J Forensic Sci, November 2007, Vol. 53, No. 6
doi: 10.1111/j.1556-4029.2008.00881.x
Available online at: www.blackwell-synergy.com

TECHNICAL NOTE

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An Updated Validation of Promega's PowerPlex® 16 System: High Throughput Databasing Under Reduced PCR Volume Conditions on Applied Biosystem's 96 Capillary 3730xl DNA Analyzer*

ABSTRACT: The PowerPlex® 16 System from Promega Corporation allows single tube multiplex amplification of sixteen short tandem repeat (STR) loci including all 13 core combined DNA index system STRs. This report presents an updated validation of the PowerPlex® 16 System on Applied Biosystem's 96 capillary 3730xl DNA Analyzer. The validation protocol developed in our laboratory allows for the analysis of 1536 loci (96×16) in *c.* 50 min. We have further optimized the assay by decreasing the reaction volume to one-quarter that recommended by the manufacturer thereby substantially reducing the total cost per sample without compromising reproducibility or specificity. This reduction in reaction volume has the ancillary benefit of dramatically increasing the sensitivity of the assay allowing for accurate analysis of lower quantities of DNA. Due to its substantially increased throughput capability, this extended validation of the PowerPlex® 16 System should be useful in reducing the backlog of unanalyzed DNA samples currently facing public DNA forensic laboratories.

KEYWORDS: forensic science, DNA typing, validation, PowerPlex® 16, reduced PCR volume, 3730xl DNA analyzer, combined DNA index system

Short tandem repeat (STR) loci are widespread throughout the human genome and show sufficient variability among individuals within populations that they have become important in several fields, including linkage analysis (1), human identity testing (2), and evolutionary studies (3). The combined DNA index system (CODIS) established by the Federal Bureau of Investigation (FBI) constitutes the core of the United States DNA database. The 13 STR loci comprising CODIS are used extensively by both forensic scientists and population geneticists and are integral components of several commercially available human identity testing kits. We were interested in applying STR analysis to ongoing population genetics studies in our laboratory that required the high throughput capability of the 96 capillary 3730xl DNA analyzer. None of the commercially available human identity testing kits, however, had been validated for use on this particular platform. The AmpFlSTR Profiler Plus™ and COfiler® multiplex systems (Applied Biosystems, Foster City, CA) which together amplify all 13 CODIS loci were previously validated for use on the single capillary ABI PRISM® Genetic Analyzer 310 (4). Higher throughput was obtained on the 16 capillary ABI PRISM® 3100, though these validations were performed on male-specific multiplex amplification kits (5,6). Koumi et al. (7) included the 96 capillary ABI PRISM® 3700 in an evaluation and validation study comparing the performance of various capillary electrophoresis platforms to the ABI PRISM® slab gel format for use with the STR multiplex AmpFlSTR SGMplus. The PRISM® 3700 however was officially discontinued by Applied Biosystems in January 2001 and replaced by the next generation 3730 and 3730xl. Promega's PowerPlex® 16 System incorporates the 13 core CODIS STR loci in addition to two pentameric loci and the Amelogenin sex-discriminating locus into a single-tube multiplex PCR. As originally validated, PCR products from a 25 μL PowerPlex® 16 amplification reaction are analyzed on the ABI single capillary PRISM® 310 Genetic Analyzer (8,9). In this study, we sought not only to increase the throughput capability of the PowerPlex® 16 System by adapting it to a 96 capillary format but also to decrease the total cost per sample by reducing the manufacturer's recommended reaction volume. STR multiplex amplifications under reduced PCR volumes have previously been reported; however, these reactions were analyzed on ABI PRISM 377 slab gel systems (10–12) or the single capillary 310 (9).

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*Poster presented at the 76th annual meeting of the American Association of Physical Anthropologists, Milwaukee, WI, 2007.
Received 28 Oct. 2007; and in revised form 1 Mar. 2008; accepted 19 Apr. 2008.

Materials and Methods

DNA Samples

Male 9948 and female 9947A DNA standards were obtained from Promega (Promega Corporation, Madison, WI) as components of the PowerPlex® Y (Lot # 216559) and PowerPlex® 16 (Lot #s 218993, 222284) kits respectively. The Genomic DNA standards

1–10 were purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD) as Standard Reference Material (SRM) 2391b. DNA sample CC was extracted from human blood using the silica dioxide method as previously described (13) and quantified on a NanoDrop® ND-3300 flourospectrometer (NanoDrop Technologies, Wilmington, DE). The field samples were collected as buccal cells via mouthwash rinse (14) and extracted and quantified as above.

