TECHNICAL NOTE

Mark S. Nelson,[1] M.S.; Eleni N. Levedakou,[1] Ph.D.; Janis R. Matthews,[1] B.S.; Buddy E. Early,[1] B.S.; David A. Freeman,[1] Ph.D.; Cynthia A. Kuhn,[1] M.S.F.S.; Cynthia J. Sprecher,[2] B.S.; Ashima S. Amin,[3] B.S.; Kevin C. McElfresh,[3] Ph.D.; and James W. Schumm,[3] Ph.D.

Detection of a Primer-Binding Site Polymorphism for the STR Locus D16S539 Using the Powerplex® 1.1 System and Validation of a Degenerate Primer to Correct for the Polymorphism

REFERENCE: Nelson MS, Levedakou EN, Matthews JR, Early BE, Freeman DA, Kuhn CA, Sprecher CJ, Amin AS, McElfresh KC, Schumm JW. Detection of a primer-binding site polymorphism for the STR locus D16S539 using the PowerPlex® 1.1 system and validation of a degenerate primer to correct for the polymorphism. J Forensic Sci 2002;47(2):345–349.

ABSTRACT: Quality assurance samples submitted from the NCSBI as part of a contract with TBTG to outsource DNA Database samples showed unexpected discrepancies for the locus D16S539 when all other loci yielded identical results. Discrepancies observed included allele drop out and an imbalance in sister alleles with samples returned from TBTG. This led to a comprehensive review of the technical procedures used between the two laboratories to determine the cause of the discrepancies noted for the locus D16S539, since both laboratories were using the PowerPlex® 1.1 typing kit from the Promega Corporation. The NCSBI and the TBTG utilize different extraction methods (organic extraction vs. FTA) and amplification conditions (AmpliTaq® vs AmpliTaq Gold®), respectively, so the exact cause of discrepancy observed was not immediately apparent. Experiments at the NCSBI associated the observed allele drop out and the imbalance of the sister alleles with the use of AmpliTaq Gold® and a hot start procedure.

Sequencing data revealed that a point mutation resides on the D16S539 primer-binding site that reaches polymorphic levels in African-American populations. This led to the development of a degenerate primer by the Promega Corporation to detect "missing" alleles when AmpliTaq Gold® is used. The degenerate primer was then thoroughly tested to show its efficacy in detecting the "true" D16S539 profile when used.

KEYWORDS: forensic science, short tandem repeat, PowerPlex® 1.1, multiplex, polymerase chain reaction, DNA typing, forensics, polymorphism, primers, point mutation, D16S539, primer sequence

Several quality assurance samples, samples previously typed in-house by the North Carolina State Bureau of Investigation (NCSBI), were submitted to The Bode Technology Group (TBTG) as part of a contract to outsource backlogged DNA Database samples at the NCSBI. Some of these quality assurance samples showed an unexpected discrepancy between the two laboratories for the locus D16S539, when all other 13 loci tested yielded identical results using identical primer pairs found in the PowerPlex® 1.1 and 2.1 (Promega Corporation, Madison, WI) systems. These discrepancies included imbalanced sister alleles and allele dropout for results returned from TBTG.

These findings led to an examination of the procedures used between the two laboratories in an effort to explain the discrepancy seen at the D16S539 locus. The NCSBI laboratory uses an organic extraction of the bloodstain (1), followed by amplification using PowerPlex® 1.1 primers and AmpliTaq® polymerase (Applied Biosystems, Foster City, CA). TBTG uses an FTA® extraction method (2) on bloodstains, followed by amplification using PowerPlex® 1.1 primers and AmpliTaq Gold® polymerase (Applied Biosystems).

