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ORIGINAL ARTICLE

Developmental Validation of the PowerPlex[®] 35GY System: An 8-Dye STR Multiplex for Human Identification Applications

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Abstract

The PowerPlex[®] 35GY System is a 35-locus, eight-dye, STR multiplex optimized for use with Spectrum CE Systems. It simultaneously amplifies all CODIS and ESS markers, along with Amelogenin and DYS391 for gender determination. Penta D, Penta E, and SE33 are included for expanded database searching capability. Ten additional nonrapidly mutating Y-STR loci are included to enable familial searching and to assist with forensic casework on sexual assault evidence. Lastly, two Quality Indicators are added to provide indications of sample quality and amplification performance. The PowerPlex[®] 35GY System is designed to amplify DNA from purified extracts as well as direct amplification of database sample substrates. The availability of 8 colors allows the inclusion of smaller, more numerous loci, thus increasing a laboratory's chance of success with challenging samples. A multilaboratory developmental validation study was performed for the PowerPlex[®] 35GY System following SWGDAM guidelines. The results of the study demonstrate the reliability and robustness of the PowerPlex[®] 35GY System for use in human identification DNA typing applications.

Keywords: 35GY, 8-dye, Spectrum CE, Short Tandem Repeat (STR), Forensic DNA, Challenging Samples

Introduction

The PowerPlex[®] 35GY System is a 35-locus, eight-dye, multiplex optimized for use with the Spectrum CE Systems (Fig. 1). The kit supports both casework and database sample workflows. The dye system consists of the blue channel (Fluorescein-8C), green channel (JOE-8C), aqua channel (AQA-8C), yellow channel (TMR-8C), red

channel (CXR-8C), purple channel (TOM-8C), and an orange channel (WEN-8C). The fragments of the internal lane standard 500 are detected in the CCO-8C dye channel (CCO ILS 500).¹

The PowerPlex[®] 35GY System allows simultaneous amplification of the autosomal loci in the CODIS and ESS core set (CSF1PO, FGA, TH01, TPOX, vWA,

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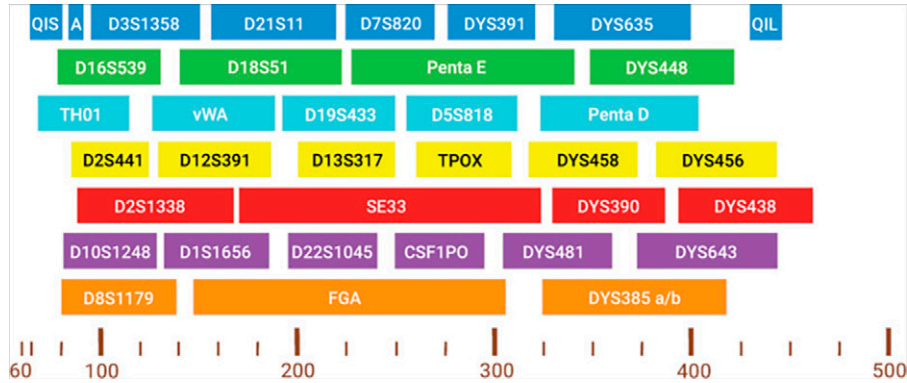


FIG. 1. Layout by dye channel and base pair size of the PowerPlex® 35GY System Loci. QIS = Quality Indicator Small; A = Amelogenin; QIL = Quality Indicator Large.

D1S1656, D2S1338, D2S441, D3S1358, D5S818, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, and D22S1045) as well as Amelogenin and DYS391 for gender determination. The Penta D, Penta E, and SE33 loci are included to increase discrimination and to enable searching of databases that encompass these loci. The expansion from six to eight dye channels allows for smaller amplicon sizes, exemplified by the presence of 15 autosomal loci smaller than 250 bp. Ten additional nonrapidly mutating Y-STR loci (DYS635, DYS448, DYS458, DYS456, DYS390, DYS438, DYS481, DYS643, DYS385a/b) are included for familial searching and to assist with the interpretation of sexual assault evidence profiles. Unlike forensic qPCR kits, which can solely quantify the concentration of Y-chromosomal DNA, the PowerPlex® 35GY System provides discriminatory power to help distinguish between male individuals, determine the number of male contributors present, or to establish paternal kinship. The system includes two Quality Indicators to enhance the analysis and interpretation of STR data. The Quality Indicator Small (QIS) and the Quality Indicator Large (QIL) cover the lower and higher base pair range of amplification products. The quality peaks aid in assessment of the overall success of the amplification reaction by helping to differentiate between degraded and inhibited samples.

A multilaboratory developmental validation study was performed following SWGDAM validation guidelines² to demonstrate the reliability and robustness of the PowerPlex® 35GY System. The laboratory sites that completed testing are New Hampshire State Police Forensic Laboratory (NHSPFL), Federal Bureau of Investigation (FBI), Forensic Genomics Innovation Hub (FGIH), Servicio de Criminalística de la Guardia Civil (SECRIM), the National Institute of Standards and Technology (NIST),

and Promega Corporation. The combined results of these laboratories are presented to demonstrate the capabilities of the PowerPlex® 35GY System for use in human identification DNA typing applications.

Materials and Methods

DNA amplification

All data described in this article were generated with components from the commercial release of the PowerPlex® 35GY System.

Extracted DNA PCR amplification was performed following the default cycling protocol as described in the PowerPlex® 35GY System technical manual.¹ Unless otherwise indicated, extracted DNA amplification reactions contained 1.0 ng DNA in a final reaction volume of 25 μ L. The reaction mix contained: 5 μ L PowerPlex® 35GY 5X Master Mix, 5 μ L PowerPlex® 35GY 5X Primer Mix, and up to 15 μ L of template DNA/Water, Amplification Grade. The thermal cycling method for extracted DNA samples was: 96°C for 1 min; 29 cycles of 98°C for 5 s, 60°C for 1 min, and 72°C for 15 s; followed by a 60°C final extension for 10 min and a 4°C soak.

The default direct amplification protocol was performed following the cycling protocol as described in the PowerPlex® 35GY System technical manual¹ with a 12.5 μ L final reaction volume. The reaction mix contained: 2.5 μ L PowerPlex® 35GY 5X Master Mix, 2.5 μ L PowerPlex® 35GY 5X Primer Mix, 1–4 μ L PowerPlex® 35GY QI Reagent and Water, Amplification Grade to balance. Unless otherwise indicated, the default thermal cycling method for direct amplification used was: 96°C for 1 min; 25 cycles of 98°C for 5 s, 60°C for 1 min, and 72°C for 15 s; followed by a 60°C final extension for 10 min and a 4°C soak. For the direct amplification of swabs, the volume of Water, Amplification Grade is adjusted to include 2 μ L of swab lysate in the reaction mixture.

A subset of lytic card samples was tested with a second direct amplification protocol designed for challenging samples. The extension phase for the challenging sample protocol is altered by reducing the temperature and extending the time. The protocol is as follows: 96°C for 1 min, 25 cycles of 98°C for 5 s, 60°C for 1 min, and 66°C for 1 min followed by 60°C for 10 min and a 4°C soak.

When conducting tests using either the default or challenging direct amplification protocols, it was necessary to modify the quantity of PowerPlex® 35GY QI Reagent added to the reaction: 4 µL for 23 cycles, 2 µL for 24 cycles, and 1 µL for 25 cycles. Incorporating additional QI reagent enhances the peak heights of the quality indicator peaks in direct amplification processes at the lower cycle numbers typically encountered.

Extracted DNA samples

Extracted human genomic DNA was purified using the Maxwell® FSC DNA IQ™ Casework Kit³ and quantified with the PowerQuant® System⁴ (Promega Corp). These samples were used for all studies, except the case-type, stutter, and concordance studies. Laboratories assessed case-type samples from their own collections representing types typically seen in forensic casework processing. Case-type samples were purified using either the Maxwell® FSC DNA IQ™ Casework Kit,³ the PrepFiler™ Forensic DNA Extraction Kit,⁵ or the EZ1&2 DNA Investigator Kit.⁶ NIPT plasma samples were extracted with the Maxwell® RSC ccfDNA Plasma Kit.⁷ The 2800M DNA was also amplified multiple times as part of the reproducibility and repeatability study. The 2800M is the human control DNA sample provided with the PowerPlex® 35GY System.¹ Stutter and concordance studies were conducted with NIST U.S. population samples. All samples used in the studies by participating laboratories were collected in accordance with the informed consent policies of each respective laboratory.

Unless otherwise specified, all amplification reactions for each study were performed in quadruplicate. A sensitivity study was conducted with two DNA donors using a two-fold serial dilution ranging from 4 ng to 7.8 pg. For the mixture study, a two-person, male:female mixture series was tested at 19:1, 9:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:9, and 1:19 ratios. In addition, a three-person, male:male:female series was tested at 1:1:10 and 1:1:20 ratios.

Various conditions were evaluated with a male DNA sample to assess the effects of changing the reaction volume (25 µL versus 12.5 µL), cycle number (28, 29, and 30 cycles), annealing temperature (58°C, 60°C, and 62°C), or PCR reaction components on the amplification reaction. Different reaction volumes were performed at 25 µL and 12.5 µL at either fixed DNA mass or fixed DNA concentration. For fixed DNA mass, 1 ng or 200

pg DNA was amplified per 25 µL or 12.5 µL reaction volume. For fixed DNA concentration, 40 pg/µL or 8 pg/µL was amplified per 25 µL or 12.5 µL reaction volume. Primer pair and master mix concentrations were assessed at 0.8X, 1.0X, and 1.2X.

The species study used commercially available nonhuman DNA purchased from ATCC (Manassas, VA), Coriell Institute (Camden, NJ), Novagen® brand, and Zyagen (San Diego, CA). Ten nanograms of each vertebrate animal or microbial species were amplified in duplicate. Four nonhuman primate species of chimpanzee, macaque, orangutan, and gorilla were amplified in duplicate at 1 ng.

The stability study evaluated the amplification of a male DNA sample with four common forensic inhibitors (Sigma-Aldrich). This was accomplished by testing different concentrations of hematin (0 µmol/L, 500 µmol/L, 750 µmol/L, and 1000 µmol/L), humic acid (0 ng/µL, 100 ng/µL, 200 ng/µL, and 300 ng/µL), tannic acid (0 ng/µL, 500 ng/µL, 750 ng/µL, and 1000 ng/µL), and EDTA (0 µmol/L, 750 µmol/L, 1000 µmol/L, and 1250 µmol/L). A male DNA sample underwent UV degradation using a Stratalinker™, with exposure levels set at 0 mJ, 50 mJ, 100 mJ, and 300 mJ.