PCR Components

The PowerPlex® 16 System includes all the components necessary for STR amplification with the exception of DNA polymerase. In addition, the kit provides reagents necessary for post-PCR analysis including an allelic ladder and the ILS600 internal lane standard. Amplifications were performed with Platinum® Taq (Invitrogen Corporation, Carlsbad, CA).

PCR Amplification and Thermal Cycling Parameters

For the PCR reagent titration, template DNA (9947A, 9948, or CC) was amplified in 25 μL (full), 12.5 μL (half) and 6.25 μL (quarter) reactions. For this and all subsequent experiments samples were amplified in MicroAmp® reaction tubes (Applied Biosytems) in a GeneAmp® PCR system 9700 thermocycler (Applied Biosystems). Cycling parameters employing a 10/19 profile (for a total of 29 cycles) were according to the PowerPlex® 16 System Technical Manual (part #TMD012) with the exception that the final extension step was increased to 45 min. All remaining experiments in the validation study were performed in quarter reaction volumes.

Sample Electrophoresis, Bin Settings, and Data Analysis

The PowerPlex® 16 System employs a three-dye set system consisting of carboxy-tetramethylrhodamine (TMR), flourescein (FL), and 6-carboxy-4′, 5-dichloro-2′, 7′dimethoxyflouroscein (JOE). PCR products were separated and detected as follows. One microliter of amplification reaction was mixed with 9.5 μL of Hi-Di™ formamide (Applied Biosystems) and 0.5 μL of carboxy-X-rhodamine (CXR) labeled internal lane standard ILS600, transferred to the wells of a MicroAmp™ optical 96 well reaction plate (Applied Biosystems) and loaded onto the 3730xl DNA Analyzer equipped with a 50 cm, 96 capillary array. Included in each run was a well containing 1 μL PowerPlex® 16 allelic ladder mix. Samples were injected for 5 sec at 3 V and electrophoresed for 50 min in Performance Optimized Polymer (POP-7™) at 60°C. All other run parameters were left at default. Data were collected using ABI Foundation Data Collection software version 3.0 and analyzed with Genemapper® version 4.0 (Applied Biosystems). Allele peaks were interpreted when ≥100 relative fluorescent units (rfu) for the sensitivity studies and 200 rfu for all other experiments. Allelic cutoff for heterozygous alleles was set to 0.2 and stutter peaks were filtered according to the locus specific ratios determined in the original developmental validation study (9).

PowerPlex® 16 allelic ladder peak sizes from five injections were averaged and the bin locations set up with a left and right offset of 0.5 base pairs. Heterozygous locus peak balance was determined by dividing the lower peak intensity of a heterozygous locus by the higher. Intracolor peak balance was calculated by dividing the lowest peak height for a given dye by the highest. Both sets of values are expressed as percentages.

Results and Discussion

Kit Titration

Our first objective was to determine if the PowerPlex® 16 System could be made more cost-effective by the scaling down of reagents. To this end amplifications were performed in full, half, and quarter reaction volumes while keeping input DNA (male 9948 standard) at 1 ng. In three individual amplifications, full profiles without spurious peaks were obtained with all three reaction volumes. In addition, decreasing reaction volume while keeping input DNA constant resulted in the increase of signal intensity at all loci (Fig. 1). On average, signal intensity increased sixfold when reaction volume was decreased a quarterfold. This result is to be expected as decreasing the reaction volume while maintaining input DNA constant effectively increases the template concentration thereby resulting in more product per unit volume. Concomitant with this increase in signal intensity we observed no adverse affect on heterozygous peak ratios with decreasing reaction volume. As indicated in Fig. 2, heterozygous peak ratios did not fall below 65% and averaged above 80% for all three reaction volumes. The effects of varying the standard 25 μL PCR reaction volume was also studied in the PowerPlex® 16 validation by Krenke et al. (9). As stated by the authors, in volumes ranging