Despite the differences in extraction procedures between the two labs, the NCSBI was able to reproduce the results from TBTG when AmpliTaq Gold® was used. Therefore, sample extraction procedures were ruled out as a potential source of the discrepant results at D16S539, pointing to differences in amplification conditions as the cause of the discrepancy. The AmpliTaq Gold® procedure uses a higher stringency "hot start" and all steps of the amplification cycle are lengthened as compared to an AmpliTaq® procedure. For these reasons, there is a potential for destabilizing the primer if a point mutation were to occur at the primer-binding site. This hypothesis could be tested by sequencing samples that gave discrepant results between the two laboratories. The Promega Corporation sequenced samples identified as giving discrepant results and found a point mutation on the D16S539 primer-binding sequence for the locus D16S539 as the cause of the discrepant results.

Primer-binding site mutations or "null alleles" have been reported for other STR loci used in forensic testing. Schumm et al.

[1] North Carolina State Bureau of Investigation (NCSBI), Raleigh, NC.
[2] Promega Corporation (PC), Madison, WI.
[3] The Bode Technology Group (TBTG), Springfield, VA.

Received 18 March 2001; and in revised form 13 June 2001; accepted 11 July 2001.

(3) described primer-dependent allele dropout for the STR locus D7S820, which was traced to a sequence polymorphism in the primer-binding region resulting in imperfect hybridization of the primer and subsequent allele dropout. The solution they used was to redesign the primer pair to amplify a larger region of the D7S820 locus. In another report, Kline et al. (4) reported a sample tested where the PowerPlex1.1® kit yielded a vWA phenotype of 16, 19, but the AmpFℓSTR® Blue kit (Applied Biosystems) yielded a vWA phenotype of 16. Walsh (5) reported that a sequence polymorphism near the 3′-end of the forward primer was the reason that the vWA 19 allele dropped out with the AmpFℓSTR® Blue kit.

The finding of point mutations that reach polymorphic levels in primer-binding sequences in core CODIS (Combined DNA Index System) loci is of concern to forensic scientists using CODIS. Even though CODIS matching algorithms allow for mismatches at low and moderate stringency (nonperfect matches are revealed i.e., a two-banded pattern at a particular locus will be returned as a match to a locus with a single band) to handle rare mutations, scientists using CODIS must be aware of the consequences of primer-binding site mutations, especially those that reach polymorphic levels in a population, as well as the consequences of using different primer pairs from different sources.

Materials and Methods

NCSBI

DNA was extracted from bloodstains prepared on S & S 903 paper (Schleicher & Schuell, Keene, NH) using either organic extraction (1) or the FTA extraction method (2) as described in Levedakou et al. (personal communication). DNA samples were quantitated using the Quanti-Blot procedure (Applied Biosystems). One ng of template DNA per 25 μL reaction was used for amplification according to the procedure outlined in the Technical Manual—PowerPlex® 1.1 System (6) supplied by the Promega Corporation. Amplification was carried out in Perkin Elmer 9600 or 9700 thermocyclers (Applied Biosystems). The conditions using Ampli Taq® are as follows (6):

96°C for 1 min, then:
94°C for 30 s
Ramp to 60°C, hold for 30 s
Ramp to 70°C, hold for 45 s
For 10 cycles, then:
90°C for 30 s
Ramp to 60°C, hold for 30 s
Ramp to 70°C, hold for 45 s
For 20 cycles, then:
60°C for 30 min, then:
4°C for infinite hold.

The amplification conditions using AmpliTaq Gold® are as follows (Levedakou et al., personal communication):

95°C for 11 min
96°C for 2 min, then:
94°C for 60 s
Ramp to 60°C, hold for 60 s
Ramp to 70°C, hold for 90 s
For 10 cycles, then:
90°C for 60 s
Ramp to 60°C, hold for 60 s
Ramp to 70°C, hold for 90 s
For 20 cycles, then:
60°C for 30 min, then:
4°C for infinite hold.

The differences between the two amplification procedures lie in the extended hot start (95°C for 11 min and at 96°C for two min) prior to the amplification cycling steps, as well as the extended length of the cycling steps when using AmpliTaq Gold®.

