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Validation of a 16-Locus Fluorescent Multiplex System*

ABSTRACT: STR multiplexes have been indispensable for the efficient genotyping of forensic samples. The PowerPlex® 16 System contains the core CODIS loci, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, CSF1PO, FGA, TH01, TPOX, vWA, the sex determinant locus, amelogenin, and two pentanucleotide STR loci, Penta D and Penta E. This multiplex satisfies the locus requirements for most national databases and is the most efficient currently available system due to its single PCR amplification. To provide the groundwork for judicial acceptance, including the publication of primer sequences, and to evaluate laboratory-to-laboratory variation, a developmental validation for casework on this commercially available system was performed in 24 laboratories and produced the following conclusions. Amplification was reliable on a variety of thermal cyclers and product could be analyzed on either an ABI PRISM® 310 Genetic Analyzer or an ABI PRISM® 377 DNA Sequencer. Genotyping using single source samples was consistent between 0.25 and 2 ng of input DNA template with a few laboratories obtaining complete genotypes at 0.0625 ng. However, heterozygote allele imbalance (<60% peak height balance) caused by stochastic effects was observed at a rate of 13% with 0.125 ng DNA and 22% at 0.0625 ng DNA. Mixture analyses were done using a total of 1 ng of DNA template. Most alleles were detected in mixtures of 4 to 1 and some minor alleles were detected in mixtures of 19 to 1. Optimum amplification cycle number was dependent on the sensitivity of the detection instrument used and could also be adjusted to accommodate larger amounts of DNA on solid supports such as FTA® paper. Reaction conditions including volume, annealing temperature, and concentrations of primer, AmpliTaq Gold®, and magnesium were shown to be optimal yet robust enough to withstand moderate variations without affecting genotype analysis. Environmental, matrix and standard source analyses revealed an ability to obtain complete genotypes in all sample types except those exposed to 80°C for 12–48 days. Finally, comparison of genotype results from the PowerPlex® 16 System with other commercially available systems on non-probative reference and forensic samples showed consistent results.

KEYWORDS: forensic science, DNA typing, short tandem repeat (STR), polymerase chain reaction (PCR), validation, PowerPlex, primers, CODIS, Penta E, Penta D

Multiplex short tandem repeat (STR) analysis has emerged as the dominant forensic DNA identification method because it is easy to interpret, can use sub-nanogram amounts of DNA, has a high degree of discrimination and can yield results in a matter of hours (1–3). In the United States, these advantages have led to the development of a national felon database employing 13 core STR loci (4–6). In May 2000, the PowerPlex® 16 System (Promega, Madison, WI) was introduced as the first multiplex system capable of simultaneously amplifying all 13 core STR, the sex determinant locus, amelogenin, and two high discrimination low stutter pentanucleotide STR loci, Penta D and Penta E (7). This product was subsequently validated by a group of forensic laboratories to demonstrate concordance in approximately 2000 samples with existing STR typing systems (8,9). Previous studies have documented the allele frequencies (10) and physical mapping data (11,12) for the 15 STR loci in the PowerPlex® 16 System.

In this study we present validation data from 24 laboratories and developmental data from Promega Corporation demonstrating that the PowerPlex® 16 System provides reliable genotyping data under a wide variety of conditions. The results obtained demonstrate the robustness of this system and the ability to successfully use the PowerPlex® 16 System with casework samples in a large number of forensic laboratories.

These studies have been performed to satisfy TWGDAM (13) and DAB guidelines in order to address concerns presented in today's legal environment. As a result of these studies, the PowerPlex® 16 System has been approved for use in providing casework and reference sample genotypes for the CODIS/NDIS national database system.

Participating Laboratories

Austin Police Department, Detroit Police Department, Federal Bureau of Investigation, Idaho State Police Forensic Services,[7] Illinois State Police[8], Kansas Bureau of Investigation, Las Vegas

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Metro Police, Michigan State Police, Mississippi Crime Laboratory,[7] Missouri State Highway Patrol,[8] Montana Department of Justice, New York State Police, Oklahoma City PoliceDepartment, Oklahoma Bureau of Investigation, Orange County Sheriff's Office, Pennsylvania State Police,[7] Promega Corporation, San Diego Sheriff's Office, South Dakota Forensic Laboratory,[7] Washoe County Sheriff's Office, Vermont Forensic Laboratory,[7] West Virginia State Police,[7] Wisconsin State Crime Laboratory-Madison,[7] and Wisconsin State Crime Laboratory-Milwaukee were the laboratories that participated in this validation study.

Materials and Methods

For the manufacturer's stressing studies, one component was altered as indicated while the other components remained constant. Unless noted otherwise, protocols in the PowerPlex® 16 System technical manual TMD012 (14) were followed.

Instrument Calibrations and Variability

All developmental work was performed with instruments and micropipetters that were tested to ensure that they were within manufacturer-specified calibration settings. Using a large number of laboratories in the validation process also allowed a statistical approach to examine the variations encountered within the forensic community.

DNA for Developmental and Single Source Studies

Human DNA was isolated using phenol/chloroform extraction (15) from the non-cell lines B10, B15, B19, C2, and H9 (Promega). DNA from the cell lines 9947A and CCRF-SB were acquired from Promega. These templates were quantitated by spectrophotometric analysis using A_{260} detection (15). Standard samples for manufacturer's consistency and multiplex versus monoplex studies were obtained from the National Institutes of Standards and Technology (NIST, Gaithersburg, MD). Genomic #1, Genomic #4, Genomic 9947A, and Genomic 9948 from the NIST Standard Reference Materials (SRM 2391a) were amplified as monoplexes and all ten DNA standards in this kit were genotyped with the PowerPlex® 16 System. Non-human species samples were obtained from the Palm Beach County Sheriff's Office Crime Laboratory (West Palm Beach, FL) and Clontech (Palo Alto, CA). Buccal swab, liquid blood, and dried blood on FTA® paper (Whatman, Clifton, NJ) for the manufacturer's standard samples study were extracted and quantitated using the DNA IQ™ System (Promega). Non-probative casework studies used phenol/chloroform and Chelex (Bio-Rad, Hercules, CA) extraction protocols. Sexual assault samples underwent a differential lysis (16) as part of the DNA extraction. Liquid blood was spotted for matrix and environmental studies. Most forensic samples were concentrated using Microcon® devices (Millipore Corp., Bedford, MA) and quantitated using the Quantiblot™ system (Applied Biosystems, Foster City, CA) or *ACES*™ system (Life Technologies, Rockville, MD).

DNA Amplification

PCR amplifications utilized the commercial release of Promega's PowerPlex® 16 System and AmpliTaq Gold® DNA Polymerase (Applied Biosystems). Amplification reactions typically were performed using a Perkin-Elmer GeneAmp™ PCR System 9600 Thermal Cycler (Foster City, CA) and the amplification cycling parameters described in the PowerPlex® 16 System technical manual (14). However, the Perkin-Elmer GeneAmp® PCR System 2400, 9700 and the Perkin-Elmer Thermal Cycler Model 480 were also examined. Amplification reactions generally contained 0.5 to 1 ng DNA in 25 μL and used 10/20 or 10/22 cycling as described in the PowerPlex® 16 System technical manual (14). The sequences of the forward and reverse primers for each of the 16 loci are shown in Table 1. In studies examining magnesium concentrations, $MgCl_2$ solution and Mg-free GoldST*R Buffer were provided by Promega.

Amplification Analysis

The PowerPlex® 16 System employs four fluorescent dyes (7). Spectral resolution was established by Promega's *GenePrint*® Matrix FL-JOE-TMR-CXR allowing evaluation of each fluorescent dye employed in the kit (17). All analyses utilized the ILS 600 size standard and Allelic Ladder Mix (Fig. 1) provided with the PowerPlex® 16 System (14,18). Initial fragment sizing was performed by the GeneScan® software (Applied Biosystems). Allele calling was performed by Promega's PowerTyper™ 16 Macro (14) operating within the Genotyper® software program (Applied Biosystems). Analysis of amplification products was performed primarily on ABI PRISM® 310 Genetic Analyzers using 3 s or 5 s injection times. Instruments used POP4 polymer and 47 cm capillaries from Applied Biosystems. Generally, 1 μL of amplified sample and ILS 600 were prepared in 24 μL deionized formamide. Samples were denatured for 3 min at 95°C followed by quick cooling on ice. Some experiments, as noted, analyzed 1 μL or 2 μL of prepared sample on the ABI PRISM® 377 DNA Sequencer with Long Ranger™ gels (BioWhittaker Molecular Applications, Rockland, ME). Sample preparations generally used 1 μL of amplified sample, 0.5 μL ILS 600 and 1.5 μL Blue Dextran Loading Solution (Promega) and were denatured for 2 min at 95°C followed by quick cooling on ice.