Concordance testing was performed at NIST using a subset ($n = 657$) of the NIST 1036 U.S. population samples^{8,9} and the four single source components present in the Standard Reference Material® (SRM) 2391d (Components A–C, and E)¹⁰ for a total of 661 samples. The same subset ($n = 661$) of the 1036 U.S. population samples was used to examine stutter ratio values. NIST samples were amplified in single replicates.

Stutter percentages (as a percentage of associated main allele) were determined for $N - 1$ and $N + 1$ repeat positions. Where applicable, stutter percentages were calculated for $N - 2$ repeat stutter positions (DYS481) and stutter positions that were 2 nucleotides shorter than the main allele (D1S1656, FGA, and SE33). Stutter peaks at the repeat position between heterozygous alleles that are two repeat units apart were not included to eliminate the additive effect of plus and minus stutter. Stutter was only calculated when the true allele peak heights were at least 5,000 RFU and did not exceed 20,000 RFU.

Direct amplification substrates

Testing was performed with a male donor for blood and buccal samples collected on FTA™ Cards (Whatman), male buccal cells collected on Bode Buccal® DNA Collectors (Bode Technology) and cotton swabs, as well as male donor blood collected on S&S 903 paper (Whatman). Laboratories evaluated donors from their own collections. Direct amplification reactions were performed using one 1.2 mm punch from cards. Bode Buccal® DNA Collector and S&S 903 paper were pretreated with

Table 1. Thermal cycler systems evaluated with the PowerPlex® 35GY System

Manufacturer brand	Thermal cycler model	Ramp rate	Additional protocol modification	Testing site
Applied Biosystems™	ProFlex™ PCR System	6°C/s		FBI, Promega
Applied Biosystems™	GeneAmp™ PCR System 9700	Max		Promega
Applied Biosystems™	Veriti™ Thermal Cycler	100 %		Guardia Civil, Promega
Applied Biosystems™	Veriti™ Fast Thermal Cycler	100 %		NHSPFL, Promega
Eppendorf	MasterCycler X50	Heating & Cooling Limiting Rate = 5°C/s; Safe Temperature Block Mode	Denature 98°C for 10 s	Promega
Bioer	GeneExplorer GE-96G Aluminum Block	Standard Speed	Tube Control	FGIH

PunchSolution™ Reagent (Promega Corp) as described in the manufacturer's technical manual.¹¹ Cotton swabs were preprocessed with SwabSolution™ Reagent (Promega Corp) as described in the manufacturer's technical manual,¹² and 2 μ L of lysate was added to the amplification.

All direct amplification sample types were evaluated at different cycle numbers (23, 24, and 25 cycles) at both the default and challenging sample thermal cycler protocol. Lytic card substrates were tested to appraise the effects of changing the reaction volume (12.5 μ L versus 6.25 μ L). Unless otherwise specified, all amplification reactions were performed in quadruplicate. Informed consent was obtained for all samples used for experimentation purposes.

Thermal cycler compatibility

The PowerPlex® 35GY System was developed and optimized on the ProFlex™ PCR System¹ (Applied Biosystems, Foster City, CA). A sensitivity series (4 ng to 7.8 pg) was amplified at 29 cycles in 25 μ L to demonstrate compatibility with additional cycler models (Table 1). An additional dilution series (20 ng to 100 pg) was amplified at 24 cycles with the direct amplification 12.5 μ L protocol.

DNA fragment and data analysis

DNA fragment detection was performed on the Spectrum CE System, 8-Capillary¹³ (Promega Corp). Calibrations were performed in accordance with the Spectrum CE System Operating Manual¹⁴ and the respective instructions for the PowerPlex® 8C Matrix Standard¹⁵ (Promega Corp). Each sample injected on the Spectrum CE System consisted of 1 μ L of amplified sample or PowerPlex® 35GY allelic ladder with 9.5 μ L of Hi-Di™ Formamide

(Applied Biosystems) and 0.5 μ L of the CCO Internal Lane Standard (ILS) 500. Samples were heat denatured at 95°C for 3 min followed by a snap cool of 3 min. All samples were injected at 2 kV for 15 s and run using the Promega 8-Dye (8C) protocol. Files generated by the Spectrum CE Systems (.promega format) were analyzed with GeneMarker® HID Software for Spectrum CE Systems¹⁶ (v3.2.0). The appropriate panel, bin, and stutter values developed by Promega were used for analysis.

Average Peak Height was calculated for all studies by adding all detected peak heights from replicates then dividing by the total number of expected peaks. Allele dropout was treated as a 0 RFU peak in the sum. Quality indicator peaks (QIS and QIL) were excluded from peak calculations. Two-person mixture analysis consisted of counting alleles unique to the minor contributor, which were presented as a percentage of the total number of unique alleles expected.

Baseline noise and threshold analysis

The 1 ng sensitivity samples were analyzed to establish detection thresholds for extracted DNA, whereas the 5 ng sensitivity samples were analyzed to establish thresholds for direct amplification. The determination of analytical threshold (AT) was conducted by setting the reading threshold to 1 RFU in the GeneMarker® HID for Spectrum software. The average height and standard deviation of the noise peaks were determined for each dye channel. Ten times the standard deviation was added to each average noise value, then rounded up to the nearest 5 RFU to determine the AT for each dye. Noise levels were compared between different Spectrum CE Systems and determined to be similar, allowing one set of thresholds for data analysis of each sample type (Table 2).

Table 2. Analytical threshold (RFU) selected for each PowerPlex® 35GY dye channel

PowerPlex® 35GY Dye Channel	Fluorescein-8C	JOE-8C	AQA-8C	TMR-8C	CXR-8C	TOM-8C	WEN-8C
Extracted DNA 25 μ L Reaction Volume	85	95	170	175	145	110	85
Direct Amplification 12.5 μ L Reaction Volume	70	80	140	155	125	95	75

Each color represents its respective dye channel in the system.

Table 3. Summary of non-primate vertebrates tested with the PowerPlex® 35GY System

Nonprimate vertebrate DNA	Amount tested	Peak over 85 RFU	Approximate artifact sizes(s)
Cat (male)	10 ng	No	
Chicken (male)	10 ng	Yes	225 nt to 227 nt/85 RFU to 90 RFU (JOE) 288 nt to 290 nt/85 RFU (TOM)
Cow (female)	10 ng	No	
Deer	10 ng	No	
Dog	10 ng	No	
Horse	10 ng	Yes	422 nt to 424 nt/98 RFU to 114 RFU (TOM)
Mouse	10 ng	No	
Pig	10 ng	Yes	371 nt to 373 nt/348 RFU to 368 RFU (JOE) 369 nt to 372 nt/184 RFU to 222 RFU (TMR)
Rabbit (male)	10 ng	No	
Rat	10 ng	No	

Direct amplification samples were further analyzed with a 20% global filter.

Results and Discussion

Species specificity

Although primers in the PowerPlex® 35GY System are specifically designed to target human DNA, the presence of large quantities of nonhuman DNA may lead to non-specific primer annealing, potentially resulting in the production of a detectable amplicon. Twelve microorganism species, ten vertebrate species (nonprimate), and four nonhuman primate species were amplified to test cross-reactivity. Due to differences regarding how laboratories determine their detection thresholds, artifact peaks were identified using the lowest observed analytical threshold across all dye channels (85 RFU, observed for FL and WEN). No amplification products were detected in the 60 to 500 nucleotide size range for any of the microbial species tested (data not shown). Chicken, horse, and pig produced peaks above threshold (Table 3, Supplementary Fig. S1). These peaks were mostly located between

panels or called off-ladder. The four nonhuman primate species yielded profile peaks due to the genetic similarities between humans and nonhuman primates. However, these profiles were distinguishable from human DNA due to the number of out-of-bin peaks, off-ladder peaks, and variant alleles (Supplementary Fig. S2).

Sensitivity study

The dynamic range of the PowerPlex® 35GY assay was evaluated on six different Spectrum CE systems. All test systems obtained 100% of alleles with DNA input concentrations as low as 125 pg. From 125 pg to 7.8 pg DNA, there was a decline in percent alleles called (Table 4). Average peak height results appeared similar across systems (Fig. 2). Peak heights were approximately 4,000 RFU to 5,000 RFU when amplifying 1 ng of DNA. Peak heights increased linearly with an increase in the DNA template. Regression line R^2 values of 0.98 or higher were obtained for template amounts up to 2 ng of DNA (data not shown). Average peak height ratios when amplifying 1 ng of DNA were greater than 78% on all systems (data not shown). A representative graph of individual peak height ratios by template amount is shown in Supplementary Figure S3. The inter-dye balance of samples amplified with 1 ng of DNA was greater than 68% on all systems (data not shown).

Thermal cycler comparison data

The PowerPlex® 35GY System was tested on additional thermal cycler models with both a 25 μ L and 12.5 μ L reaction volume series. A direct comparison of average sample peak height between cycler models was examined from one Spectrum CE System. Average peak heights were similar between the tested thermal cycler models when amplifying 1 ng of DNA for the 25 μ L PCR reaction volume, ranging between 4,000 RFU to 5,000 RFU (Fig. 3). One hundred percent of alleles were called on all thermal cyclers at 125 pg or higher (data not shown). These results are consistent with the previous sensitivity

Table 4. Average percent profile heat map of sensitivity series for the PowerPlex® 35GY System

Concentration of Template DNA	Promega ProFlex™	FGIH Bioer GeneExplorer	NHSPFL Spectrum 1 Veriti™ Fast	NHSPFL Spectrum 2 Veriti™ Fast	FBI ProFlex™	Guardia Civil Veriti™
4 ng	100 %	100 %	100 %	100 %	100 %	100 %
2 ng	100 %	100 %	100 %	100 %	100 %	100 %
1 ng	100 %	100 %	100 %	100 %	100 %	100 %
500 pg	100 %	100 %	100 %	100 %	100 %	100 %
250 pg	100 %	100 %	100 %	100 %	100 %	100 %
125 pg	100 %	100 %	100 %	100 %	100 %	100 %
62.5 pg	91 %	78 %	90 %	95 %	92 %	93 %
31.25 pg	65 %	59 %	48 %	65 %	65 %	60 %
15.6 pg	27 %	18 %	14 %	27 %	23 %	24 %
7.8 pg	7 %	6 %	3 %	9 %	6 %	8 %

Each color represents its respective dye channel in the system.