Samples were amplified using the following primers provided by the Promega Corporation:

1. Primer mixture provided in the PowerPlex® 1.1 kit (which includes D16S539)
2. A monoplex D16S539 (which would amplify only the D16S539 locus) that was designed for PowerPlex® 16
3. A degenerate D16S539 primer designed by the Promega Corporation as part of this study. A degenerate primer is identical to the primary primer sequence, except that there is one or more base substitutions to cover point mutations, which have been detected at the primer-binding sequence. This degenerate primer was added to the primer mixture of the PowerPlex® 1.1 kit.

Samples were then denatured for 2 min at 95°C and analyzed by gel electrophoresis using a 5% Long Ranger® gels (BioWhittaker Molecular Applications, Rockland, ME), and a Tris- Borate-EDTA electrophoresis buffer (7). At the end of electrophoresis, the gels were scanned on a Hitachi FMBIO® II (MiraiBio Inc., Alameda, CA) platform and analyzed by StarCall software (MiraiBio, Inc.).

Promega Corporation

Primers that flank the D16S539 primer sequences in the PowerPlex® 1.1 Kit were designed to amplify and sequence genomic DNA samples that showed allele dropout, as follows:

D16S539 Forward Sequencing Primer
TGACTGTGTTCCCACTCTCAG

D16S539 Reverse Sequencing Primer
GCCATAGACTTAAAAACCTAATGACA

Amplification—Using the primers above, amplification was performed in 1X GoldST★R® Buffer (Promega Corporation), 1 μM primer, 0.25 units AmpliTaq Gold® (Perkin Elmer), and approximately 1 ng template in a 25 μL total reaction volume. Cycling was performed using a GeneAmp® PCR System 9600 Thermal Cycler (Perkin Elmer) following the cycling conditions for PowerPlex® 1.1 (6) including a 95°C 11 min initial incubation step.

DNA Purification—Genomic DNA amplification products were separated by electrophoresis through a 0.4 mm thick 6% denaturing polyacrylamide gel, containing 0.5X TBE and 7M urea, following protocols outlined in the *GenePrint*® STR Systems (Silver Stain Detection) Technical Manual TMD004 (Promega Corporation). Silver stained DNA bands (alleles) in acrylamide were scraped off the fixed, stained, polyacrylamide gel. The gel containing the alleles was soaked overnight at room temperature in TE (10 mM Tris pH 7.5, 1 mM EDTA), then microcentrifuged in ULTRAFREE®-MC 0.45 μm filter units (Millipore, Bedford, MA). The purified alleles were amplified following the same cycling protocol (see above) for genomic DNA except in 100 μL reaction volumes using 1X GoldST★R® Buffer, 1 μM primer, and 1 unit AmpliTaq Gold®. Following amplification, the excess primers were removed using Wizard® PCR Preps DNA Purification System (Promega Corporation).

DNA Sequencing—Sequence analysis was completed with four of the samples displaying an allele-deficient (allelic drop out/imbalance in sister alleles) D16S539 genotype and two samples that did not display the allele-deficient D16S539 genotype for a total of twelve alleles. Sequencing was performed using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems), 8 μL Terminator Ready Reaction Mix (Applied Biosystems), 3.2 pm primer, and 200 to 500 ng template in a 20 μL total reaction volume. Cycling was performed in a GeneAmp® PCR System 9600 Thermal Cycler: 5 min at 95°C, then 30 cycles of 95°C for 30 s, 50°C for 10 s, and 60°C for 4 min. Sequencing reactions were purified using Centri-Sep™ spin columns (Princeton Separations, Adelphia, NJ) and detection was done on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) using a 47-cm capillary, POP6 polymer, and run module "Seq POP6 (1 mL) Rapid E" (all from Applied Biosystems).