Stutter Calculation

Sixty-two human templates were amplified using the 10/22 cycling protocol and were analyzed on the ABI PRISM® 310 Genetic Analyzer. Stutter peaks (19–21) were determined as four bases smaller (n-4) than tetranucleotide repeats and five bases smaller (n-5) than pentanucleotide repeats (±0.5 bases). A high level of stringency was imposed to collect accurate stutter data. Stutter peaks were required to return to baseline (data with highly variable backgrounds were not used) and stutter peaks that fell in the repeat position between sister alleles two repeat units apart were not used (additive effect of $n + 4$ and $n - 4$ was seen in some loci). The actual peak heights did not exceed 5000RFU. Stutter was calculated by dividing the peak height of the stutter peak by the peak height of the true allele.

Results and Discussion

The performance of PCR-based STR assays can be influenced by numerous factors including the differences in instrumentation, variability in the quality and quantity of the DNA being amplified, precision of sizing standard and variations in the reaction and analysis conditions. Each of these factors was evaluated for its affect on the completeness and correctness of genotypes provided with the PowerPlex® 16 System. Additionally, the average stutter for each locus and the cross reactivity with non-human species were evaluated for the PowerPlex® 16 System.

[7] Data from these laboratories were evaluated for non-probative case studies.
[8] Indicates laboratories participating in the database validation (8,9) that supplied single source study and/or mixture data only.

TABLE 1—*PowerPlex® 16 System primer sequences.*

Locus	Orientation	Oligonucleotide Sequences	5′ end
FGA	Forward	GGCTGCAGGGCATAACATTA	TMR
	Reverse	ATTCTATGACTTTGCGCTTCAGGA	OH
TPOX	Forward	GCACAGAACAGGCACTTAGG	OH
	Reverse	CGCTCAAACGTGAGGTTG	TMR
D8S1179	Forward	ATTGCAACTTATATGTATTTTTGTATTTCATG	OH
	Reverse	ACCAAATTGTGTTCATGAGTATAGTTTC	TMR
vWA	Forward	GCCCTAGTGGATGATAAGAATAATCAGTATGTG	OH
	Reverse	GGACAGATGATAAATACATAGGATGGATGG	TMR
Amelogenin	Forward	CCCTGGGCTCTGTAAAGAA	TMR
	Reverse	ATCAGAGCTTAAACTGGGAAGCTG	OH
Penta E	Forward	ATTACCAACATGAAAGGGTACCAATA	OH
	Reverse	TGGGTTATTAATTGAGAAAACTCCTTACAATTT	FL
D18S51	Forward	TTCTTGAGCCCAGAAGGTTA	FL
	Reverse	ATTCTACCAGCAACAACACAAATAAAC	OH
D21S11	Forward	ATATGTGAGTCAATTCCCCAAG	OH
	Reverse	TGTATTAGTCAATGTTCTCCAGAGAC	FL
TH01	Forward	GTGATTCCCATTGGCCTGTTC	FL
	Reverse	ATTCCTGTGGGCTGAAAAGCTC	OH
D3S1358	Forward	ACTGCAGTCCAATCTGGGT	OH
	Reverse	ATGAAATCAACAGAGGCTTGC	FL
Penta D	Forward	GAAGGTCGAAGCTGAAGTG	JOE
	Reverse	ATTAGAATTCTTTAATCTGGACACAAG	OH
CSF1PO	Forward	CCGGAGGTAAAGGTGTCTTAAAGT	JOE
	Reverse	ATTTCCTGTGTCAGACCCTGTT	OH
D16S539	Forward	GGGGGTCTAAGAGCTTGTAAAAAG	OH
	Reverse	GTTTGTGTGTGCATCTGTAAGCATGTATC	JOE
D7S820	Forward	ATGTTGGTCAGGCTGACTATG	JOE
	Reverse	GATTCCACATTTATCCTCATTGAC	OH
D13S317	Forward	ATTACAGAAGTCTGGGATGTGGAGGA	OH
	Reverse	GGCAGCCCAAAAAGACAGA	JOE
D5S818	Forward	GGTGATTTTCCTCTTTGGTATCC	OH
	Reverse	AGCCACAGTTTACAACATTTGTATCT	JOE

FL = fluorescein; JOE = 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein; TMR = carboxy-tetramethylrhodamine.

Variation of Cycle Number and Injection Time

A level of flexibility can be provided in an STR typing system by alteration of amplification cycle number. Higher cycle number can increase signal while decrease in cycle number can achieve a balanced signal when DNA template is in excess (>1 ng). As PCR cycling conditions have a major affect on the amount of product and the balance between loci, variations in the number of PCR cycles around the recommended 10/20 to 10/22 cycle protocols were evaluated (Fig. 2). Cycle protocols between 10/18 and 10/26 (28 and 36 cycles, respectively) were evaluated using a titration of templates between 0.2 and 5 ng. As expected, a decrease in cycle number produced decreased peak heights with yield of the smaller loci being affected most. Locus dropout was observed with 0.5 ng template or less at 28 cycles. Five nanograms of template countered the effect of 28 cycles. Consequently, amplifications that inherently use excess templates, such as FTA punches, produce balanced peak heights across loci with decreased cycle number (personal communication, Susanne Brenneke, Missouri State Highway Patrol). A good balance was produced with 10/20 and 10/22 cycle protocols with roughly a 40% difference in signal intensity between conditions. Increases in cycle number (34 and 36 cycles) produced only a slight increase in yield for the largest loci while the smaller loci displayed a substantial increase in signal leading to notable imbalance between loci. Although increased cycle number may be useful in low copy number situations, the stochastic effects inherent in these amplifications increase the likelihood of heterozygote allele imbalance (see below).

An examination of peak heights under various cycling and injection times was performed by 19 different laboratories to address the issue of differing sensitivities of ABI PRISM® 310 Genetic Analyzers. Figure 3 demonstrates a five-fold variation in average peak heights using 0.5 ng of template, 10/22 cycling, and 3 s injections. This variation does not appear to be the result of differences in thermal cyclers as the variation is also reflected in the internal lane standard, which is not amplified. To overcome this inherent variation, each laboratory selected the best one of four conditions between 10/20 and 10/22 cycling at either 3 or 5 s injections to obtain the peak heights recommended in the PowerPlex® 16 System technical manual. Most laboratories selected 10/20 cycling with 5 sec injection or 10/22 cycling with 3 s injection.

Variation of Reaction Volume

Reduction of reaction volume, which is often used as a cost-saving measure, can alter the concentration of PCR reaction components. Varied reaction volumes were evaluated to determine the effects on amplification (Fig. 4). Two approaches can be used in changing volume, keeping the template concentration constant or keeping the quantity of template constant. We elected to keep the concentration constant to reduce the effects of using high template concentrations, such as high background and saturation of the CCD pixels that may lead to too much or incomplete spectral subtraction resulting in phenomena known as pull down or pull up. Reaction volumes ranged from 5 μL to 50 μL (25 μL volume standard) with template titrations between 0.2 and 5 ng/25 μL. As expected, sig-