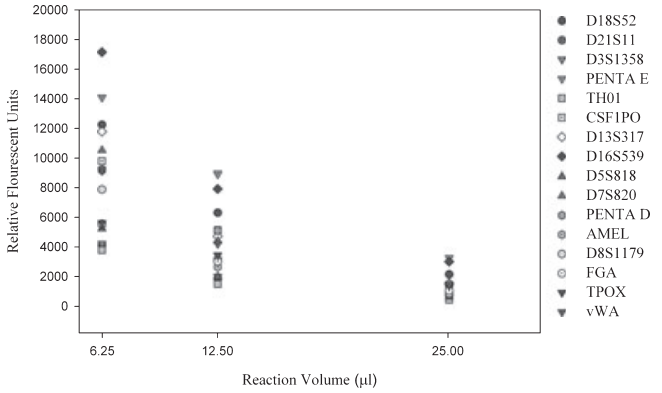

FIG. 1—*Peak intensities of three reaction volumes. The average peak heights for all 16 PowerPlex® 16 System loci from three amplifications of 1 ng of male 9948 DNA standard in the indicated reaction volumes.*

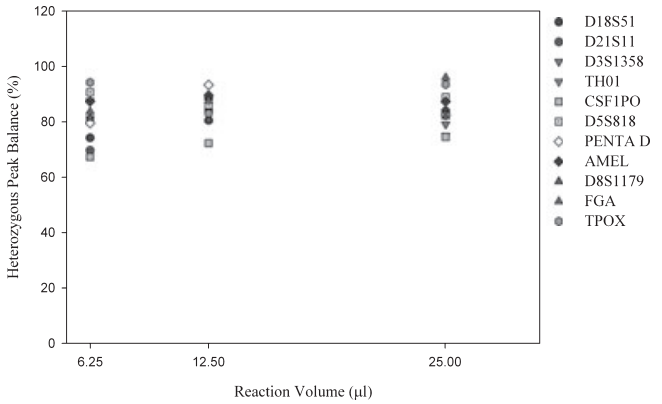

FIG. 2—*Heterozygous locus peak balance of three reaction volumes. Average (n = 3) peak ratios were determined for the 11 heterozygous loci of the 9948 male DNA standard amplified from 1 ng DNA in the indicated reaction volumes.*

from 5 to 50 μL the concentration of input template was kept constant (0.02 ng/μL) to reduce background and saturation of the charge coupled device (CCD) pixels which tend to occur on the ABI PRISM® 310 Genetic Analyzer with high template concentrations. Employing the 3730xl, it was unnecessary to similarly maintain the DNA concentration below a certain level, and we did not observe any pull down/push up phenomena in the full, half, or quarter reaction amplifications (0.04, 0.08, and 0.16 ng/μL template, respectively) thereby demonstrating the greater dynamic range of this platform as compared to the 310. Krenke also noted a concomitant increase of imbalance at heterozygous loci accompanying reduction in reaction volume; however, heterozygous peak ratios were not reported thus making it difficult to directly compare this aspect of reaction volume reduction to our results. Intracolor peak balance was low, consistently surpassing 50% only at the TMR labeled loci. The original PowerPlex® 16 validation did not include an analysis of locus to locus balance, therefore it is not possible to perform a comparison with our results. Furthermore, to our knowledge there have been no studies to date evaluating locus to locus balance on the 3730xl platform. Limiting locus to locus imbalance is most essential in mixture studies intrinsic to forensic case work analyses. However, as the purpose of this study is the validation for standard single source databasing, maintaining locus to locus balance is not deemed to be as critical a factor.

Sensitivity

To determine the detection limits of single source DNA in the quarter reaction volume, amplifications were performed using input DNA (9947A and 9948) template levels of 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, and 0.039 ng, respectively (Fig. 3). Full profiles were obtained with all input templates down to 0.078 ng. At this minimum amount of template DNA, complete and accurate profiles peaks were readily detectable; remaining above 100 rfu and well above background with no allelic dropout observed (Fig. 4). Intralocus peak imbalance, especially pronounced between homozygous and heterozygous loci, does not interfere with accurate allele calling and as mentioned earlier would not be problematic for databasing purposes. Allelic dropout was detected with 0.039 ng DNA, but was not extensive occurring only at a single locus for both of the DNA samples analyzed. At this lowest amount of DNA template, we also detected a spurious peak at D5S818 in the female control sample leading to a heterozygous miscall at this locus. These sensitivity data are similar to results obtained by Krenke et al. who reported obtaining complete genotypes using 0.0625 ng input DNA.