TBTG

All DNA samples submitted to TBTG for outsourcing were extracted using an FTA extraction procedure (2) and were originally amplified using AmpliTaq Gold® and the PowerPlex® 1.1 System (6). The subsequent products were analyzed as described (6) using a Hitachi FMBIO® II platform and the StarCall software. In subsequent testing by TBTG, 423 samples were amplified using AmpliTaq Gold® and the AmpFℓSTR® COfiler™ PCR Amplification Kit (Applied Biosystems) to determine if identical results for the locus D16S539 were obtained using the AmpFℓSTR® COfiler™ and the PowerPlex® 1.1 kits. Samples displaying a weak or missing allele at the locus D16S539 were also amplified using a prerelease version of the PowerPlex® 16 System primer mix (Promega Corporation, Madison, WI) containing the D16S539 primer 5′-GTTTGTGTGTGCATCTGTAAGCATGTATC-3′ (see Fig. 1) which is identical with the monoplex D16S539 primer used for this study. Amplified products were separated and detected using the ABi Prism® 3700 Genetic Analyzer (Applied Biosystems) and analyzed using Genotyper Version 3.6 software (Applied Biosystems).

Results and Discussion

To determine if a difference in amplification was responsible for the discrepancy observed at the D16S539 locus for some samples, 210 apparently homozygous D16S539 results returned by TBTG were retested by the NCSBI using both AmpliTaq® and AmpliTaq Gold® amplification conditions and PowerPlex® 1.1 primers. Using AmpliTaq®, 26 of the 210 apparent homozygotes returned from TBTG were identified as heterozygotes by the NCSBI, a rate of 12.4%. When these same samples were amplified with AmpliTaq Gold®, the NCSBI noted either an imbalance in sister alleles or a dropout of one band (allele), when compared with the AmpliTaq® amplification procedure. The NCSBI also conducted experiments where AmpliTaq Gold® was used with the amplification conditions for AmpliTaq® described in the Materials and Methods and the same genotypes were returned as with the amplification conditions using a "hot start" and longer annealing and extension times.

These same 210 samples were sent to TBTG, where they were amplified using the AmpFℓSTP® COfiler™ PCR Amplification Kit with AmpliTaq Gold® polymerase. TBTG also tested those samples that displayed missing or weak alleles for the locus D16S539 using a prerelease version of the PowerPlex® 16 System