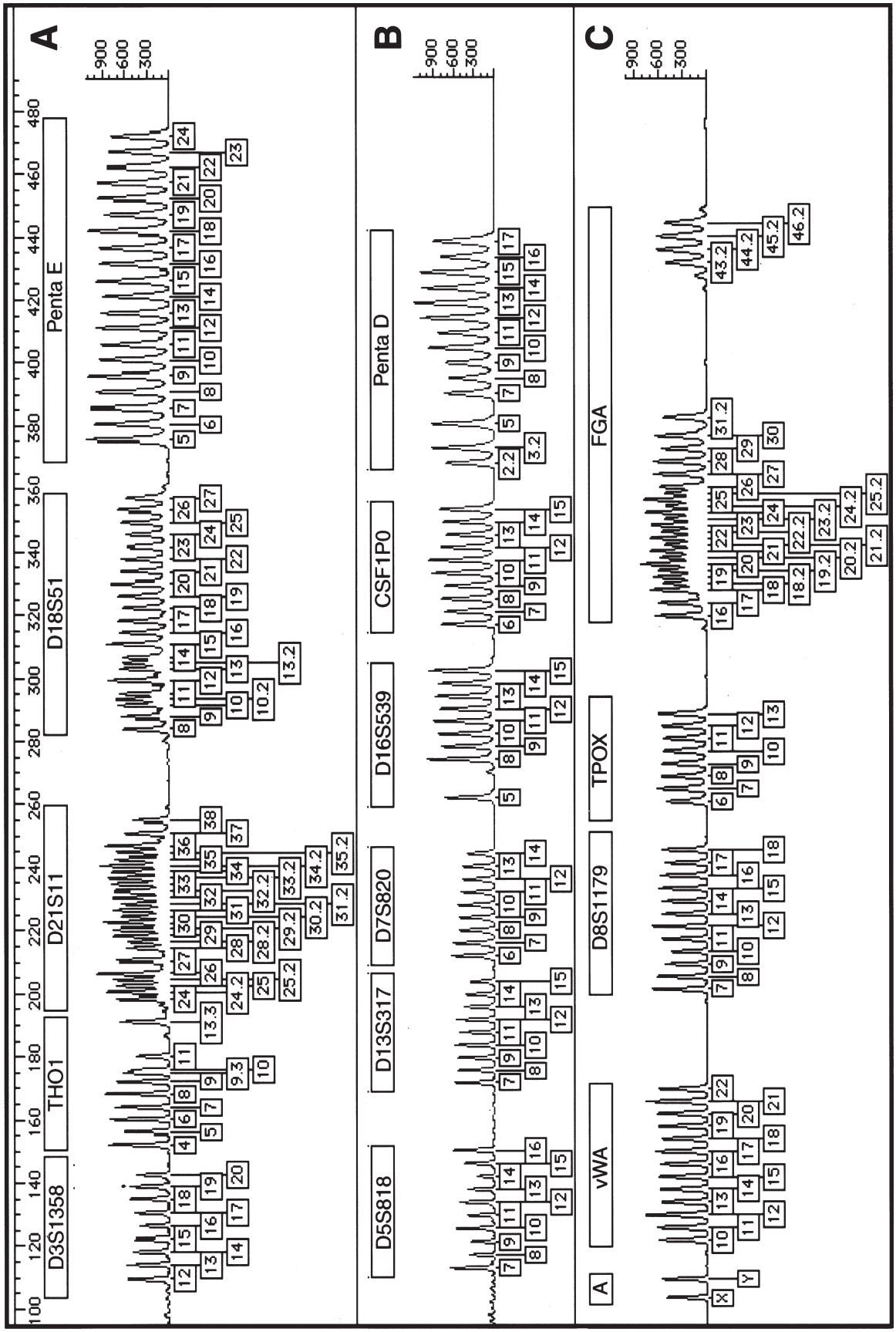

FIG. 1—*Allelic standard provided with the PowerPlex® 16 System. Panels A–C display PowerTyper™ 16 Macro analyses of the Allelic Ladder Mix. Panel A is the Blue Channel displaying the fluorescein-labeled loci. Panel B is the Green Channel displaying the JOE-labeled loci. Panel C is the Yellow Channel displaying the TMR-labeled loci.*

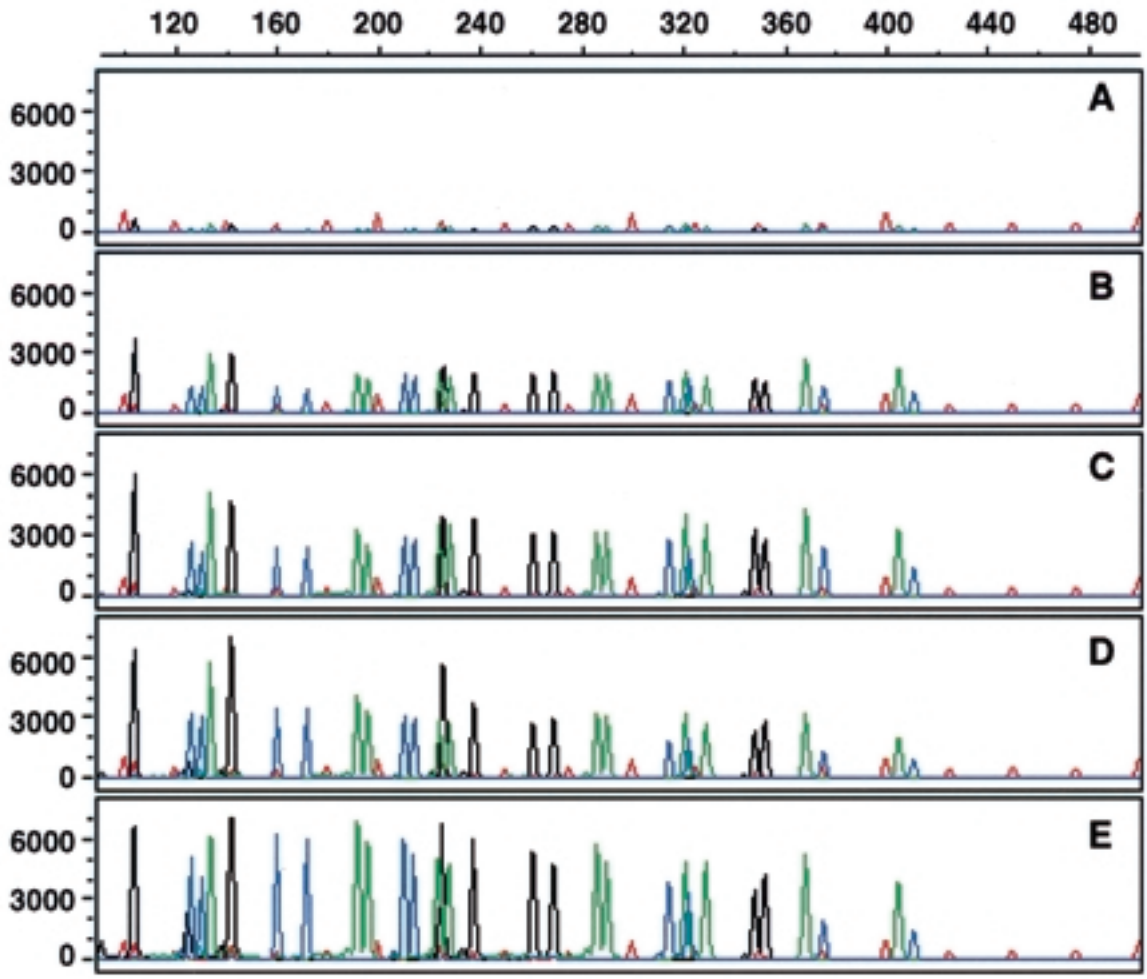

FIG. 2—*Variation of cycle number. Amplifications of 1 ng B-10 genomic DNA is shown here analyzed on the ABI PRISM® 310 Genetic Analyzer. Cycle number of each reaction is as follows: Panel A is 28 cycles (10/18), Panel B is 30 cycles (10/20), Panel C is 32 cycles (10/22), Panel D is 34 cycles (10/24), Panel E is 36 cycles (10/26). The standard cycle number is 30 or 32 cycles (10/20 or 10/22, respectively).*

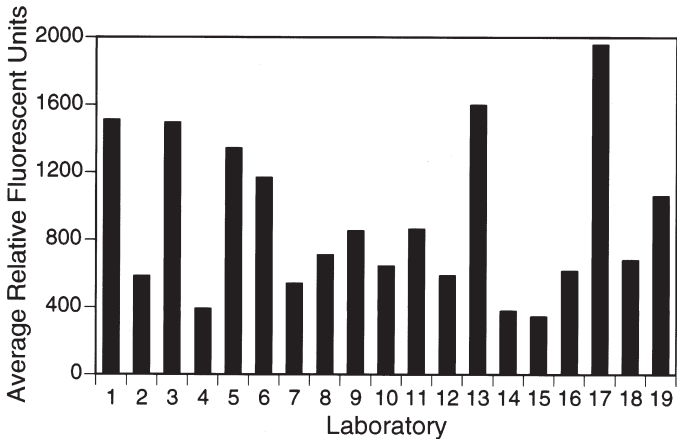

FIG. 3—*Variation in the sensitivity of ABI PRISM® 310 Genetic Analyzers. 0.5ng of the single source sample B15 was amplified according to the Promega standard protocol using 10/22 cycling conditions on Perkin Elmer GeneAmp 9600 thermal cyclers. The amplified DNA was analyzed on ABI PRISM® 310 Genetic Analyzers using 3 s injection times. A comparison of the average peak heights from 19 different laboratories showed over a five-fold difference in sensitivity.*

nal intensity remained similar at all volumes tested. However, increased heterozygote allele imbalance was seen with reaction volumes of 10 μL (0.2 ng) or less. This is likely due to stochastic effects resulting from the low amount of template. Decreased reaction volume may be a useful adaptation for very low template protocols as it effectively increases the concentration of a given amount of template. However, stochastic effects and corresponding concentration of inhibitors are all potential concerns.