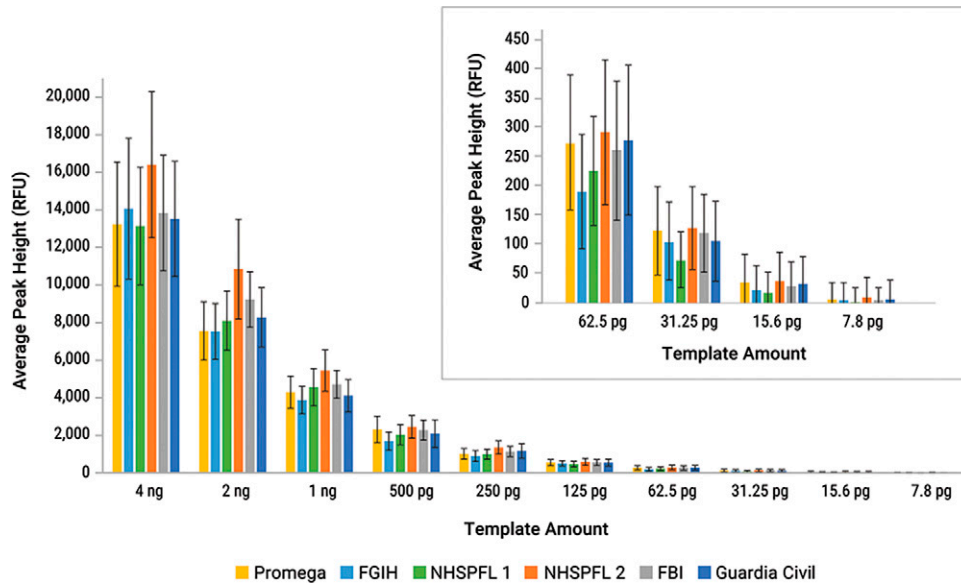


FIG. 2. Average peak heights of sensitivity series amplified at 29 cycles with the PowerPlex[®] 35GY System at the 25 μ L reaction volume. Error bars represent ± 1 standard deviation ($n = 8$).

study results. Representative data from one male donor are presented. A similar pattern was observed for the data from the second male donor. Locus *DYS391* exhibited roughly half the peak heights for non-cell-line DNA when amplified on the MasterCycler X50 as compared with other cyclers models. An example of peak height variation by locus for 1 ng samples amplified on various thermal cyclers can be found in Supplementary Figure S4.

Average peak heights were similar between the tested thermal cycler models for the 12.5 μ L PCR reaction volume, ranging between 4,200 RFU to 5,500 RFU when amplifying 20 ng of DNA (Fig. 4). Representative data from one male donor are presented. One hundred percent of alleles were called on all thermal cyclers at 2.5 ng or higher (data not shown). Locus *DYS391* exhibited roughly half the peak heights for non-cell-line DNA when amplified on the MasterCycler X50 as compared with other cycler models when using the 12.5 μ L PCR reaction volume.

Stability study

Degraded samples. Exposure to heat and sun can often degrade DNA of forensic casework samples. The PowerPlex[®] 35GY results affirm that an increase in exposure to UV radiation results in a decrease of average peak height and percent profile (Fig. 5). The samples exposed to 100 mJ and 300 mJ of UV radiation resulted in partial DNA profiles of 90% and 75%, respectively. Preferential loss of the high-molecular-weight loci was observed. The average quality indicator ratio (QIL Peak

Height/QIS Peak Height) remained at or above 1.0 for all degraded samples. A graph depicting the average peak heights related to percent profile for the degradation series is shown in Supplementary Figure S5.

Inhibited samples. Environmental inhibitors can affect amplification performance when obtaining profiles from forensic samples. The PowerPlex[®] 35GY System demonstrates tolerance at a wide range of inhibitor concentrations and types. Complete profiles were generated in the presence of 750 μ mol/L EDTA, 500 μ mol/L hematin, and 500 ng/ μ L and 750 ng/ μ L of tannic acid. On average, 99% of alleles were detected at the humic acid 100 ng/ μ L sample (Fig. 6). The remaining concentrations resulted in partial DNA profiles. EDTA had the greatest effect on the percent profile, while tannic acid had the least effect. High-molecular-weight loci along with the *TH01* and *D7S820* loci were typically affected first.

The PowerPlex[®] 35GY Quality Indicators are designed to aid in distinguishing degraded samples from inhibited samples. The QIL peak height is reduced when a sample contains higher levels of inhibition. A complete loss of the QIL peak was observed in the following inhibited sample concentrations: EDTA 1000 μ mol/L and 1250 μ mol/L, humic acid 200 ng/ μ L and 300 ng/ μ L, and hematin 750 μ mol/L and 1000 μ mol/L. Generally, dropout of the QIL peak coincided with dropout of higher-molecular-weight loci in the multiplex. A representative electropherogram of the Fluorescein-8C dye channel for hematin-inhibited samples amplified with PowerPlex[®] 35GY is shown in Supplementary Figure S6.

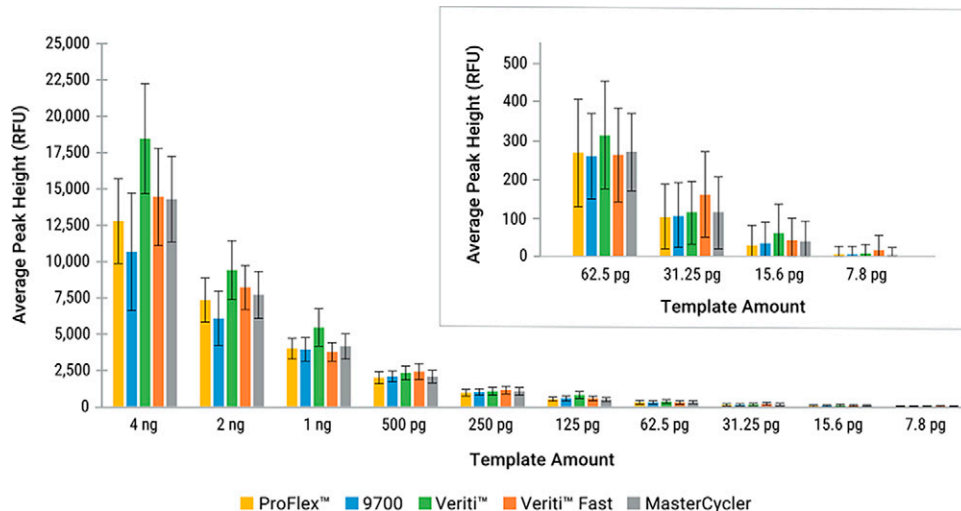


FIG. 3. Average peak heights of additional thermal cycler sensitivity series amplified at 29 cycles with the PowerPlex® 35GY System at the 25 µL reaction volume. Error bars represent ±1 standard deviation (n = 4).

Precision and accuracy

Allelic ladder precision. Sizing precision is critical for accurate genotyping. The data from 24 PowerPlex® 35GY allelic ladders injected on five different Spectrum CE Systems were evaluated. All resulted in a standard deviation of ≤0.09 nt and size range ≤0.30 nt for all CE systems (Table 5). The maximum standard deviations observed were less than 0.16 nt, which is the maximum

value to be within three standard deviations of a 0.5 nt binning window. This means that when PowerPlex® 35GY allelic ladders are included on a plate, sample alleles will fall within appropriate allele bins.

The PowerPlex® 35GY allelic ladder contains four loci with peaks separated by a single nucleotide: TH01, D2S441, D12S391, and D1S1656. Single-nucleotide resolution was achieved 100% of the time for each of these alleles.

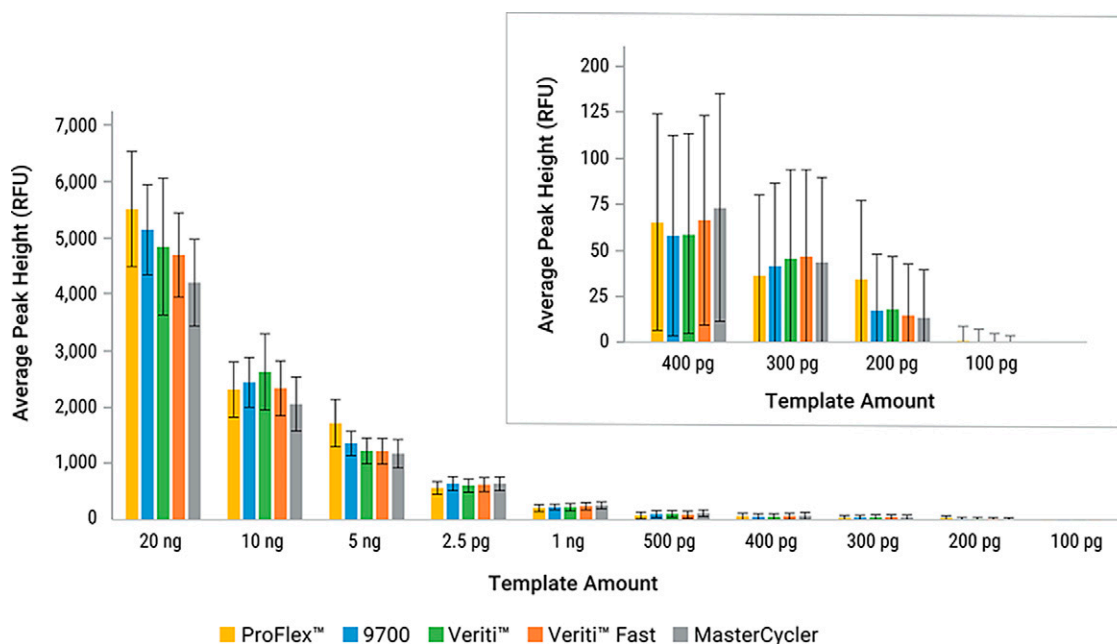


FIG. 4. Average peak heights of additional thermal cycler sensitivity series amplified at 24 cycles with the PowerPlex® 35GY System at the 12.5 µL reaction volume. Error bars represent ±1 standard deviation (n = 4).