Figure 1A

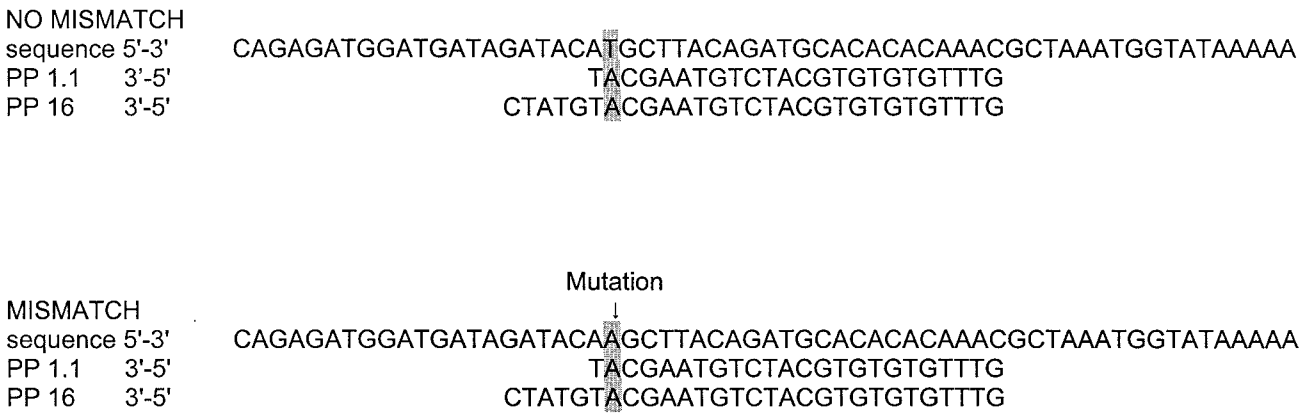

Figure 1B

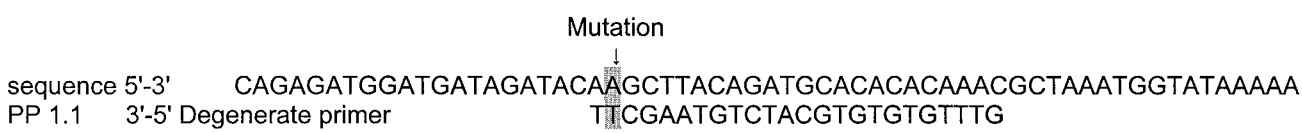

FIG. 1—*Sequencing data showing the region of the D16S539 primer-binding site harboring the mutation. A—The D16S539 primer sequence of PowerPlex® 1.1 (PP1.1), the D16S539 primer sequence developed for PowerPlex® 16 (PP16), and the binding location, when the "wild type" sequence is encountered (no mismatch), and when the mutation occurs (mismatch). B—Sequence of the degenerate primer developed to anneal with the D16S539 region carrying the primer-binding site mutation.*

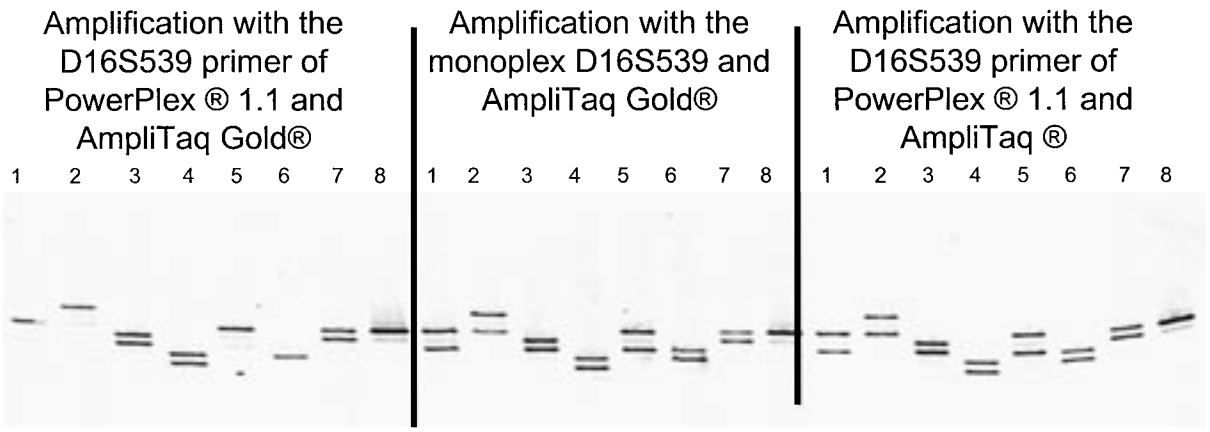

FIG. 2—*D16S539 amplification results for the same set of samples (1 to 8) using two different primers and two different DNA polymerases, which demonstrate allelic drop out when PowerPlex® 1.1 primers are used with AmpliTaq Gold®. Samples 1, 2, 5, and 6 were identified from this study as giving discrepant D16S539 results, whereas Samples 3 and 4 gave consistent results between two different labs. Sample 7 is the cell line 9947A and Sample 8 is a control sample used by the Promega Corporation.*

primer mix and AmpliTaq Gold® polymerase. The D16S539 primer in the prerelease version of the PowerPlex® 16 System is different from the D16S539 primer in the PowerPlex® 1.1 system, but has identical sequence with the monoplex D16S539 primer used in this study (see Fig. 1 for details). Identical results were noted for these two tests to those obtained by the NCSBI with the AmpliTaq® procedure and PowerPlex® 1.1 primers.

The data generated led the NCSBI and TBTG to surmise that a primer-binding site polymorphism might exist at D16S539, and the Promega Corporation was notified of these results. Further testing by the Promega Corporation confirmed the findings of the NCSBI and TBTG as demonstrated in Fig. 2 and Table 1.