AmpliTaq Gold® DNA Polymerase Titration

The DNA polymerase plays a key role in determining the amount of amplification that can occur. Therefore, variations in the concentration of this enzyme were evaluated (Fig. 5). Between 2 and 16U/25 μL (0.5X–4X) were examined with 10/20 and 10/22 cycling and template ranges between 0.2–5 ng. Optimal balance of signal between loci was seen at 1X enzyme (4U). No locus dropout was observed with reduced enzyme but lower yield was noted in the larger loci. An increase in enzyme concentration produced a minimal increase in signal of several loci with 1ng or less template and a notable increase in the signal of most loci with 5 ng template (data not shown).

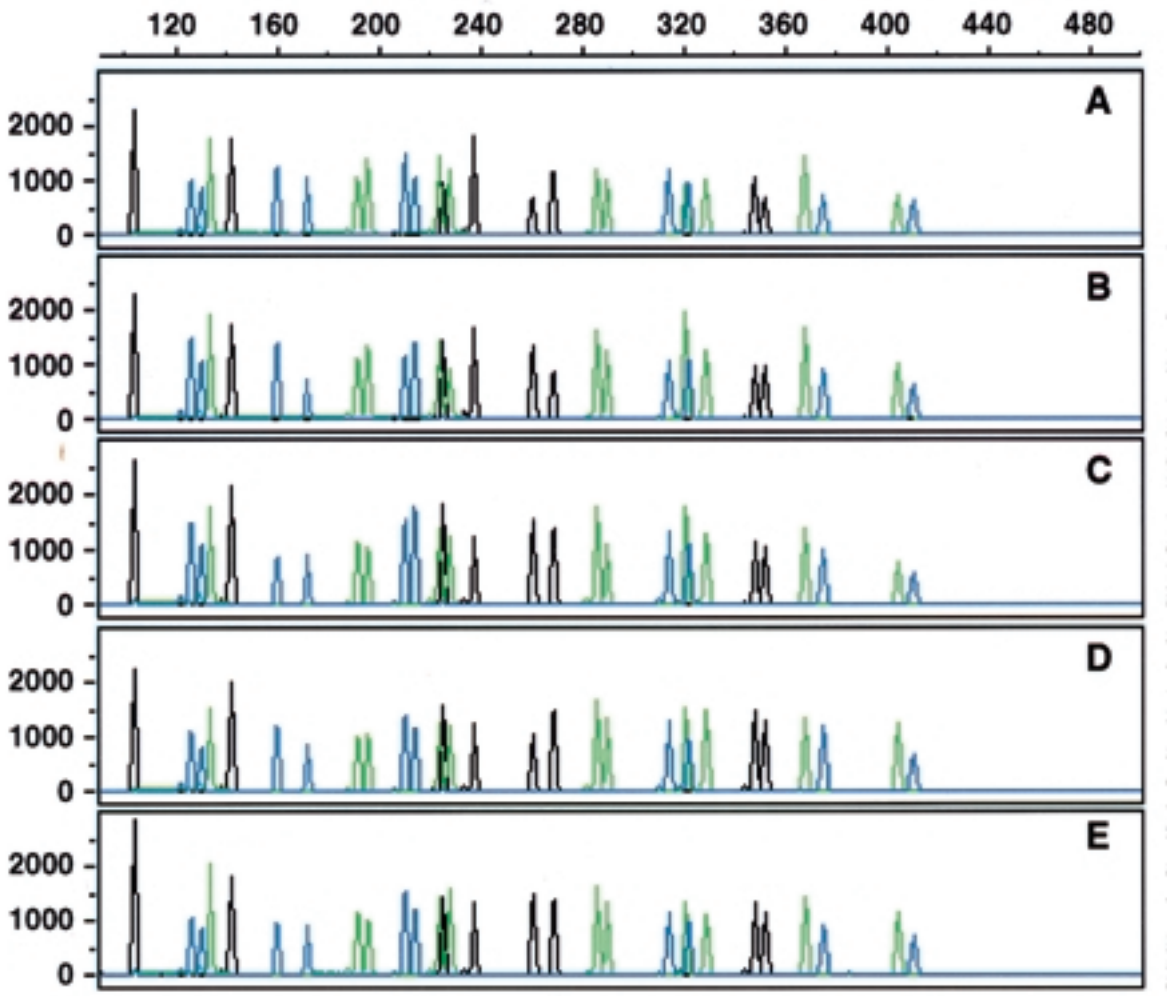

FIG. 4—*Variation of reaction volume. Amplifications (10/22 cycling) of 0.5ng/25μL B-10 genomic DNA is shown here analyzed on the ABI PRISM® 310 Genetic Analyzer. Volume of each reaction is as follows: Panel A is 5 μL, Panel B is 10 μL, Panel C is 12.5 μL, Panel D is 25 μL, Panel E is 50 μL. The standard reaction volume is 25 μL.*

Primer Pair Titration

The concentrations of the 32 primers play a critical role in the amplification efficiency of each locus. This required the manufacturer to adjust the primer concentrations to produce equivalent signals for each locus. Primer concentrations between 0.5X and 2X were tested with 10/20 and 10/22 cycles and template between 0.2 and 5ng to determine the effects on peak height and balance between loci (Fig. 6). When primer concentration was increased above 1X, smaller loci (in bases) were preferentially amplified. As primer concentration was decreased below 1X, peak heights decreased in small loci and increased in larger loci. Locus dropout was seen at 0.5X primer concentration and low template (0.2 ng) (data not shown). These data suggest that normal variability in pipetting precision will have minimal effect but intentional changes in primer concentration should not be made.

Titration of Magnesium

Magnesium plays an important role in polymerase activity and specificity (22). Although magnesium is part of the reaction buffer, EDTA in the DNA sample can inadvertently alter effective concentrations of magnesium. To examine the effect of magnesium variations, concentrations between 1–2 mM (1.5 mM is standard) were examined using 10/20 and 10/22 cycles and 1 ng of template (Fig. 7). Optimal balance between loci was seen at 1.5 mM magnesium. Increased magnesium concentration produced a slight increase in the yield of smaller loci. A magnesium concentration of 1.25 mM resulted in locus-to-locus imbalance with 10/22 cycling and actual dropout of D3S1358, D18S51, and D5S818 loci with 10/20 cycling in some reactions (data not shown). Dropout of over 12 loci was seen in reactions using 1 mM magnesium. These results indicate that small increases in the magnesium concentration had little effect but a decrease in the magnesium concentration (i.e., adding large amounts of EDTA with sample DNA) could adversely affect results by chelating magnesium. For this reason, the manufacturer recommends storing samples in water or TE^{-4} (Tris, pH 8.0, 0.1 mM EDTA).

Variation of Annealing Temperature

Changes to the annealing temperature of the amplification reaction can affect the specificity and balance of the amplified loci. To

examine these effects, amplification reactions were performed using annealing temperatures up to 4°C above and below the 60°C recommended annealing temperature with both 10/20 and 10/22 cycling conditions and DNA between 0.2 and 5 ng per reaction (Fig. 8). Locus dropout and additional artifacts were not observed with lower annealing temperature but an increase in the yield of smaller loci causing locus to locus imbalance was noted. An increase to 62°C produced a decrease in yield of D3S1358, D5S818, D7S820 and amelogenin relative to other loci in 0.5–5ng reactions. At 64°C, dropout or near dropout of D3S1358, D18S51, D5S818, D7S820, Penta E, amelogenin, and D8S1179 was observed. These data suggest that the chosen annealing temperature has a 2°C leeway above and at least a 4°C range below.

Comparison of Thermal Cyclers

Different models of thermal cyclers have slightly different heating and cooling properties. To see what affect these differences have on amplification using the PowerPlex® 16 System, four different thermal cycler models—the Perkin-Elmer GeneAmp® PCR System 2400, 9600, 9700 and the Perkin-Elmer Thermal Cycler Model 480 using the 10/22 cycling protocols described in the technical manual were examined. No consistent performance differences were observed (data not shown).