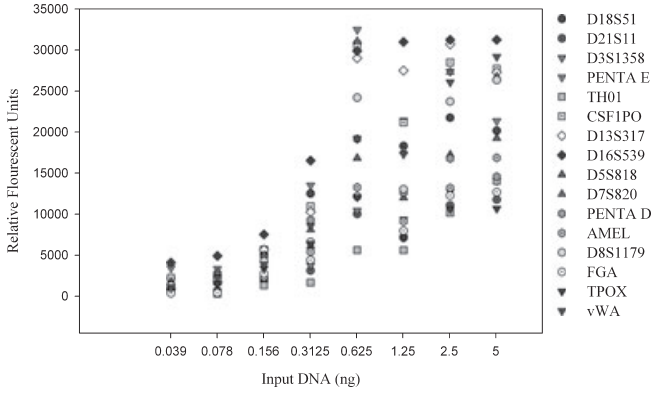

FIG. 3—*Peak intensities as a function of DNA template quantity. Serially diluted DNA template 9948 was amplified and peak heights plotted as a function of input DNA template.*

Peak saturation was first detected at relative fluorescent intensities of 27,000 and above, but not all peaks exceeding this rfu were flagged by the GeneMapper® software as off-scale. Flagged off-scale peaks resulting in bleed-through also exhibited split-tops characteristic of incomplete adenylation due to excess DNA template in the amplification reaction and so are visually identifiable. Bleed-through spurious peaks with rfu above the preset cutoff occur at the same size location as the saturated peak and are similarly tagged, thus allele mis-calling can easily be avoided by simultaneously viewing the electropherograms of all three dye labels. These artifacts associated with excess template, while undesirable, did not compromise accurate calling of the full panel of alleles. With respect to heterozygous peak ratios, values dropped below 60% with 0.3125 ng input template but only at a single locus. Below this level of input template, imbalance of heterozygous alleles was more extensive, with ratios falling below 60% at multiple loci. We likewise amplified serially diluted DNA (0.5, 0.25, 0.125, 0.0625, and 0.0313 ng) from blood (CC DNA) spanning the lower range of the sensitivity curve. Similar to the male and female standards, allelic dropout was not observed above 0.0313 ng of template. We also obtained comparable results when comparing heterozygous peak balance where ratios did not fall below 70% until input DNA was lowered to 0.25 ng. The increase in imbalance of heterozygous peaks resulting from decreasing input template was also observed in the original PowerPlex® 16 System validation and documented by Promega in reference manual "Internal Validation of STR Systems" (part # GE053). While this imbalance of heterozygous loci accompanying lower template amounts would be problematic for mixture analysis integral to forensic casework studies, it did not interfere with our ability to accurately call the full panel of alleles at the 0.078 ng template level for the male and female standards and at the 0.0625 ng level for DNA CC. At these template levels and below, peaks falling below the 0.2 cutoff were not automatically called by the software. However, with peak heights averaging over 1000 rfu at even the lowest template levels, we had no difficulties manually calling the alleles by visual inspection. In the instances where allelic dropout did occur the peaks were indiscernible above background. Results from these titration experiments indicate that amplification of DNA in the range of 0.4–1 ng yields optimal results with respect to peak height and quality, and balance of heterozygous loci.

Intercapillary Variation

To evaluate the degree of intercapillary variation, 0.5 μL of the PowerPlex® 16 allelic ladder mix was injected into each of the 96 capillaries of the 3730xl. The mean fragment size of the alleles was determined and plotted against the standard deviation for each allele (Fig. 5). Seventy-eight percent of the alleles demonstrated standard deviations in the range of 0.03–0.06 bases and only 10 of the 210 alleles exceeded 0.10. This sizing consistency is an improvement over the original validation performed on the single capillary 310 where 13 individual ladder injections yielded slightly higher standard deviation values. Similar to the original validation the alleles demonstrating increased deviation were in the D18S51 and Penta D loci. Results from this experiment reconfirm the utility of the ILS600 as a sizing standard and demonstrate consistency across the 96 capillaries.