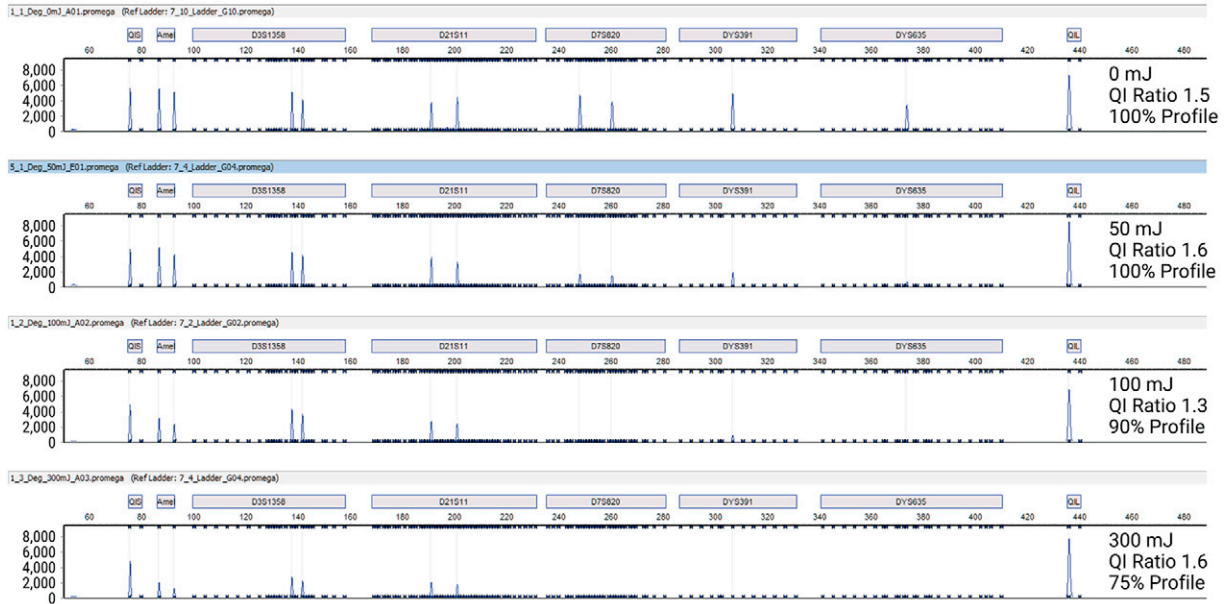


FIG. 5. Representative electropherogram of the Fluorescein-8C dye channel for a 1 ng sample exposed to increasing amounts of UV radiation amplified with the PowerPlex[®] 35GY System. The QI Ratio = QIL Peak Height/QIS Peak Height.

Representative loci demonstrating single-nucleotide resolution for the PowerPlex[®] 35GY System are displayed in Figure 7.

Reproducibility and repeatability. Forty-eight 2800M samples were evaluated for genotype concordance. All 2800M samples showed 100% concordance across instruments and were within plus or minus 0.5 nt of the corresponding allele in the PowerPlex[®] 35GY allelic ladder. All profiles obtained for the 2800M samples also

matched the profile in the PowerPlex[®] 35GY technical manual.¹

Genetic marker characterization and concordance. Allele frequencies,^{17–22} Y-haplotype, and physical mapping^{23–26} have been well characterized and are available for the loci included in the PowerPlex[®] 35GY System. PowerPlex[®] 35GY genotypes obtained from the NIST U.S. population set and single-source Components A–C, and E of SRM 2391d were compared in a large

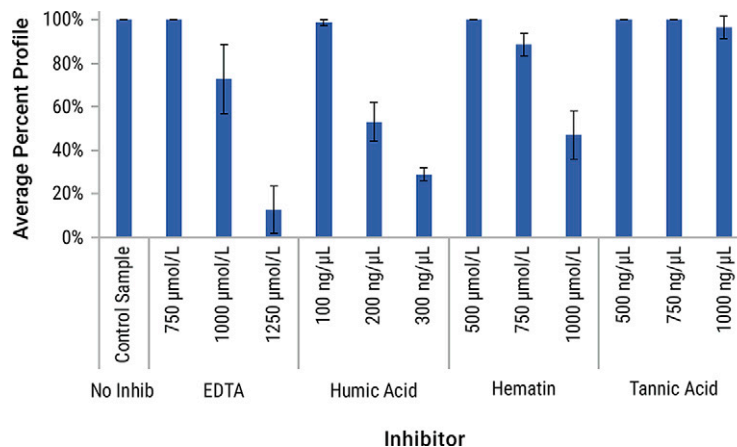


FIG. 6. Average percent profile of inhibited samples with the PowerPlex[®] 35GY System. Error bars represent ± 1 standard deviation ($n = 4$).

Table 5. Standard deviation and allele size range of PowerPlex® 35GY allelic ladder peaks per Spectrum CE System

<i>Spectrum CE systems (n = 24 ladders per System)</i>	<i>Maximum standard deviation (nt)</i>	<i>Maximum allele size range (nt)</i>
Promega	0.06	0.20
FBI	0.08	0.30
FGIH	0.09	0.30
NHSPFL, Spectrum 1	0.06	0.20
NHSPFL, Spectrum 2	0.07	0.30

concordance study with previously generated genotypes from other STR systems. Out of 38,999 alleles compared, 9 discordant calls were observed resulting in a 99.98% concordance between the PowerPlex® 35GY System and the NIST data set.

The first discordant sample generated a null genotype at DYS635 with the PowerPlex® 35GY System, while other systems produced a 24 genotype. Sequencing revealed a primer binding-site mutation as the cause for this discrepancy.

The second discordant sample generated an 8, 9.3 genotype at D7S820 with the PowerPlex® 35GY System as well as PowerPlex® Fusion 6C and Identifiler®, whereas other systems produced an 8, 11 genotype. Sequencing revealed the cause of this discrepancy to be a five-base deletion in the PowerPlex® 35GY System amplicon that is located outside the primer binding sites used in several other STR chemistries.

The remaining seven discordant allele calls were observed in SE33, including Component B of SRM 2391d and were caused by either a TTTT (6 occurrences) or a TTG deletion (1 occurrence) in the DNA region outside the primer binding sites used to generate the PowerPlex® 35GY System amplicon but included within the region amplified by several other STR chemistries.

All NIST SRM 2391d samples demonstrated 100% concordance between test sites. The PowerPlex® 35GY System is concordant at SE33 for Component B when using the NIST certified sequence of 18 repeats.¹⁰ A 17 allele is reported for Component B at SE33 using other commercial CE multiplex kits; however, the certified sequence reveals 18 repeats with a 4-base pair (AAAA) deletion 85 base pairs upstream from the repeat, as noted in the Genetic Marker Characterization and Concordance section.

Stutter. Stutter peaks are common artifacts observed during the PCR amplification process. The stutter filter at each locus for the GeneMarker® HID for Spectrum CE Systems software was calculated using the average stutter ratio plus three standard deviations. The results of the stutter analysis can be found in Table 6. The table summarizes the minus and plus stutter for each locus in the PowerPlex®

35GY multiplex. The number of stutter peaks counted (n) along with the average stutter percentage, standard deviation (SD), and maximum observed stutter are shown. Additionally, DYS481 exhibited N – 2 repeat stutters, while SE33, D1S1656, and FGA exhibited N – 2 nt stutter.

Case-type samples

Forensic casework samples exhibit a wide range of different quantities, qualities, substrate types, and biological sample categories. Three laboratories evaluated 73 case-type samples from their own collections. A variety of sample types were successfully amplified using the PowerPlex® 35GY System (Table 7). Profiles generated were consistent with the expected source of DNA and the quantity. Full profiles were obtained from multiple sample types, including blood, bones and teeth, bullet scraping, cigarette butt, differential extraction fractions, envelopes, hair, saliva, mixed stain samples, and wearer DNA. When partial profiles were generated, genotype information was still attained, particularly at loci approximately 250 bp or less. Many partial profiles exhibited signs of degradation with sloping pattern, loss of high-molecular-weight loci, and balanced Quality Indicators. A few samples exhibited inhibition depicted by a sloping pattern with a reduced or completely lost QIL peak. Several wearer DNA samples included multiple contributors, or produced no profile, as expected with this sample type. Representative electropherograms for single-source hair and degraded blood can be found in Supplementary Figures S7–S8. DNA-dependent and-independent amplification artifacts are defined in the PowerPlex® 35GY System technical manual.¹

Mixture study

Forensic samples often contain DNA from more than one source. Two-person and three-person contributor mixtures were evaluated for the ability to resolve minor contributor alleles with the PowerPlex® 35GY System. Minor allele dropout was observed in the 19:1, 9:1, 1:9, and 1:19 two-person mixture ratios (Fig. 8). An average of 82% of unique minor contributor alleles were distinguishable in the 19:1 mixture ratio, 97% in 9:1, 97% in 1:9, and 81% in the 1:19 ratio. The minor donor contribution in these samples was 100 pg (9:1 and 1:9) and 50 pg (19:1 and 1:19), respectively. Multiple contributors were detected in the three-person mixture samples. On average, 98% of total alleles were detected in the 1:1:10 ratio samples and 89% of alleles of the 1:1:20 ratio (Fig. 8).

PCR-based studies extracted DNA

Cycle number. One hundred percent of alleles were successfully genotyped across all test systems and cycle

