in mixtures.*

Lab	DNA Mixtures	Body Fluid Mixtures
VDFS	20:80/30:70	1:1 vaginal/semen
PC/BT	20:80	N/A
NCSBI	1:10	N/A
PBSO	20:80	1:1 blood/blood
PSP*	N/A	1:2 blood/saliva

* The vaginal/semen mixture gave a complete profile of the sperm fraction at all ratios (from 1:20 to 20:1).

TABLE 6—*Family study summary.*

Lab	Family	Locus	Potential Mutation Source	Actual Parental Alleles	Offspring Mutation
PBSO	A	D21S11	M	30, 32	31
		D21S11	M	28, 31	30
		*CSF1PO	M or F	13 or 15	14
TXDPS	B	D8S1179	F	12, 14	13
		D21S11	F	29, 31	30
		D7S820	M	12, 14	13
VDFS	D	FGA	F	22, 25	21

(M): Mother.
(F): Father.
* The donor mother is 11, 13, the donor father is 11, 15. It is not certain which parental allele mutated to the offspring (11, 14).

transfer mutations. Three generations of Family A consisting of 35 individuals, four generations of Family B consisting of 22 individuals and three generations of Family C consisting of 18 individuals, were analyzed for the 13 core STR loci. Three parent/child mutations were detected in Family A and three parent/child mutations in Family B, but no mutations were detected in Family C. As shown in Table 6, mutations were observed for three individuals at D21S11, one individual at CSF1PO, one individual at D8S1179 and one individual at D7S820 (offspring mutations). Also the parental allele which is most likely the contributor for each of these mutations is also indicated in Table 6. All alleles were verified using both ABI and Promega STR multiplex systems. Data obtained from the VDFS laboratory include four families consisting of at least five members each. In one of these families one mutation was observed where an FGA 22 or 25 allele from the father was possibly mutated to a 21 in his son's profile (Table 6, Family D). Sequencing of the amplified products in these studies may determine the origin of these mutations. Generally, single-step mutations account for approximately 90% of STR mutation events (30). These data have demonstrated that mutations can occur from a parent to a child and may provide information to paternity laboratories where current practice suggests that a difference in two or more loci of the DNA profiles should be observed before an alleged parent could be eliminated as a biological parent. Mutation rates vary at each STR locus as it has been currently estimated (30–32) and they are approximately on the order of 1.2×10^{-3} per locus per gamete per generation, with the FGA locus exhibiting a four-fold higher rate of mutation (4.01×10^{-3}). To confirm mutation rates, a greater number of families need to be examined. Generally, mutation rates do not affect casework interpretation of analysis of forensic evidence samples using STRs because comparisons are being made between samples either originating (in the case of an inclusion) or not originating (in the case of an exclusion) from the same source.

Conclusion

The validation studies presented in this report demonstrate that the fluorescent PowerPlex™ 2.1 multiplex STR system is a sensitive, reliable forensic PCR DNA typing technique. PowerPlex™ 2.1 has been shown to be species-specific and robust when samples have been exposed to environmental insults and contaminants. Regardless of sample preparation protocols, interlaboratory and intralaboratory genotypic comparisons show great concordance. Detection and allele designation of microvariants is confidently determined using the internal lane standard and appropriate window sizing in the StarCall^{MT} software.

Through population database studies performed by NCSBI, PBSO, VDFS, and PC/BT, the multiplex was demonstrated to be highly discriminating (33). The PowerPlex™ 2.1/Penta D system and the STR loci primer sequences presented here facilitate successful application in the forensic field.

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References

1. Edwards A, Civitello A. Hammond HA, Caskey CT. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am J Hum Genet 1991;49:746–56.
2. Edwards A, Hammond HA, Jin L, Caskey CT, Chakraborty R. Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 1992;12:241–53.
3. Erlich HA. PCR technology: Principles and applications for DNA amplification. Stockton Press, New York 1989.
4. Warne D, Watkins C, Bodfish P, Nyberg K, Spurr NK. Tetranucleotide repeat polymorphism at the human beta-actin related pseudogene 2 (ACTBP2) detected using the polymerase chain reaction. Nucl Acids Res 1991;19:6980.
5. Kimpton CP, Gill P, Walton A, Urquhart A, Millican ES, Adams M. Automated DNA profiling employing multiplex amplification of short tandem repeat loci. PCR Methods Appl 1993;3:13–22.
6. Lins AM, Sprecher CJ, Puers C, Schumm JW. Multiplex sets for the amplification of polymorphic short tandem repeat loci-silver stain and fluorescence detection. Biotechniques 1996;20:882–9.
7. Buel E, Schwartz M, LaFountain MJ. Capillary electrophoresis STR analysis: Comparison to gel-based systems. J Forensic Sci 1998;43:164–70.
8. Isenberg AR, Allen RO, Keys KM, Smerick JB, Budowle B, McCord BR. Analysis of two multiplexed short tandem repeat systems using capillary electrophoresis with multiwavelength fluorescence detection. Electrophoresis 1998;19:94–100.
9. Budowle B, Koons BW, Keys KM, Smerick JB. Methods for typing the STR triplex CSF1PO, TPOX, and HUMTH01 that enable compatibility among DNA typing laboratories. Adv Forensic Haemogenet 1996;6:107–14.
10. Higuchi R, von Beroldingen CH, Sensabaugh GF, Erlich HA. DNA typing from single hairs. Nature 1988;332:543–6.
11. Paabo S. Ancient DNA: extraction, characterization, molecular cloning and enzymatic amplification. Proc Natl Acad Sci USA 1989;86:1939–43.
12. Hammond HA, Jin L, Zhong Y, Caskey CT, Chakraborty R. Evaluation of 13 short tandem repeat loci for use in personal identification applications. Am J Hum Genet 1994;55:175–89.