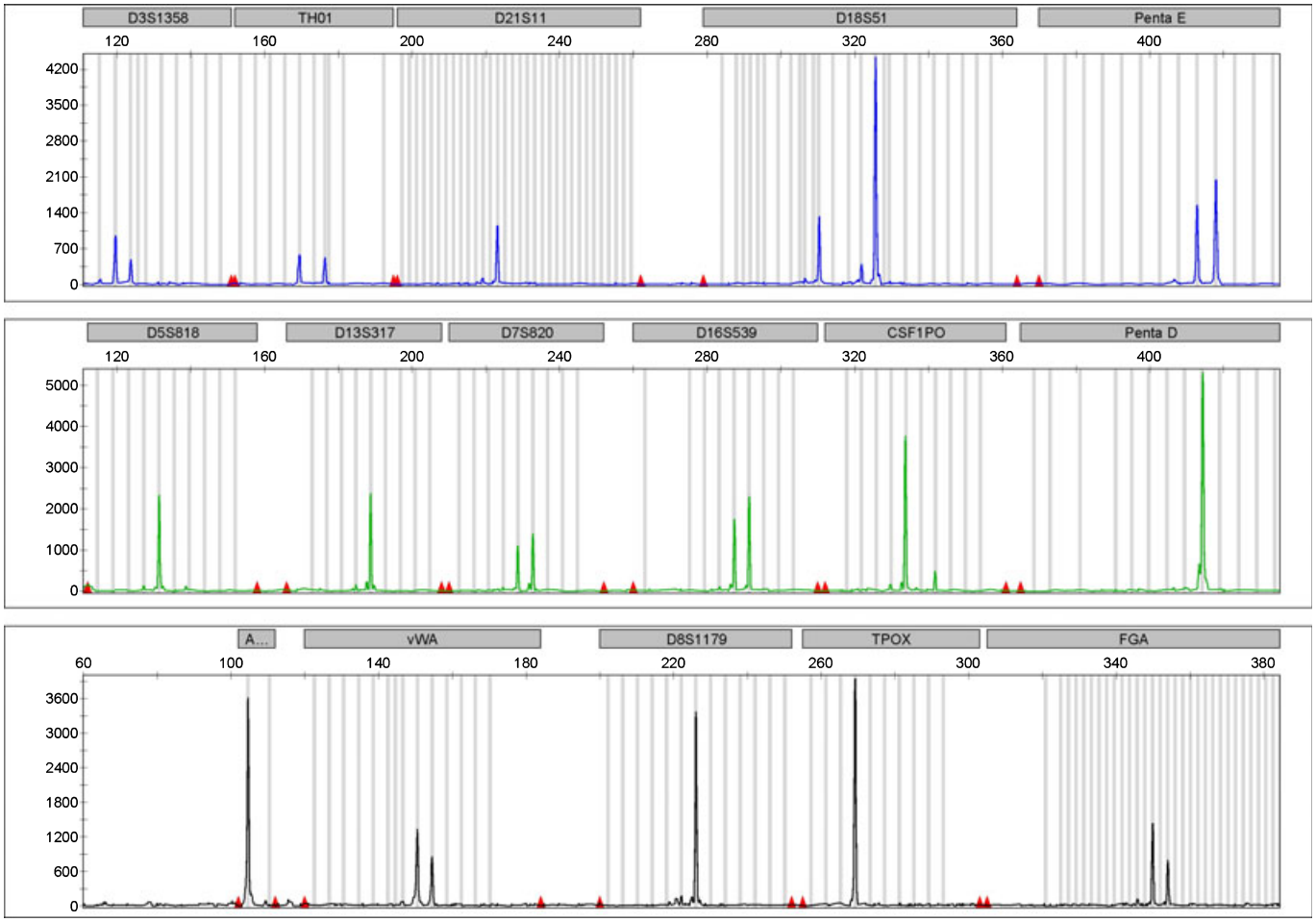

FIG. 4—*Sample electropherograms from amplification of 0.078 ng of 9947A female standard DNA. Dye labels from top to bottom are FL, JOE, and TMR. The far left locus in the TMR panel is Amelogenin.*

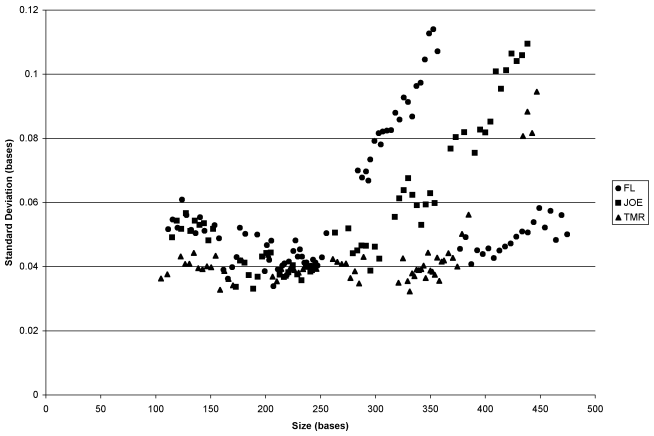

FIG. 5—*Intercapillary variation. The average fragment size of alleles in the PowerPlex® 16 allelic ladder are plotted against the standard deviation observed across the 96 capillaries of the 3730xl.*