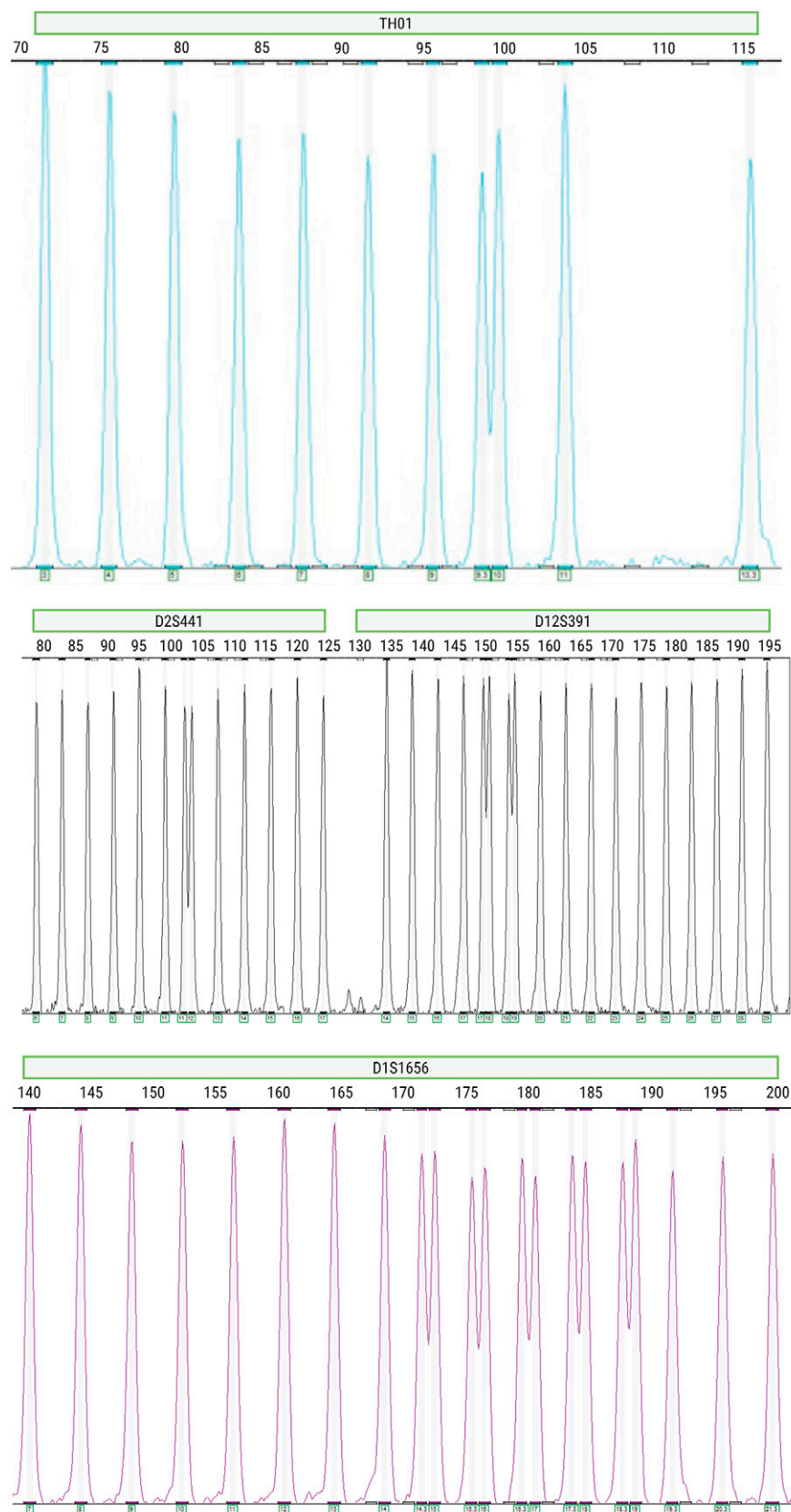


FIG. 7. PowerPlex[®] 35GY System single-nucleotide allelic ladder resolution. The top panel is TH01 (73 nt to 116 nt). The second panel is D2S441 (81 nt to 125 nt) and D12S391 (134 nt to 194 nt). The bottom panel is D1S1656 (140 nt to 199 nt).

Table 6. PowerPlex® 35GY System stutter summary

	<i>N-1 repeat stutter</i>					<i>N + 1 repeat stutter</i>				
	<i>n</i>	<i>Average</i>	<i>SD</i>	<i>Max</i>	<i>Average + 3SD</i>	<i>n</i>	<i>Average</i>	<i>SD</i>	<i>Max</i>	<i>Average + 3SD</i>
35GY locus										
D3S1358	409	10.2	1.5	16.1	14.6	74	1.3	0.3	2.8	2.3
D21S11	644	9.3	1.5	17.3	13.9	586	1.3	0.3	3.1	2.2
D7S820	447	5.8	2.1	11.3	12.0	80	1.3	0.3	2.6	2.2
DYS391	558	7.5	0.9	11.5	10.2	26	1.6	0.6	3.3	3.5
DYS635	541	7.1	1.7	12.8	12.1	61	1.4	0.5	3.5	2.8
D16S539	430	7.0	2.0	12.1	13.1	265	1.3	0.3	3.3	2.2
D18S51	685	9.3	2.4	16.8	16.7	190	1.4	0.4	3.7	2.8
Penta E	735	3.8	1.9	11.3	9.5	32	1.5	0.4	2.8	2.7
DYS448	551	3.2	0.5	6.4	4.7	13	1.3	0.3	2.1	2.2
TH01	563	2.5	0.8	5.7	5.0	27	1.3	0.3	2.0	2.1
vWA	480	8.3	2.8	14.2	16.8	60	1.4	0.4	3.0	2.7
D19S433	563	8.8	1.9	15.8	14.4	134	1.6	0.5	3.4	3.0
D5S818	382	6.6	1.8	11.5	12.0	374	1.5	0.4	3.8	2.6
Penta D	469	2.1	0.7	7.0	4.2	77	1.6	0.7	3.4	3.6
D2S441	643	5.8	1.8	10.7	11.1	542	1.6	0.4	4.2	2.9
D12S391	701	10.9	3.2	21.1	20.6	72	1.5	0.4	2.9	2.8
D13S317	446	5.7	2.1	11.1	12.2	291	1.5	0.4	4.0	2.8
TPOX	454	3.3	1.1	7.1	6.7	18	1.4	0.3	1.9	2.3
DYS458	554	10.1	1.2	13.8	13.8	121	1.5	0.5	4.4	3.1
DYS456	537	10.8	1.3	15.1	14.7	534	2.0	0.5	4.5	3.4
D2S1338	787	11.2	2.2	16.9	17.7	68	1.3	0.4	3.6	2.6
SE33	873	11.7	2.3	21.6	18.7	371	1.6	0.5	3.3	3.1
DYS390	549	8.3	1.5	13.6	12.7	46	1.3	0.4	3.6	2.5
DYS438	519	2.8	0.8	5.1	5.0	27	1.3	0.5	3.2	2.8
D10S1248	422	10.8	2.2	17.1	17.5	47	1.4	0.4	3.3	2.6
D1S1656	713	10.3	2.4	17.3	17.5	446	1.3	0.3	5.3	2.4
D22S1045	527	7.4	3.0	14.4	16.5	585	3.4	0.9	5.9	6.2
CSF1PO	396	6.5	1.8	11.8	11.9	407	1.6	0.6	5.4	3.3
DYS481	527	20.0	2.9	28.4	28.6	526	2.8	0.6	6.5	4.7
DYS643	538	2.7	0.7	4.7	4.8	16	1.4	0.7	3.7	3.4
D8S1179	441	8.6	1.7	13.5	13.8	130	1.4	0.3	3.4	2.4
FGA	640	8.6	2.0	13.4	14.5	162	1.4	0.3	2.9	2.4
DYS385a/b	689	8.9	2.3	17.0	15.8	50	1.3	0.4	2.8	2.4
	<i>N-2 Repeat Stutter</i>					<i>N-2 nt Stutter</i>				
	<i>n</i>	<i>Average</i>	<i>SD</i>	<i>Max</i>	<i>Average + 3SD</i>	<i>n</i>	<i>Average</i>	<i>SD</i>	<i>Max</i>	<i>Average + 3SD</i>
35GY Locus										
SE33						870	5.2	0.4	6.9	6.5
D1S1656						832	2.0	0.5	4.8	3.5
DYS481	533	4.0	1.0	7.2	6.9					
FGA						177	1.7	0.6	4.2	3.6

numbers (28, 29, and 30) for the sample amounts of 1 ng and 200 pg, except for 200 pg amplified at 28 cycles. An average of 98% of alleles was obtained for 200 pg at all test sites when amplified at 28 cycles (data not shown). An increase in cycle numbers resulted in an increase in average peak height for both target amounts. Peak heights increased by about a factor of two with each increment of the cycle number. Representative data obtained for a 1 ng sample from one test site is displayed in Figure 9.

Annealing temperature. One hundred percent of alleles were successfully genotyped across all test systems and annealing temperatures (58°C, 60°C, and 62°C) for the 1 ng samples (data not shown). In general, average peak heights were consistent across all temperatures tested, apart from *DYS635* and *TH01*, which showed reduced peak heights at 58°C. *TH01* exhibited

increased peak heights at 62°C. Representative data obtained for a 1 ng sample from one test site are displayed in Figure 10.

Reaction volume. Amplification reactions were conducted using volumes of 25 μL and 12.5 μL , either with a constant DNA mass or a constant DNA concentration. When the DNA mass was fixed, peak heights at the 12.5 μL volume were observed to double in comparison to those at 25 μL (Fig. 11). This increase is anticipated since the DNA concentration effectively doubles when the reaction volume is halved while maintaining the same total DNA mass.

Conversely, when the DNA concentration was held constant, the peak heights showed no significant variation between the two reaction volumes (Fig. 12). Additionally, the balance between different loci remained

Table 7. Case-type samples PowerPlex® 35GY System Summary

<i>Sample description</i>	<i>[Auto] (ng/μL)</i>	<i>PowerPlex® 35GY amplification target (ng)</i>	<i>PowerPlex® 35GY profile description</i>	<i>PowerPlex® 35GY QIL/QIS ratio</i>
Blood on Fabric (Mixed)	4.2739	1.50	Mixture with Major Female and a Male Contributor	1.09
Blood on Tissue	0.3055	1.50	Single-Source Male	1.22
Blood on Denim	0.1674	1.50	Single-Source Partial Female	1.22
Blood on Tissue	0.3312	1.50	Single-Source Partial Male	1.14
Blood on T-shirt	0.5775	1.50	Single-Source Male	1.30
Blood on FTA®	0.2657	1.50	Single-Source Partial Male	1.02
Blood Swab	1.2896	1.50	Single-Source Partial Male	1.31
Blood on Tissue	0.4696	1.50	Single-Source Female	1.39
Blood Stain (Mixed)	0.0140	0.21	Partial Male with Low-Level Contributor	1.74
Blood Stain	0.0660	0.99	Single-Source Female	1.70
Blood Swab	0.0080	0.12	Single-Source Partial Female	1.54
Bone	0.1200	0.97	Partial Male with Low-Level Contributor	0.00
Bone	27.8900	1.00	Single-Source Female	1.40
Bone	0.2920	0.99	Single-Source Male	1.04
Bullet Scrapings	22.1500	1.00	Single-Source Male	1.07
Cigarette	0.2043	1.50	Single-Source Partial Female	1.10
Cigarette	0.0150	0.23	Single-Source Partial Female	1.00
Cigarette	0.0000	0.00	No Profile	1.48
Cigarette	0.0000	0.00	No Profile	1.56
Cigarette	0.0210	0.32	Single-Source Male	1.45
Yellow Stain (NS Fraction)	0.3180	0.99	Single-Source Male	1.41
Yellow Stain (S Fraction)	0.0140	0.21	Single-Source Male	1.29
Differential Swab (NS Fraction)	0.0270	0.41	Single-Source Female	1.71
Differential Swab (S Fraction)	0.1330	1.00	Single-Source Male	1.40
Differential Swab (NS Fraction)	0.0550	0.83	Mixture with Major Female and Low-Level Male Contributor	1.38
Differential Swab (S Fraction)	0.0790	1.00	Single-Source Male	1.32
Envelope	0.0409	0.61	Single-Source Partial Male	1.45
Envelope	0.0340	0.51	Single-Source Female	1.46
Envelope	0.0030	0.05	Single-Source Partial Female	1.07
Envelope	0.0440	0.66	Single-Source Female	1.53
Envelope	0.0100	0.15	Single-Source Male	1.18
Hair	0.0408	0.61	Single-Source Male	1.12
Hair	0.4120	1.50	Single-Source Male	1.01
Hair	0.3978	1.50	Single-Source Male	1.79
Hair	0.1190	1.00	Single-Source Female	1.27
Hair	0.4590	1.01	Single-Source Female	0.90
Hair	0.2290	1.01	Single-Source Female	1.22
NIPT Plasma	0.8200	1.00	Partial Female with Low-Level Contributor	0.95
NIPT Plasma	0.2780	1.00	Mixture with Major Female and Male Contributor	1.03
NIPT Plasma	0.2220	1.00	Mixture with Major Female and Low-Level Male Contributor	0.27
NIPT Plasma	0.2430	1.00	Mixture with Major Female and Low-Level Male Contributor	0.64
NIPT Plasma	0.1630	0.99	Partial Female	0.00
NIPT Plasma	0.3180	0.99	Female with Low-Level Contributor	1.52
NIPT Plasma	0.4570	1.01	Partial Female with Low-Level Contributor	1.40
Saliva Swab from Cup	0.3527	1.50	Single-Source Male	1.48
Toothbrush	0.0852	1.28	Single-Source Female	1.13
Swab from Coffee Lid	5.2937	1.50	Single-Source Male	1.40
Swab from Juice Cup	0.1358	1.50	Single-Source Male	1.05
Plastic Utensil from Trash	0.2450	1.00	Single-Source Female	1.02
Plastic Utensil from Trash	0.0010	0.02	Low-Level Partial Profile	1.31
Bottle from Trash	0.1790	1.00	Mixture with Female and Male Contributor	1.50
Bottle from Trash	0.0210	0.32	Single-Source Male	1.09
Can from Trash	0.1590	1.00	Single-Source Male	1.51
Coffee Lid from Trash	0.2230	1.00	Single-Source Female	1.31
Semen on Washcloth	5.4366	1.50	Single-Source Male	1.00
Semen/Vaginal Fluid on Tissue	6.6459	1.50	Mixture with Major Male and a Female Contributor	1.20
Semen on Underwear Crotch	6.7781	1.50	Mixture with Major Female and a Male Contributor	1.49
Semen on Vaginal Swab	57.3380	1.50	Mixture with Major Female and a Low-Level Male Contributor	1.01
Saliva on External Genital Swab	1.6587	1.50	Mixture with Major Female and a Male Contributor	1.10
Blood/Semen Stain	1.8500	1.50	Mixture with ≥ Two Male Contributors	1.54
Blood/Semen Stain	6.3638	1.50	Mixture with ≥ Two Male Contributors	1.18
Vaginal Swab	0.0220	0.33	Single-Source Partial Female	0.81