Sequencing results generated by the Promega Corporation for samples that showed D16S539 anomalies established that there was a T to A mutation corresponding to the next to the last base in the 3′ primer-binding sequence for these samples (see Fig. 1A). Apparently, under the AmpliTaq Gold® amplification procedures, the D16S539 3′ primer in the PowerPlex® 1.1 kit becomes sufficiently unstable. As Fig. 1A also demonstrates, the monoplex D16S539 primer designed for PowerPlex® 16, has an additional five bases at the 3′ end that helps to stabilize the primer under the AmpliTaq Gold® amplification conditions.

The Promega Corporation then designed a degenerate primer to be added to the PowerPlex® 1.1 primers to compensate for the sequence mutation detected when AmpliTaq Gold® amplification procedures are used. The sequence of this new primer is shown in Fig. 1B where a T has been placed at the next to last base position of the 3′ end of the primer.

In order to have a large enough sample size to test the efficacy of the degenerate primer and to determine the extent of the mutation in the population, the NCSBI then screened another 213 apparently homozygous samples returned from TBTG using PowerPlex® 1.1 primers with AmpliTaq® and AmpliTaq Gold® amplification procedures. This study detected another 16 individuals who have the polymorphism, or 7.5% of the population of samples screened. These same samples were also tested by TBTG using PowerPlex® 16, as well as the primer set in the AmpFℓSTR® COfiler™ PCR Amplification Kit and AmpliTaq Gold® amplification procedures, and found to return concordant results to those returned by the NCSBI.

The next phase of the study was to validate the efficacy of the degenerate D16S539 primer. All 423 samples in the combined test set (of which 42 displayed allele drop out or sister allele imbalance) were then tested by the NCSBI using AmpliTaq Gold® polymerase with both the degenerate D16S539 primer added to the PowerPlex® 1.1 primers, and the monoplex primer for D16S539 developed for the prerelease version of the PowerPlex® 16 system. The D16S539 primer has not changed between the prerelease and final version of the PowerPlex® 16 system. Both primer sets yielded identical results to those originally developed using PowerPlex® 1.1 primers and the AmpliTaq® procedure. It can be noted that samples having the D16S539 primer-binding site mutation when tested with the PowerPlex® 1.1 primers and AmpliTaq Gold® amplification procedures at the NCSBI, most often resulted in an imbalance of sister alleles instead of allelic drop out.

TABLE 1—*Genotypes of the samples depicted in Fig. 2.*

Genotype with the Monoplex D16S539 Primer from PowerPlex® 16 using AmpliTaq® Gold and with PowerPlex® 1.1 using AmpliTaq®	Genotype with PowerPlex® 1.1 using AmpliTaq® Gold	Sample # on Gel
10, 12	12	1
12, 14	14	2
10, 11	10, 11	3
8, 9	8, 9	4
10, 12	12	5
9, **10**	9	6
11, 12	11, 12	7
12, 12	12, 12	8

NOTE: Bold numbers indicate the new allele.

The samples in this study were chosen because they were apparent homozygotes and came from a sequential block of samples sent to TBTG for DNA testing. This group of samples included individuals primarily of Caucasian and African-American origin. However, samples from individuals identified as Lumbee Indians and Hispanics were also present but in such low numbers that no inferences can be drawn as to the frequency of the primer-binding site mutation in these two populations.

A total of 429 Caucasians were represented in this block of samples of which 79% were typed as heterozygotes for D16S539 by

TBTG, and 21% were typed as homozygotes. All those apparent homozygotes were retested and only one individual was found to have the primer-binding site mutation. From this sample set, one can assign a frequency of the D16S539 primer-binding site mutation of approximately 0.002 in the Caucasian population, which is a rare event.

A total of 1310 African-American individuals were represented in this block of samples, of which 77% were typed as heterozygotes for D16S539 by TBTG, and 23% were typed as homozygotes. All those apparent homozygotes were retested and 41 individuals were found to have the primer-binding site mutation. From this sample set, one can assign a frequency of the D16S539 primer-binding site mutation of 0.0313 to the African-American population. Any frequency above 0.01 is considered polymorphic in a population (8).