13. Oldroyd NJ, Urquhart AJ, Kimpton CP, Millican ES, Watson SK, Downes T, et al. A highly discriminating octoplex short tandem repeat polymerase chain reaction system suitable for human individual identification. Electrophoresis 1995;16:334–7.
14. Schumm JW, Lins AM, Sprecher CJ, Micka KA. High throughput systems for analysis of STR loci. Proceedings from the Sixth International Symposium on Human Identification 1996;10–9. Scottsdale (AZ).
15. Lins AM, Micka KA, Sprecher CJ, Taylor JA, Bacher JW, Rabbach DR, et al. Development and population study of an eight-locus short tandem repeat (STR) multiplex system. J Forensic Sci 1998;43:1–13.
16. Budowle B, Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM. Population data on the thirteen CODIS core short tandem repeat loci in African Americans, U.S. Caucasians, Hispanics, Bahamians, Jamaicans, and Trinidadians. J Forensic Sci 1999;44:1277–86.
17. Moller 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.
18. Brinkmann B, Moller A, Wiegand P. Structure of new mutations in 2 STR systems. Int J Leg Med 1995;107:201–3.
19. Federal Bureau of Investigation. Quality Assurance Standards for Forensic DNA Testing Laboratories. October 1998.
20. Promega Corporation. GenePrint® PowerPlex™ 2.1 System Technical Manual, Part #TMD011. Madison, WI 1999.
21. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratories Press, Cold Spring Harbor 1989.
22. Gill P, Jeffreys AJ, Werrett DJ. Forensic application of DNA "fingerprints." Nature 1985;318:577–9.
23. Gill P, Sparkes R, Fereday L, Werrett DJ. Report of the European Network of Forensic Science Institutes (ENSFI): formulation and testing of principles to evaluate STR complexes. Forensic Sci Int 2000;108:1–29.
24. Smith JR, Carpten JD, Brownstein MJ, Ghosh S, Magnuson VL, Gilbert DA, et al. Approach to genotyping errors caused by nontemplated nucleotide addition by *Taq* DNA polymerase. Genome Res 1995;5:312–7.
25. Magnuson VL, Ally DS, Nylund SJ, Karanjawala ZE, Rayman JB, Knapp JI, et al. Substrate nucleotide-determined non-templated addition of adenine by *Taq* DNA polymerase: implications for PCR-based genotyping and cloning. Biotechniques 1996;21:700–9.
26. Levinson G, Gutman GA. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol Biol Evol 1987;4:203–21.
27. Schlotterer C, Tautz D. Slippage synthesis of simple sequence DNA. Nucl Acids Res 1992;20:211–5.
28. 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.
29. Comey CT, Budowle B. Validation studies on the analysis of the HLA DQ alpha locus using the polymerase chain reaction. J Forensic Sci 1991;36:1633–48.
30. Brinkmann B, Klintschar M, Neuhuber F, Huhne J, Rolf B. Mutation rate in human microsatellites: influence of the structure and length of the tandem repeat. Am J Hum Genet 1998;62:1408–15.
31. Charlesworth B, Sniegowski P, Stephan W. The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 1994;371:215–20.
32. Jeffreys AJ, Barber R, Bois P, Buard J, Dubrova YE, Grant G, et al. Human minisatellites, repeat DNA instability and meiotic recombination. Electrophoresis 1999;20:1665–75.
33. Levedakou EN, Freeman DA, Budzynski MJ, Early BE, McElfresh KC, Schumm JW, et al. Allele frequencies for fourteen STR loci of the PowerPlex™ 1.1 and 2.1 multiplex systems and Penta D locus in Caucasians, African-Americans, Hispanics and other populations of the United States of America and Brazil. J Forensic Sci 2001;46:736–61.

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