(continued)

Table 7. Continued.

Sample description	[Auto] (ng/ μ L)	PowerPlex® 35GY amplification target (ng)	PowerPlex® 35GY profile description	PowerPlex® 35GY QIL/QIS ratio
Tooth	0.1600	1.00	Single-Source Female	0.97
Tooth	0.4880	0.98	Single-Source Male	0.93
Tooth	0.3370	1.01	Mixture with Major Male and Female Contributor	1.15
Tooth	18.2150	1.00	Single-Source Female	1.42
Tooth	0.2260	0.99	Mixture with Major Male and Female Contributor	1.13
Tooth	3.7750	1.00	Single-Source Male	1.23
Wearer Swab	0.0464	0.70	Mixture with Major Female and a Male Contributor	1.54
Wearer Swab	0.0280	0.42	Mixture with \geq Three Contributors	1.59
Wearer Swab	0.0130	0.20	Mixture with Major Female and Low-Level Contributor	1.24
Wearer Swab	0.0010	0.02	No Profile	0.99
Wearer Swab	0.0000	0.00	No Profile	1.22

stable across these conditions. One hundred percent of alleles were successfully genotyped across all test systems and reaction volumes.

Reaction components. Full profiles were obtained for all samples (data not shown). When varying the primer pair concentrations, the overall average peak height remained consistent between 1 ng samples. Representative data obtained for a 1 ng sample are displayed in Figure 13.

When varying the master mix concentrations, the overall average peak height at some loci increased slightly with the 0.8X master mix concentration and decreased slightly with the 1.2X master mix concentration. Representative data obtained for a 1 ng sample are displayed in Figure 14.

PCR-based studies direct amplification

Cycle number. Replicates with complete loss of profile were considered an outlier due to sampling error

and not included in calculations. As a result, one replicate was removed from buccal on FTA® for all cycle numbers at both the regular and challenging sample protocol.

For Bode Buccal® Collectors, using the default protocol, 100% of alleles were successfully identified across all tested cycle numbers. Similarly, for blood samples on 903 paper, all alleles were called at every cycle number, with the exception of the 24 cycle condition (Fig. 15). Given that 100% of alleles were seen at 23 and 25 cycles, this reduction from 100% at 24 cycles likely reflects sampling variation.

One hundred percent of alleles were successfully genotyped with the default protocol for buccal on FTA®, except for 23 cycles (Fig. 15). Eighty-six percent, 99%, and 95% alleles were recovered for blood on FTA® at 23, 24, and 25 cycles, respectively (Fig. 15). For blood samples on FTA® cards, the variation in the number of allele calls did not show a correlation with the cycle number (Fig. 15). This indicates that sampling variation

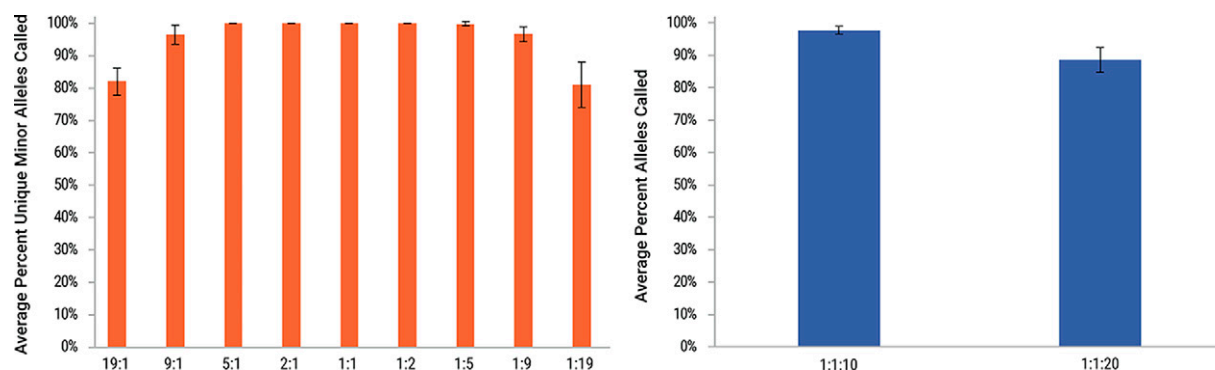


FIG. 8. Average profiles obtained for two- and three-person mixtures with PowerPlex® 35GY System. The panel on the left represents the percentage of unique minor contributor alleles obtained from male to female two-person mixtures. The panel on the right represents the percentage of total alleles obtained from male to male to female three-person mixtures. X-axis represents the mixture ratio. Error bars represent ± 1 standard deviation ($n = 20$).

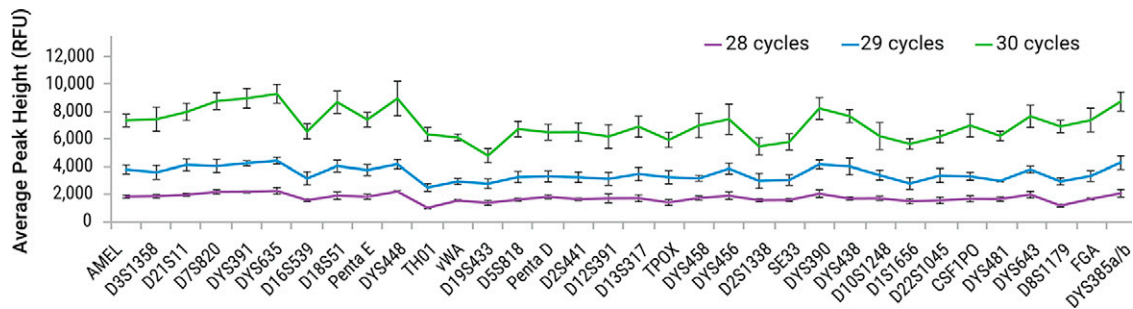


FIG. 9. Average peak heights by locus obtained for 1 ng sample amplified at different cycle numbers with PowerPlex[®] 35GY System. Samples were amplified at 28 (Purple), 29 (Blue), and 30 (Green) cycles. Error bars represent ± 1 standard deviation ($n = 4$).

plays a partial role in the percent recovery of alleles from this type of sample.

One hundred percent of alleles were successfully genotyped for the buccal swabs at all cycle numbers and with both the default and challenging protocol (data not shown). One hundred percent of alleles were successfully genotyped with the challenging sample protocol for all sample types at all cycle numbers tested, apart from buccal on FTA[®] at 23 cycles (Fig. 16).

Overall, increasing the cycle number increased the average peak height with both cycling protocols. An increase in performance of the large molecular weight loci was observed when using the challenging protocol. Smaller molecular weight loci were less affected when using the challenging protocol, except for D8S1179, which showed a slight decrease in peak heights relative to the other loci in the same dye channel (data not shown). The direct amplification data show that different cycle numbers and thermal cycler protocols used with the PowerPlex[®] 35GY System can accommodate a range of outcomes based on material sources.

Reaction volume. Replicates with complete loss of profile were considered an outlier due to sampling variation and not included in calculations. As a result, one replicate was removed from blood and buccal on FTA[®] for both reaction volumes.

Full profiles were obtained from all 12.5 μL reactions. All buccal FTA[®] samples at 6.25 μL reaction volume generated a full profile (Fig. 17). Blood on FTA[®] at the 6.25 μL reaction volume showed a reduction in alleles called, ranging from 16% to 100%. Testing at another site, also with blood on FTA[®], resulted in partial profiles, ranging from 9% to 32% alleles called (data not shown). In general, average peak heights increased for buccal on FTA[®] when amplified at a reduced volume of 6.25 μL . Average peak heights for blood on FTA[®] were variable due to PCR inhibition as indicated by the loss of the QIL peak in these samples (data not shown.)

Conclusions

The PowerPlex[®] 35GY System is an 8-color multiplex designed for the Spectrum CE System. The system

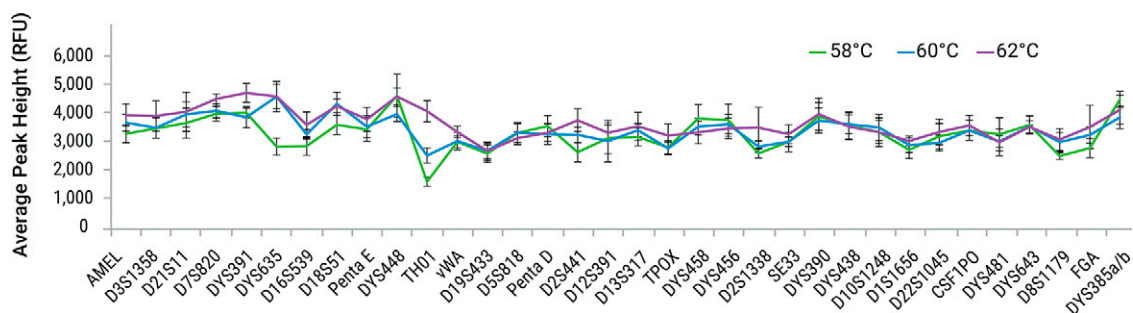


FIG. 10. Average peak heights by locus obtained for 1 ng sample amplified at different annealing temperatures with PowerPlex[®] 35GY System. Samples were amplified with annealing temperatures at 58°C (Green), 60°C (Blue), and 62°C (Purple). Error bars represent ± 1 standard deviation ($n = 4$).