Genotype Consistency/Reproducibility

One key aspect of any amplification system is the ability to provide reproducible results in a variety of laboratory settings. To test this, 24 laboratories genotyped single source samples at 1.0, 0.5, and 0.25 ng of input DNA. After optimizing cycle number and injection times for their instruments, all laboratories were able to reliably genotype samples containing 1.0 and 0.5 ng of DNA. Only two laboratories had difficulty detecting two alleles using 0.25 ng of DNA due in part to low instrument sensitivity combined with signal thresholds of 150 RFU. Thus, even with the variations in ABI PRISM® 310 Genetic Analyzer sensitivities, correct genotyping under a variety of input DNA concentrations was reliably obtained by a large group of laboratories (data not shown).

In addition to genotyping analyses between laboratories, monoplex and multiplex amplification reactions were performed using standard samples from NIST. All monoplex and PowerPlex® 16 System derived genotypes were consistent with the NIST determined genotypes (data not shown).

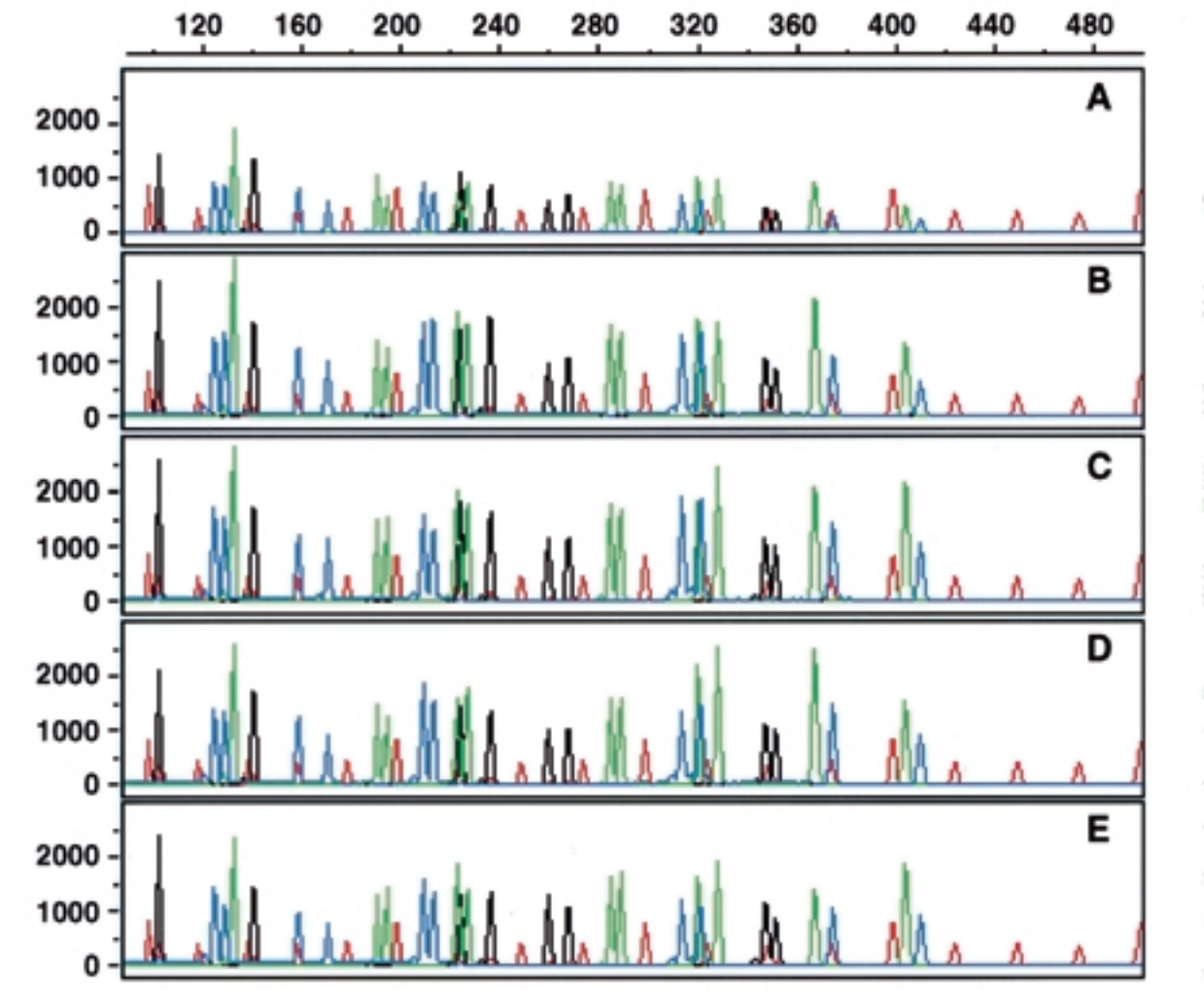

FIG. 5—*Titration of AmpliTaq Gold® DNA Polymerase. Amplifications (10/22 cycling) of 0.5 ng B-10 genomic DNA is shown here analyzed on the ABI PRISM® 310 Genetic Analyzer. Panel A is 0.5X polymerase, Panel B is 1X polymerase, Panel C is 1.5X polymerase, Panel D is 2X polymerase, and Panel E is 4X polymerase.*

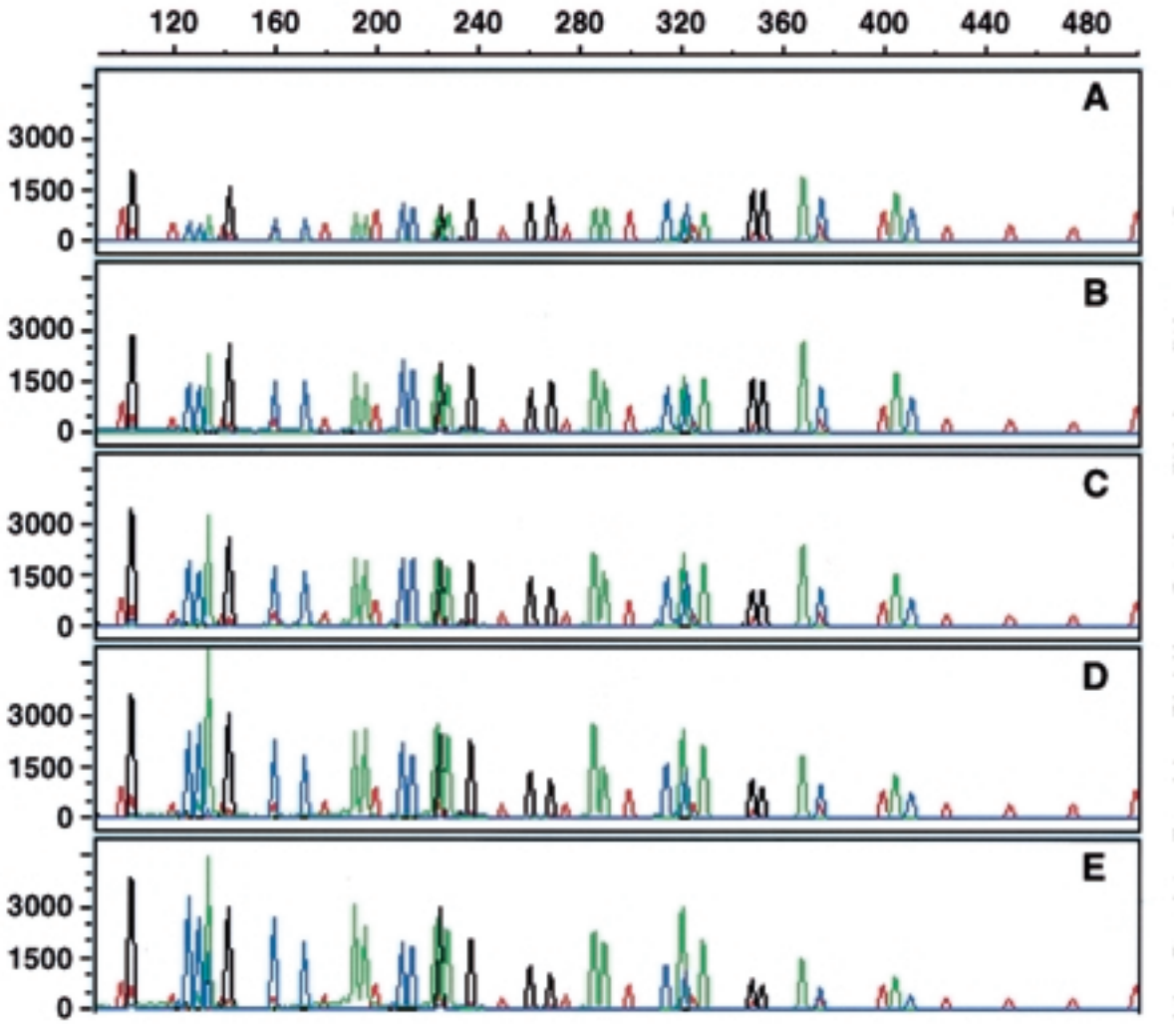

FIG. 6—*Titration of primer pair. Amplifications (10/22 cycling) of 1 ng B-10 genomic DNA is shown here analyzed on the ABI PRISM® 310 Genetic Analyzer. Panel A is 0.5X primer, Panel B is 0.75X primer pair, Panel C is 1X primer pair, Panel D is 1.5X primer pair, Panel E is 2X primer pair.*