Conclusions

Many laboratories over the past few years have switched from using AmpliTaq® to AmpliTaq Gold®. AmpliTaq Gold® allows better timing of the initiation of the amplification, giving analysts time to set up large numbers of samples and increase efficiency, as well as increasing the yield of PCR products (9).

This study shows the importance in thoroughly characterizing the performance of primer sets prior to changing amplification parameters or DNA polymerases. It is also very important that a significant number of individuals from different racial groups be tested when designing primers to avoid polymorphic regions for primer-binding sites.

It is expected that single base mutations will present themselves on rare occasions in the population, and that occasionally they will occur at primer binding sequences, resulting in imbalanced sister alleles or allelic dropout. The use of STR markers for comparison of convicted offender samples with forensic case samples is the heart of the national DNA Database (CODIS). The matching algorithm in CODIS is capable of dealing with one vs. two band "matches" at moderate stringency so that samples affected by primer-binding site mutations would still be candidates for a match. For example, if one obtained a 12 loci high stringency (perfect) match and one moderate stringency locus match, further studies would be necessary, and one possible explanation would be the presence of a primer-binding site mutation. This would be especially true if the two "matching" DNA profiles were generated in different labs using different primer sets or DNA polymerases. The ideal situation would be for all labs to return identical results based on the use of identical primer sets. Although rare primer-binding site mutations are expected to appear in a population, the finding of a polymorphic binding site in a racial/ethnic group is less than ideal in a national database. For this reason, the development of a degenerate primer for the locus D16S539 was necessary to be used with the PowerPlex® 1.1 kit and AmpliTaq Gold® polymerase. This degenerate primer will also be added by the manufacturer to future PowerPlex® 1.1 kits. This study has validated the efficacy of this primer in providing identical results to those obtained for other D16S539 primer sets currently available on the market.

This study can also serve as a model to be followed, should further polymorphic primer-binding site mutations appear in either current or new mega-plex DNA testing kits used in forensic testing.

References

1. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a Laboratory Manual. Cold Spring Harbor Laboratories Press, Cold Spring Harbor, 1989.
2. Eisenberg AJ, Hochmeister MN, Hammer L, Hausherr C, Burgoyne L, Sherman JM. High throughput automated DNA sample analysis for both RFLP and PCR using FTA™ paper and the Rosys Plato robotic microplate processor. Proceedings from the First European Symposium on Human Identification. Promega Corporation;1996;20.
3. Schumm JW, Lins AM, Micka KA, Sprecher CJ, Rabbach DR, Bacher JW. Fluorescent detection of STR multiplexes—development of the GenePrint TM PowerPlex TM and FFL Multiplexes for forensic and paternity applications. Proceedings from the Seventh International Symposium on Human Identification, Promega Corporation;1997;70–88.
4. Kline MC, Jenkins B, Rogers S. Non-amplification of a vWA allele. J Forensic Sci 1998;43:250.
5. Walsh PS. Commentary on Kline MC, Jenkins B, Rogers S. Non-amplification of a vWA allele. J Forensic Sci 1998;43:1103–4.
6. Promega Corporation Technical Manual—PowerPlex® 1.1 System. Promega Corporation, Madison, WI, 1999.
7. Promega Corporation Technical Manual—PowerPlex® 2.1 System. Promega Corporation, Madison, WI, 2000.
8. Wang DG, Fan JB, Siao CJ, Berno A, Young P, Sapolosky R, et al. Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. Science 1998;280:1077–82.
9. Moretti T, Koons B, Budowle B. Enhancement of PCR amplification yield and specificity using AmpliTaq Gold TM DNA polymerase. BioTechniques 1998;25(4):716–22.

Additional information and reprint requests:
Mark S. Nelson
North Carolina State Bureau of Investigation
Molecular Genetics Section
121 E. Tryon Rd., Raleigh, NC 27603
Ph: (919) 662-4509 ext. 2527
E-mail: Mnelson@mail.jus.state.nc.us