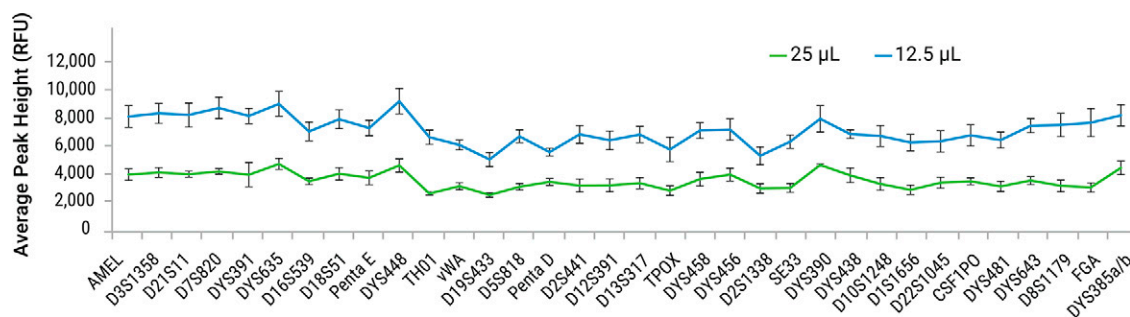


FIG. 11. Average peak heights by locus obtained for 1 ng sample amplified at different reaction volumes with PowerPlex® 35GY System. Samples were amplified at a 25 µL reaction volume (Green) and 12.5 µL volume (Blue). Error bars represent ± 1 standard deviation ($n = 4$).

leverages its multicolor capacity to produce smaller amplicons for autosomal STR loci, thus significantly improving performance with degraded and inhibited samples. Additionally, the system incorporates 11Y-STR loci, allowing data generation for familial searching or mixture interpretation within a single amplification reaction.

The performance of the PowerPlex® 35GY System was examined by five forensic laboratories with adherence to SWGDAM validation guidelines. The strengths and limitations of multiple kit parameters were determined with extracted DNA and a variety of direct amplification substrates. PowerPlex® 35GY performance characteristics were established by experiments performed for this developmental validation study. The results indicate that the PowerPlex® 35GY System has specificity for human DNA, a large dynamic range for template amounts, is precise and accurate, and is robust enough to allow for compatibility with different thermal cycler models. It has also demonstrated the ability to

overcome inhibitors, and to perform with a variety of sample types. Variability in results obtained from direct amplification storage cards may be significantly influenced by storage conditions, techniques of collection, the number and density of donor cells, and the location from which samples are punched. The results of the developmental validation study provide context for forensic laboratories when executing their own internal validation studies. The information documented in this work also verifies the reliability of the PowerPlex® 35GY System, making it suitable for use in a forensic DNA laboratory.

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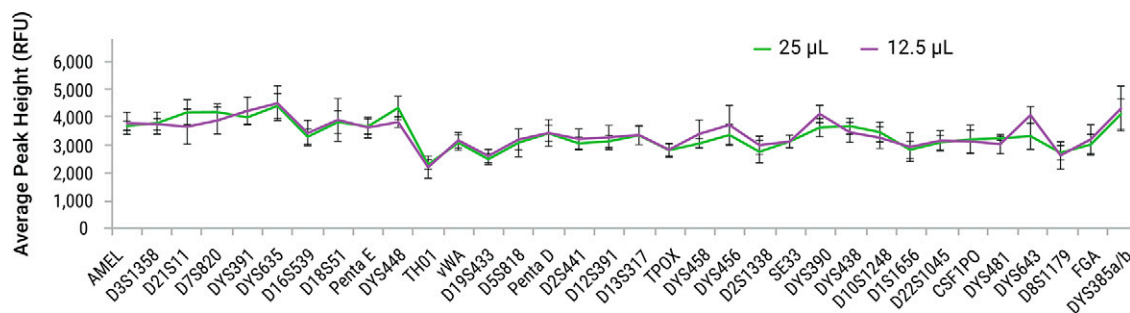


FIG. 12. Average peak heights by locus obtained for 40 pg/µL sample amplified at different reaction volumes with PowerPlex® 35GY System. Samples were amplified at a 25 µL reaction volume (Green) and 12.5 µL volume (Purple). Error bars represent ± 1 standard deviation ($n = 4$).

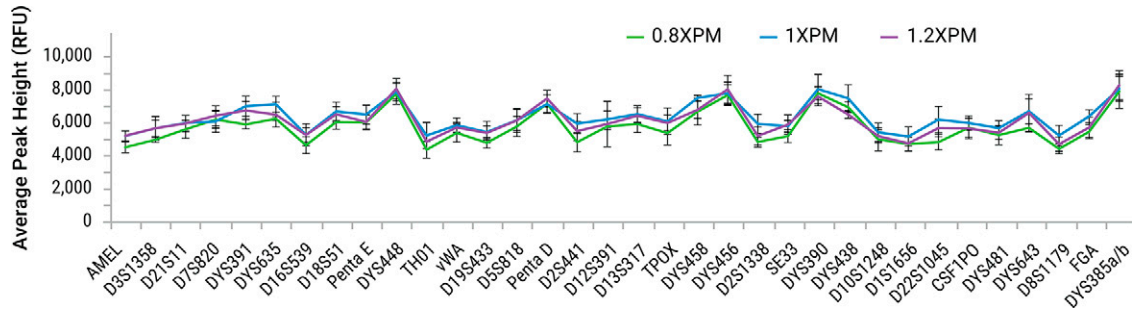


FIG. 13. Average peak heights by locus obtained for a 1 ng sample amplified at different primer pair concentrations with PowerPlex® 35GY System. Samples were amplified with primer pair concentrations of 0.8X (Green), 1.0X (Blue) and 1.2X (Purple). Error bars represent ± 1 standard deviation ($n = 4$).

Authors' Contributions

E.K.G.: Data Curation; Formal Analysis; Investigation; Visualization; and Writing—Original Draft. M.L.: Formal Analysis; Investigation; Visualization; and Writing—Review and Editing. J.A.P.: Formal Analysis; Visualization; and Writing—Review and Editing. J.J.S.: Investigation; and Writing—Review and Editing. J.M.D.: Investigation; and Writing—Review and Editing. P.M.V.: Investigation; Formal Analysis; and Writing—Review and Editing. C.R.S.: Investigation; Formal Analysis; and Writing—Review and Editing. M.O.'D.: Investigation. N.P.S.: Investigation. C.P.: Investigation. L.I.M.: Investigation. M.Z.: Investigation. L.F.: Investigation. J.J.F.-S.: Investigation. J.M.-G.: Investigation. K.A.L.: Investigation. S.L.M.: Investigation. D.R.R.: Investigation. K.D.: Investigation. R.S.M.: Investigation; and Writing—Review and Editing. D.R.S.: Conceptualization. C.J.S.: Conceptualization; and Investigation. J.M.T.: Methodology; Project Administration; Resources; Supervision; and Writing—Review and Editing.

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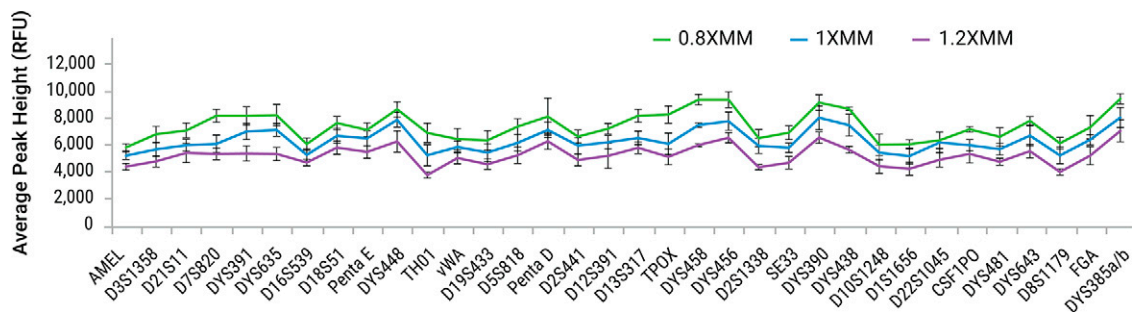


FIG. 14. Average peak heights by locus obtained for a 1 ng sample amplified at different master mix concentrations with PowerPlex® 35GY System. Samples were amplified with master mix concentrations of 0.8X (Green), 1.0X (Blue), and 1.2X (Purple). Error bars represent ± 1 standard deviation ($n = 4$).

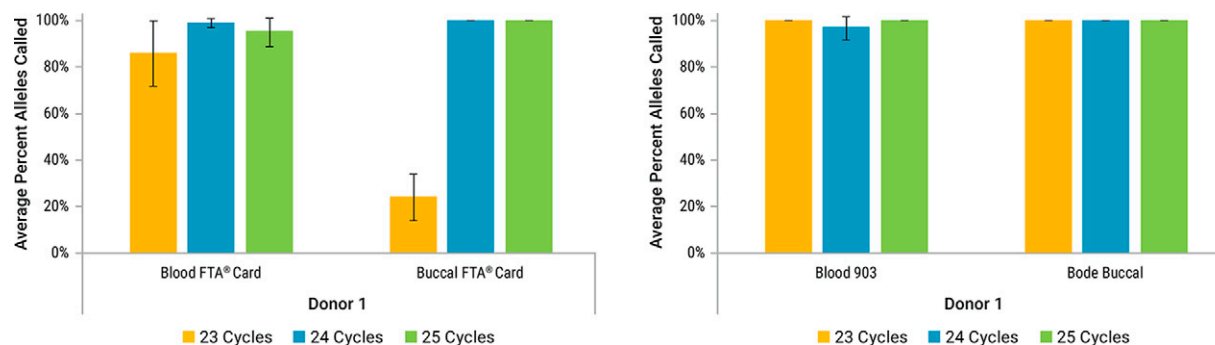


FIG. 15. Average percent profile obtained for lytic storage cards (left panel) and non-lytic storage cards (right panel) amplified at different cycle numbers with PowerPlex® 35GY System using the default protocol. Samples were amplified at 23 (Yellow), 24 (Blue), and 25 (Green) cycles. Error bars represent ± 1 standard deviation ($n = 4$; Buccal FTA® $n = 3$).

commercial software, instruments, and materials are identified to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by NIST, nor does it imply that any of the materials, instruments, or equipment identified are necessarily the best available for the purpose.