Heterozygote Allele Balance, Sensitivity and Stochastic Effects

Accurate genotyping requires balanced heterozygous alleles. Imbalance can result from one allele amplifying slightly better than another or from sampling variations (stochastic effects) in samples with very few template molecules. Twenty-four laboratories examined the peak height balance of 16 heterozygote allele pairs (384 total pairs) using 0.25–1 ng single source DNA samples. At 1 ng, the mean ratio of heterozygote peak heights was 0.90 with a standard deviation of 0.085 Table (Table 2). This balance ratio dropped to 0.87 and 0.84 as the amount of input DNA was reduced to 0.5 ng and 0.25 ng, respectively.

Seven laboratories examined general heterozygote allele balance, allele dropout, sensitivity, and stochastic effects using single source samples from 0.0625–2 ng. Six of the seven laboratories were able to accurately genotype all 32 alleles using 0.125 ng DNA. Even at 0.0625 ng DNA, three of the laboratories were able to obtain a complete genotype (data not shown). However, as noted below, stochastic effects became significant at the lower DNA concentrations and could present difficulties analyzing samples containing DNA from more than one individual.

Figure 9 shows the effect of DNA quantity on heterozygote allele balance. The degree of imbalance was divided into three categories: severe imbalance (ratio of peak heights of heterozygous alleles <0.5), moderate imbalance (ratio between 0.5 and 0.6), and slight imbalance (ratio between 0.6 and 0.7). Values greater than 0.7 were considered balanced. Above 0.25 ng template, slight to moderate imbalance was noted at a very low level. At 0.25 ng, three laboratories observed at least one moderately imbalanced allele pair while at 0.125 ng and below, most laboratories observed at least one imbalance. Although the majority of allele pairs remained balanced at 0.0625 ng, each laboratory should examine where imbalances begin to occur and be aware of this phenomena when using low amounts of input DNA (so-called stochastic threshold).

Mixture Analysis

Mixture analysis plays an important role in many casework studies. To analyze the resolving power of the PowerPlex® 16 System, fifteen laboratories analyzed mixture sets of B19 and H9 DNA (provided by Promega Corporation) where the ratio of one sample to the other was changed from 19:1 to 1:19. Total DNA was kept constant at 1 ng. Interpretation and minimum RFU cutoff values were set by each laboratory and followed their established guidelines. Most of the laboratories could identify all of the minor alle-

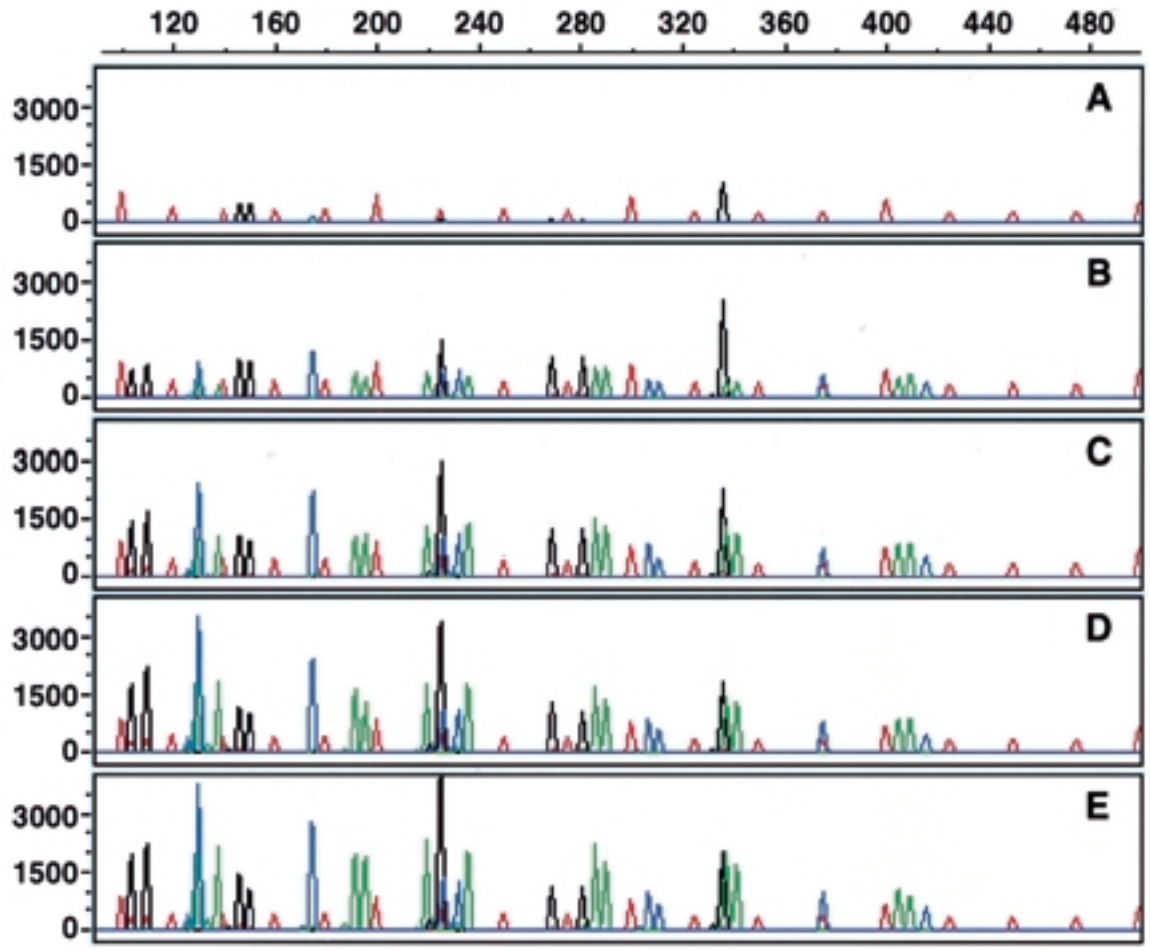

FIG. 7—*Titration of magnesium. Amplifications (10/22 cycling) of 1 ng C-2 genomic DNA is shown here analyzed on the ABI PRISM® 310 Genetic Analyzer. Final concentration of Mg in each reaction is as follows: Panel A is 1 mM Mg, Panel B is 1.25 mM Mg, Panel C is 1.5 mM Mg, Panel D is 1.75 mM Mg, Panel E is 2 mM Mg. The standard final Mg concentration (provided in Gold ST*R 10X Buffer) is 1.5 mM.*

les with ratios between 2:1 and 1:2 (Fig. 10). As the ratios became more extreme, the percentage of minor alleles detected decreased, averaging about 50% at 9:1 and 1:9 and about 17% at 19:1 and 1:19. However, there was a great deal of variation from laboratory to laboratory depending on the sensitivity of their ABI PRISM® 310 Genetic Analyzer, the amount of DNA used, and their RFU cutoff limit.

Analysis Using the ABI PRISM® 377 DNA Sequencer

Most of the developmental work was performed using ABI PRISM® 310 Genetic Analyzers. However, some developmental, validation and non-probative work was performed on the ABI PRISM® 377 DNA Sequencer. The results obtained on the two instruments were similar with the major difference being some locus balance differences. The large loci on the ABI PRISM® 377 DNA Sequencer had consistently higher peak heights than the small loci (data not shown). This difference did not interfere with the interpretation of the data. Laboratories utilizing the ABI PRISM® 377 DNA Sequencer should evaluate different loading volumes (1 μL or 2 μL) to produce preferred signal intensity.

Average Stutter

Stutter has a significant affect on genotype analysis, especially in mixtures (19–21). For this reason, each STR locus in the PowerPlex® 16 System was evaluated for stutter (Fig. 11). Average stutter calculated was similar to a report by Finis (23) with low average stutter being observed in Penta D, Penta E, THO1, and TPOX.