Reproducibility

Eight nonprobative human DNA samples obtained from the NIST were genotyped using this modified PowerPlex® 16 protocol. The SRM obtained from the NIST also includes the male 9948 and female 9947A standards provided by Promega as components of their PowerPlex® 16 and PowerPlex® Y kits. One nanogram of DNA from each of the above mentioned DNA samples was amplified in triplicate for a total of 30 multiplexed amplifications. Of the 464 (one PCR failed) loci analyzed all but one matched the NIST determined genotypes (data not shown). The single exception occurred at the D18S5 1ocus of one amplification of sample genomic DNA 1. In this case, a homozygous allele was miscalled as a heterozygote due to saturation bleed-through from the JOE labeled CSF1PO locus. In four instances, out-of-bin alleles necessitated manual calling. Because GeneMapper® does not reset the bins according to the allelic ladder from each individual run, drift, due to run-to-run variations such as polymer lot or ambient temperature is not accounted for. These out-of-bin alleles were manually called by comparing their sizes to alleles in the corresponding locus of the concurrently run allelic ladder. In all cases, out-of-bin alleles corresponded to ladder alleles with their sizes falling within the predefined ±0.5 bp offset range. Such drift underscores the need for the inclusion of an allelic ladder with each run. Nevertheless in a high volume laboratory such manual calls could result in a significant slowdown in the analysis process. For this reason, when considering high-throughput databasing, the genotyping software of choice should be one which automatically recalculates the bin offsets with every run. Such is the case with Applied Biosystems' GeneMapper® ID which genotypes samples according to the allelic bins calculated from ladders within the same run folder. With the exception of these out-of-bin alleles, all remaining alleles were correctly called by the GeneMapper® software. Peak intensities

were robust, averaging 9645 rfu and comparable to values obtained in the DNA titration experiment (13,377 average rfu for 1.25 ng input DNA). In addition to the robust peak intensities, heterozygous peak ratios remained high. Of the 350 heterozygous loci, 340 or 97% had peak ratios of greater than 60%. These ratios are well within levels customarily deemed acceptable for databasing (Joseph Bessetti, Promega Corporation, personal communication). In addition to the NIST standards, we also genotyped a full 96 well plate of DNA collected from field samples as part of an ongoing population-based study in our laboratory. Input DNA was in the range of 0.5–1 ng. A complete panel of alleles was successfully obtained for each sample with peak heights and ratios at heterozygous loci averaging over 5000 rfu and 80% respectively (data not shown). The results obtained with these nonprobative DNA samples indicate that our revised PowerPlex® 16 validation using the 3730xl DNA Analyzer platform is ideal for DNA databasing where high throughput, reliability, and reproducibility are desired.

Conclusion

We have presented an extended validation of the PowerPlex® 16 System on the ABI 3730xl 96 capillary DNA Analyzer. Our goal in this study was the development of a modified validation with increased throughput and cost effectiveness without compromising robustness, reliability, or reproducibility of the assay. As validated on the ABI 310, 96 samples would require *c.* 3 days to be completely processed. The extended validation developed in our laboratory results in a protocol that can analyze a 96 well plate in *c.* 1 hour and at a quarter of the reagent cost of the original validation (4). The reduction in reaction volume combined with the greater dynamic range of the 3730xl platform in comparison to previous generation DNA analyzers resulted in an overall increase in signal and assay sensitivity. Moreover, the capacity of this extended validation to yield accurate profiles over a wide range of input template means fewer reruns due to too little or too much DNA template, further increasing the throughput capability of the assay. Furthermore, these modifications did not adversely affect heterozygous locus peak ratios, which remained above 60%. The quality of the data is reflected in the ability to reproducibly obtain complete and accurate profiles using nonprobative samples with a 99.8% (463/464 loci correctly identified) success rate. Coupled with the DNA concentration titration experiment, these data suggest that a wide range of field-collected samples would also result in accurate profiles. Thus, the 3730xl validated PowerPlex® 16 System is robust and accurate and optimally suited to DNA databasing and addressing the backlog of DNA samples facing many state laboratories.

Acknowledgments

This study was funded by a start-up grant from the Research Foundation of SUNY Binghamton to JKL. We wish to thank Joseph Bessetti (Promega Corporation) for providing the PowerPlex® 16 System and PowerPlex® Y System kits and for his invaluable technical support.

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