The work contributed by NIST using U.S. population samples has been reviewed and approved by the NIST Research Protections Office. This study was determined to be “not human subjects research” (often referred to as research not involving human subjects) as defined in the U.S. Department of Commerce Regulations, 15 CFR 27, also known as the Common Rule (45 CFR 46, Subpart A), for the Protection of Human Subjects by the NIST Human Research Protections Office and therefore not subject to oversight by the NIST Institutional

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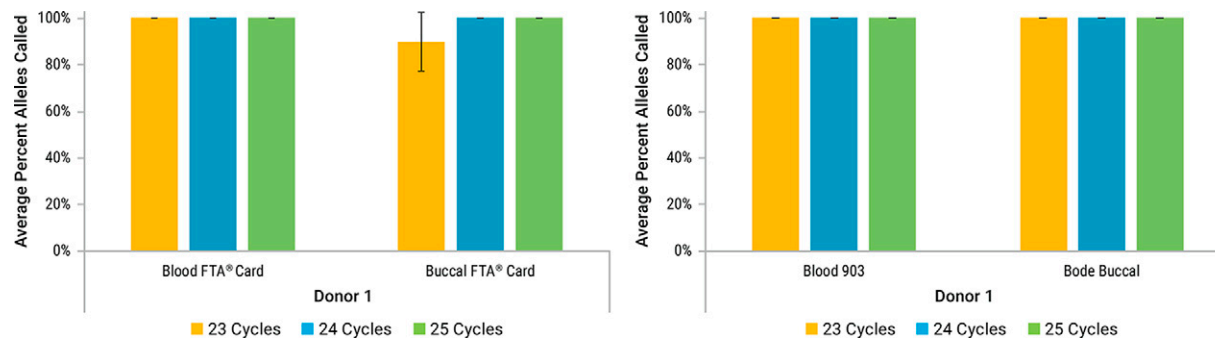


FIG. 16. Average percent profile obtained for lytic storage cards (left panel) and non-lytic storage cards (right panel) amplified at different cycle numbers with PowerPlex® 35GY System using the challenging sample protocol. Samples were amplified at 23 (Yellow), 24 (Blue), and 25 (Green) cycles. Error bars represent ± 1 standard deviation ($n = 4$; Buccal FTA® $n = 3$).

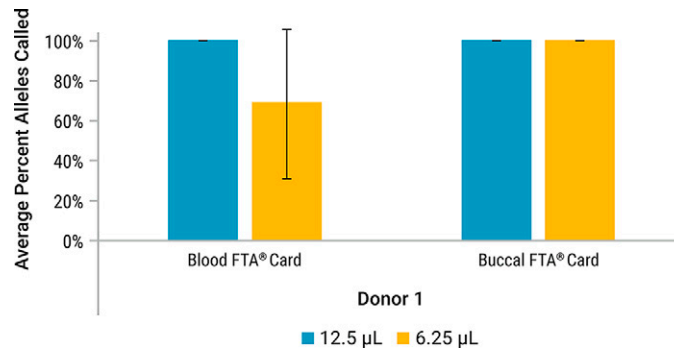


FIG. 17. Average percent profile obtained for lytic storage cards amplified at different reaction volumes with PowerPlex® 35GY System using the default protocol. Samples were amplified at a 12.5 µL reaction (Blue) and a 6.25 µL reaction (Yellow). Error bars represent ± 1 standard deviation ($n = 3$).

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Supplementary Material

Supplementary Figure S1
 Supplementary Figure S2
 Supplementary Figure S3
 Supplementary Figure S4
 Supplementary Figure S5
 Supplementary Figure S6
 Supplementary Figure S7
 Supplementary Figure S8

References

- Promega Corporation. PowerPlex® 35GY System for Use on the Spectrum CE System Technical Manual #TMD077. Madison, WI; 2023, July. Available from: <https://www.promega.com/resources/protocols/technical-manuals/d0/powerplex-35gy-system-for-use-on-the-spectrum-ce-system-protocol/> [Last accessed: July, 2023].
- Scientific Working Group on DNA Analysis Methods. Validation Guidelines for DNA Analysis Methods. May 12, 2016. Available from: https://www.swgdam.org/_files/ugd/4344b0_813b241e8944497e99b9c45b163b76bd.pdf [Last accessed: February, 2023].
- Promega Corporation. Maxwell® FSC DNA IQ™ Casework Kit Technical Manual #TM499. Madison, WI; August, 2021. Available from: <https://www.promega.com/resources/protocols/technical-manuals/101/maxwell-fsc-dna-iq-casework-kit-protocol/> [Last accessed: February, 2023].
- Promega Corporation. PowerQuant® System Technical Manual #TMD047. Madison, WI; revised August, 2022. Available from: <https://www.promega.com/resources/protocols/technical-manuals/d0/powerquant-system-protocol/> [Last accessed: February, 2023].
- Applied Biosystems. PrepFiler™ and PrepFiler™ BTA Automated Forensic DNA Extractions Kits User Guide, Publication 4463349. ThermoFisher Scientific, Warrington, UK; revision E. Available from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_096097.pdf [Last accessed: June, 2023].
- Qiagen. EZ1&2® DNA Investigator® Kit Handbook. August, 2022. Available from: <https://www.qiagen.com/us/resources/download.aspx?id=46064856-1b88-4b27-a825-d3f61e06c08&lang=en> [Last accessed: May, 2023].
- Promega Corporation. Maxwell® RSC ccfDNA Plasma Kit Technical Manual #TM454. Madison, WI; January, 2021. Available from: https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/maxwell-rsc-ccfdna-plasma-kit-protocol.pdf?rev=8315d29ece2840809b84065d9ecc6056&sc_lang=en [Last accessed: June, 2023].
- Steffen CR, Coble MD, Gettings KB, et al. U.S. population data for 29 autosomal STR loci. *Forensic Sci Int Genet* 2013;31(3):e36–e40; doi: 10.1016/j.fsigen.2012.12.004
- Steffen CR, Coble MD, Gettings KB, et al. Corrigendum to 'U.S. population data for 29 autosomal STR loci'. *Forensic Sci Int Genet* 2017;31: E36–E40; doi: 10.1016/j.fsigen.2017.08.011
- National Institute of Standards and Technology. Standard Reference Material® 2391d PCR-Based DNA Profiling Standard Certificate of Analysis. Gaithersburg, MD; May 12, 2023. Available from: <https://tsapps.nist.gov/srmext/certificates/2391d.pdf> [Last accessed: August, 2023].
- Promega Corporation. PunchSolution™ Kit Technical Manual #TMD038. Madison, WI; September, 2016. Available from: <https://www.promega.com/resources/protocols/technical-manuals/d0/punchsolution-kit-protocol/> [Last accessed: February, 2023].
- Promega Corporation. SwabSolution™ Kit Technical Manual #TM037. Madison, WI; April, 2021. Available from: <https://www.promega.com/resources/protocols/technical-manuals/d0/swabsolution-kit-protocol/> [Last accessed: February, 2023].
- Loten ML, Ewing MM, Picciano JA, et al. Developmental Validation of the Spectrum CE System for Fragment Analysis. Promega Corporation White Paper #WP130, June, 2023. Available from: <https://promega.widen.net/s/t8mxmk2kq/spectrum-developmental-validation-white-paper-wp130> [Last accessed: January, 2024].
- Promega Corporation. Spectrum CE System Operating Manual #TMD052. Madison, WI; July, 2022. Available from: <https://www.promega.com/resources/protocols/technical-manuals/d0/spectrum-ce-system-operating-manual/> [Last accessed: February, 2023].
- Promega Corporation. PowerPlex® Matrix Standards for Use on the Spectrum CE System Technical Manual #TMD068. Madison, WI; January, 2023. Available from: <https://www.promega.com/resources/protocols/technical-manuals/d0/powerplex-matrix-standards-spectrum-ce-system-protocol/> [Last accessed: February, 2023].
- Promega Corporation. GeneMarker® HID Software for Spectrum CE Systems User Manual #TM555. Madison, WI; January, 2023. Available from: <https://www.promega.com/resources/protocols/technical-manuals/500/genemarker-hid-software-for-spectrum-ce-systems-user-manual/> [Last accessed: February, 2023].
- Gettings KB, Borsuk LA, Steffen CR, et al. Sequence-based U.S. population data for 27 autosomal STR loci. *Forensic Sci Int Genet* 2018;37: 106–115; doi: 10.1016/j.fsigen.2018.07.013
- Butler JM, Schoske R, Vallone PM, et al. Allele frequencies for 15 autosomal STR loci on U.S. Caucasian, African American, and Hispanic populations. *J Forensic Sci* 2003;48(4):908–911.
- Levedakou EN, Freeman DA, Budzynski MJ, 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(3):736–761.
- Lareu MV, Barral S, Salas A, et al. Sequence variation of a hypervariable short tandem repeat at the D1S1656 locus. *Int J Legal Med* 1998;111(5): 244–247; doi: 10.1007/s004140050161

21. Butler JM, Hill CR, Kline MC, et al. The single most polymorphic STR Locus: SE33 performance in U.S. populations. *Forensic Sci Int Genet Supplement Series* 2009;2(1):23–24; doi: 10.1016/j.fsigss.2009.08.173
22. Butler JM, Hill CR, Coble MD. Variability of New STR Loci and Kits in US Population Groups. Promega Corporation Profiles in DNA. 2012. Available from: <https://www.promega.com/resources/profiles-in-dna/2012/variability-of-new-str-loci-and-kits-in-us-population-groups/>
23. Steffen CR, Huszar TI, Borsuk LA, et al. A multi-dimensional evaluation of the 'NIST 1032' sample set across four forensic Y-STR multiplexes. *Forensic Sci Int Genet* 2022;57:102655; doi: 10.1016/j.fsigen.2021.102655
24. Geppert M, Edelmann J, Lessig R. The Y-chromosomal STRs DYS481, DYS570, DYS576 and DYS643. *Legal Medicine* 2009;11:S109–S110; doi: 10.1016/j.legalmed.2009.01.063
25. Rodig H, Roewer L, Gross A, et al. Evaluation of haplotype discrimination capacity of 35 Y-chromosomal short tandem repeat loci. *Forensic Sci Int* 2008;174(2–3):182–188; doi: 10.1016/j.forsciint.2007.04.223
26. Willuweit S, Roewer L. Y chromosome haplotype reference database (YHRD): update. *Forensic Sci Int Genet* 2007;1(2):83–87; doi: 10.1016/j.fsigen.2007.01.017