Sizing Precision of ILS 600

The consistency of sizing provided by the ILS 600 size standard was assessed through comparing the sizing of alleles from 13 ladder injections. Sizes of each allele were determined using GeneScan® Software and the Local Southern Method. The sizes were averaged and a standard deviation for each allele was plotted against allele size (bases, Fig. 12). Increased deviation (SD > 0.1 bases) was observed in Penta D, E, FGA, and D18S51 but never exceeded a SD of 0.2 bases. ILS 600 demonstrated the precision across the assay range necessary for sizing off-ladder variants.

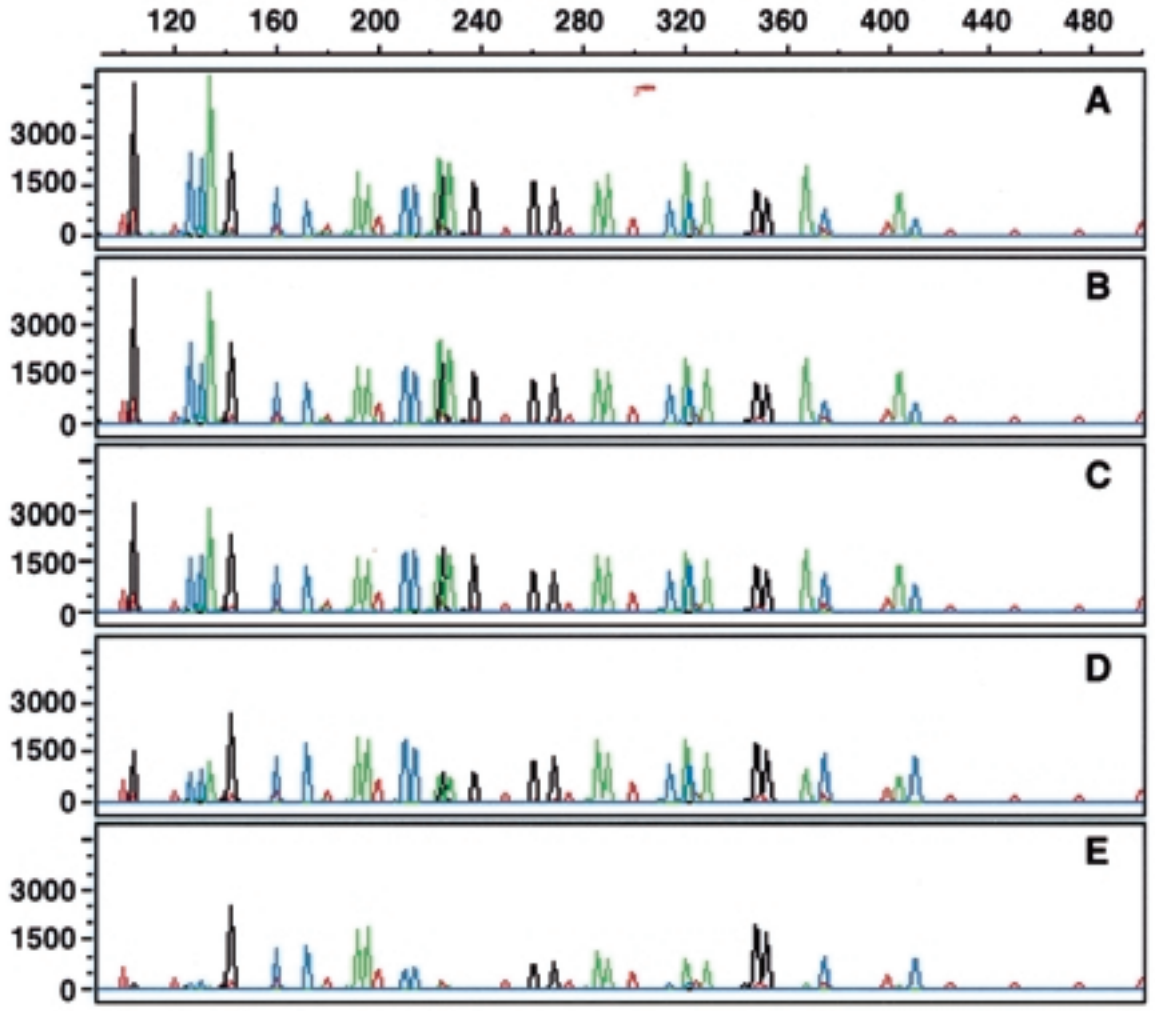

FIG. 8—*Variation of annealing temperature. Amplifications (10/22 cycling) of 1 ng B-10 genomic DNA is shown here analyzed on the ABI PRISM® 310 Genetic Analyzer. Annealing temperature of each reaction is as follows: Panel A is 56°C, Panel B is 58°C, Panel C is 60°C, Panel D is 62°C, Panel E is 64°C. The standard annealing temperature is 60°C.*

TABLE 2—*Heterozygote peak height ratios at various amplification conditions.*

Cycles	Injection Time (s)	1 ng Template		0.5 ng Template		0.25 ng Template	
		Peak Ratio	SD	Peak Ratio	SD	Peak Ratio	SD
10/20	3	0.91	0.08	0.87	0.12	0.84	0.13
10/20	5	0.91	0.09	0.87	0.10	0.85	0.13
10/22	3	0.89	0.08	0.87	0.11	0.83	0.13
10/22	5	0.90	0.09	0.89	0.10	0.82	0.13

Nonhuman Studies

STR analysis of forensic samples relies on the specificity of primers to humans. This prevents confusion when analyzing samples containing animal or microbial DNA. To ensure that the PowerPlex® 16 System demonstrates this specificity, a variety of species were examined. No amplification was seen in mouse, rat, rabbit, chicken, *E. faecalis, E. coli, P. aeruginosa, S. aureus,* Hepatitus B virus, Human Papilloma virus, or *C. albicans* and a low level of amplification was seen in the amelogenin size range for dog and cow (data not shown). Amplification throughout the assay size range was seen in primates (Table 3). Similar results were seen when cross reactive templates were assayed with monoplexes. Except for primates, none of the samples tested of animal, bacteria, or viral origin yielded any amplified products for the STR loci examined. Furthermore, the primates tested did not amplify at all 16 loci and often produced alleles that had migration patterns inconsistent with known human alleles. These results are consistent with results obtained with other forensic STR systems (24).

Standard Specimens

To demonstrate that the sampling method does not alter genotype patterns, liquid blood, dried bloodstain cards (FTA® paper),

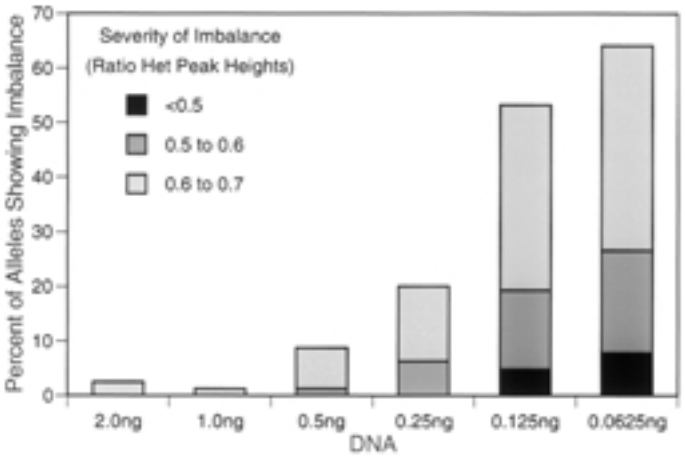

FIG. 9—*Stochastic effect on heterozygote peak heights. Eight laboratories amplified and analyzed the indicated DNA amounts of single source sample B15 using optimized conditions for their ABI PRISM® 310 Genetic Analyzers. The ratio of peak heights of each of the 16 loci (all were heterozygous) were determined and divided into four groups, <0.5, 0.5 to 0.6, 0.6 to 0.7 and >0.7. Peak height ratios below 0.7 are plotted as a function of the amount of DNA amplified. Ratios above 0.7 are considered balanced alleles.*

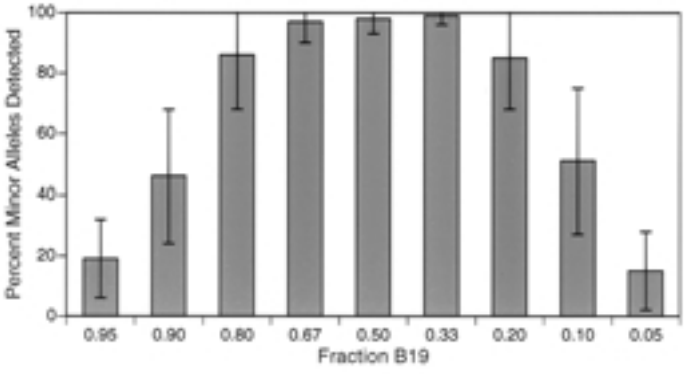

FIG. 10—*Mixture analysis. Fifteen laboratories analyzed mixtures of the single source DNA samples B19 and H9 using optimized conditions determined for each laboratory. Conditions for identifying alleles followed established procedures of each laboratory. The average percentage of the minor alleles detected is plotted as a function of the fraction of B19 DNA. The error bars depict standard deviations, which vary considerably due to variations in the sensitivity of the ABI PRISM® 310 Genetic Analyzers and to different minimum RFU values used.*

and buccal samples were taken from a single subject and genotyped with the PowerPlex® 16 System. Complete, correct, and identical genotypes were obtained from DNA extracted from the three sources (data not shown). Additionally, common database samples were used extensively in the concordance studies for the PowerPlex® 16 System (8,9).

Environmental Studies

Exposure to temperature and sunlight exposure can affect the quality of DNA resulting in degradation of larger templates and thus decreasing yield of larger amplicons (24). To evaluate the PowerPlex® 16 System, DNA was extracted from blood spots that were exposed to summertime light and temperature (Pennsylvania)

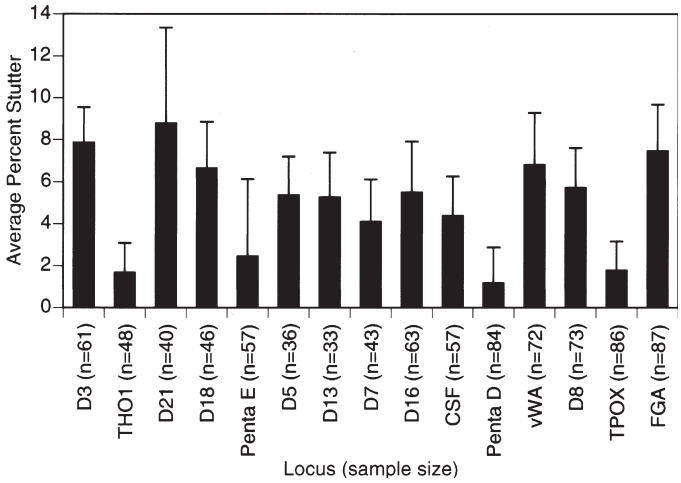

FIG. 11—*Average stutter for each STR locus. The sample size (n) indicates the number of alleles for which stutter was calculated. The bar indicates the average plus one standard deviation.*

and laboratory-controlled temperatures of 80, 50, room temperature, and 4°C. All spots were sampled on a time course of 3, 6, 12, 25, 48, and 85 days. Only samples exposed to 80°C displayed partial loss of genotype with Penta E dropout at 12 days, and additional dropout of CSF1PO, Penta D, and FGA after 48 days. All samples exposed to 50°C or lower temperature and all samples that experienced the light exposure portion of the study produced complete genotypes (data not shown).

Matrix Studies

Presence of polymerase inhibitors in substrate materials can affect the performance of PCR-based assays (24). To evaluate the PowerPlex® 16 System, DNA samples extracted from blood spotted on medium blue denim with a texture of normal blue jeans, a navy blue leather shoe, stacked plywood, glass, a rusty bolt, a rusty pliers, an oily rag, a soiled automotive tire, and a maple tree leaf were analyzed. Complete and correct amplifications were produced on both the ABI PRISM® 310 Genetic Analyzer and ABI PRISM® 377 DNA Sequencer (data not shown).

Non-Probative Case Studies

Acceptance of a new genotyping system necessitates reevaluation of previously typed forensic samples. To evaluate the PowerPlex® 16 System concordance with previously released products, 95 samples from 26 cases collectively were examined by seven laboratories. These analyses included several samples that contained DNA from more than one individual. These non-probative samples had been previously genotyped with RFLP, and Applied Biosystems' DQ/PM, Ampf*ℓ*STR® Profiler Plus™, and/or Ampf*ℓ*STR® COfiler™. Consistent results were found when compared with all previous analyses and all crime scene samples produced genotypes that were consistent with the appropriate reference samples. Heterozygote peak height balance was examined in 80 samples containing a single profile (36 crime scene samples). The average heterozygote peak height ratio was 0.87 with a standard deviation of 0.089 and a range of 0.84–0.91. There was no significant difference in peak height ratio between crime scene samples and reference samples (data not shown).

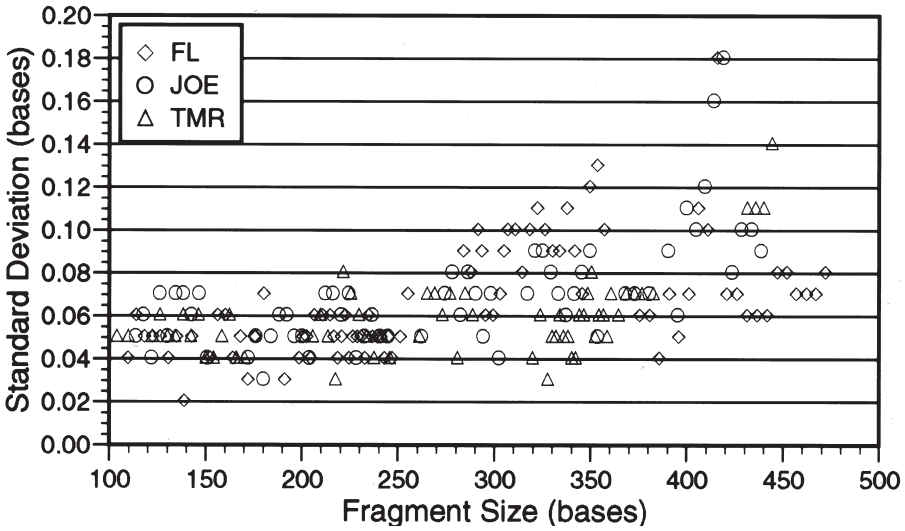

FIG. 12—*Precision of ILS 600. The average fragment size (bases) of each allele was plotted against the standard deviation observed across 13 Allelic Ladder injections.*

TABLE 3—*Cross reactivity of PowerPlex® 16 System primers to higher primates.*

Primate	D3	THO1	D21	D18	Penta E	D5	D13	D7	D16	CSF	Penta D	Amel.	vWA	D8	TPOX	FGA
Orang.-Renari	+	+			+	+	+	+	+		Between	+				+
Orang.-Iri	+	+			+	+	+	+	+		Between	+				+
Orang.-1	+	+			+	+	+	+	+		Between	+				+
Orang.-2	+	+			+	+	+	+	+		Between	+				+
Gorilla-P	+	+				+		+	+	+	+	+	+	+	+	+
Gorilla-K	+	+				+		+	+	+	+	+	+	+	+	+
Gorilla-5	+	+	Between			+		+	+		+	+	+	+	+	+
Chimp.-Teppie	+	Between		+		+	+	+	+	+	+	+	+	+	+	+
Chimp.-Wood		+	+	+		+	+	+	+	+	+	+	+	+	+	+
Chimp.-3	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Chimp.-4	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+

+ = Indicates amplicons migrating in locus size range (bases).
Between = indicates amplicons produced that migrated between the size ranges of two loci.
NOTE: See Fig. 1 for locus size ranges.

Conclusion

Availability of validation data following TWGDAM guidelines reduces the burden of the individual laboratory to repeat a majority of the developmental findings. This allows a focus on determining optimal analysis parameters for each laboratory's instrumentation. The findings of this study, performed with commercially released reagents, suggest that the PowerPlex® 16 System is robust in handling moderate changes to the preferred amplification protocol and to various sample sources. This multiplex system demonstrated a high level of sensitivity in obtaining complete genotypes and is primarily limited by stochastic effects inherent in PCR amplification. Reliability in the performance of the PowerPlex® 16 System was demonstrated by the generation of consistent results both between 24 laboratories using standard samples and within seven laboratories using previously genotyped, non-probative evidence.

Previous studies of this multiplex system documented allele frequencies, characterized the loci, and obtained CODIS/NDIS approval for database and casework samples. This report, in addition to these previous studies, represents assessable documentation necessary to implement the PowerPlex® 16 System's general use in the forensic community.

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dreas, Wisconsin State Crime Laboratory—Madison; Gretchen DeGroot, Daniel Hasse, Dirk Janssen, Wisconsin State Crime Laboratory—Milwaukee.

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