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Developmental validation of the PowerPlex® 18D System, a rapid STR multiplex for analysis of reference samples

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ABSTRACT

As short tandem repeat markers remain the foundation of human identification throughout the world, new STR multiplexes require rigorous testing to ensure the assays are sufficiently robust and reliable for genotyping purposes. The PowerPlex® 18D System was created for the direct amplification of buccal and blood samples from FTA® storage cards and reliably accommodates other sample materials. The PowerPlex® 18D System allows simultaneous amplification of the 13 CODIS loci and amelogenin along with four additional loci: Penta E, Penta D, D2S1338, and D19S433. To demonstrate suitability for human identification testing, the PowerPlex® 18D System was tested for sensitivity, concordance, inhibitor tolerance, and performance with thermal cycling and reaction condition variation following SWGDAM developmental validation guidelines. Given these results, PowerPlex® 18D System can confidently be used for forensic and human identification testing.

1. Introduction

To achieve greater efficiency, offender databasing laboratories have sought technology improvements to handle greater throughput requirements. Automation, megaplex STR assays, and multicapillary electrophoresis instruments have become common over the last decade, allowing significant increases in throughput. More recently, direct amplification has become attractive to laboratories because it can dramatically shorten processing times for genotyping laboratories. By removing the DNA purification step significant reductions in labor and reagent costs can be realized. Because of its long-term storage capabilities at room temperature, FTA® paper (GE Healthcare/Whatman, Waukesha, WI) has been a popular storage method and material for direct amplification. However, chemicals in the paper intended to lyse cells and stabilize DNA can inhibit PCR and typically must be removed for successful STR amplification. The PowerPlex® 18D System was developed to address the challenges associated with direct amplification, especially from FTA® paper, and furthers efficiency through a rapid thermal cycling protocol.

The PowerPlex® 18D System allows analysis of the 13 CODIS loci and amelogenin along with four additional loci: Penta E, Penta D, D2S1338, and D19S433. The Penta E and Penta D or D2S1338 and D19S433 loci have been routinely used alongside the 13 CODIS loci for over ten years as part of the PowerPlex® 16 System (Promega, Madison, WI) and the AmpFℓSTR® Identifiler® PCR Amplification Kit (Life Technologies/Applied Biosystems, Carlsbad, CA). Inclusion of all four of these loci increases discrimination as well as supports data sharing between current databases. The

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PowerPlex® 18D System enables direct amplification of buccal and blood samples from FTA® paper, and reliably accommodates other sample materials. Additionally, the rapid thermal cycling protocol reduces the cycling time to approximately one and a half hours. The five-color system is compatible with the Applied Biosystems 3130 and 3500 Genetic Analyzers, and 3730 DNA Analyzer.

Validation of the PowerPlex® 18D System followed SWGDAM developmental validation guidelines [1]. Studies were performed to demonstrate the effectiveness and performance of the PowerPlex® 18D System for sensitivity, concordance, robustness with varied thermal cycling and reaction conditions, and resilience against several contaminating substances. The PowerPlex® 18D System was demonstrated to be a robust and reliable identification assay fit for human identification purposes.

2. Materials, methods, and techniques

2.1. Samples

Testing was performed with buccal FTA®, blood FTA®, and extracted DNA samples. Buccal FTA® samples were created from buccal swabs collected from volunteers or unidentified offenders and transferred by contact to Indicating FTA® paper (GE Healthcare/Whatman). Blood FTA® samples were created following the product instructions for FTA® Micro Cards. A 60 μl or 125 μl volume of whole blood anti-coagulated with K_2EDTA was spotted on FTA® paper. Extracted DNA was purified from whole blood by organic extraction, and quantified using A^{260} absorbance.

Concordance testing was performed using a total of 418 samples of various types. Samples included FTA® paper, S&S 903 paper (GE Healthcare/Whatman), Bode Buccal DNA Collectors™ (Bode Technologies, Lorton, VA), Omni Swabs™ (GE Healthcare/Whatman) and extracted DNA. Direct amplifications were performed using 1.2 mm disks punched from FTA® paper and S&S 903 paper and added directly into reactions or 1.2 mm disks from Bode Buccal DNA Collectors™ pretreated with Bode PunchPrep™ (Bode Technologies). Alternatively, reactions were performed using 2 μl of an Omni Swab™ extract generated with prototype SwabSolution™ (Promega) or 1 μl of extracted DNA.

Species studies were conducted using purified DNA from higher primates, domestic animals, and common microorganisms amplified for 30 cycles. One nanogram was used for primates (privately obtained collection) and bacteria (ATCC, Manassas, VA), ten nanograms for domestic animals (Novagen, Madison, WI) (Zyagen, San Diego, CA), and two nanograms for yeast (ATCC). Species include: chimpanzee, gorilla, orangutan, cow, dog, chicken, rabbit, cat, deer, horse, pig, *Candida albicans*, *Saccharomyces cervisae*, *Escherichia coli*, *Streptococcus salivarius*, *Acinetobacter lwoffii*, *Streptococcus mutans*, *Streptococcus mitis*, *Fusobacterium nucleatum*, *Enterococcus faecalis*, *Lactobacillis acidophilis*, *Staphylococcus epidermidis*, and *Micrococcus luteus*.

Several common forensic inhibitors were tested including hematin (Sigma–Aldrich, St. Louis, MO), humic acid (Sigma–Aldrich), tannic acid (Sigma–Aldrich), chocolate, tobacco, sugar, and coffee as well as common purification contaminants including EDTA (Sigma–Aldrich), ethanol, and SDS (Fisher Scientific, Pittsburgh, PA). No weights were available for the chocolate, tobacco, or sugar samples.

2.2. Amplification methods

Amplifications were performed on a GeneAmp® PCR System 9700 (Life Technologies/Applied Biosystems) using commercially available PowerPlex® 18D reagents. Final reaction volume was 25 μl. Each direct amplification used a 1.2 mm diameter punch. Except where noted, buccal FTA® reactions used two punches and blood FTA® reactions used one punch. When amplifying extracted DNA 5ng was typically used. Negative controls were included in all experiments, and consisted of reaction reagents only (Supplemental Fig. 1). The thermal cycling method listed in the manufacturer's technical manual [2] was followed, unless specifically mentioned otherwise: 96 °C for 2 min, 94 °C for 10 s and 60 °C for 1 min for 27 cycles, a final extension at 60 °C for 20 min, and a 4 °C soak.

2.3. Electrophoresis and analysis

Electrophoresis and detection methods followed the manufacturer's technical manual. Samples were prepared for capillary electrophoresis using 1 μl CC5 ILS 500, 10 μl Hi-Di™ formamide (Life Technologies/Applied Biosystems), and 1 μl sample or allelic ladder. Injection conditions for the 3130*xl* Genetic Analyzer and 3730 DNA Analyzer (Life Technologies/Applied Biosystems) instruments were 3 kV and 5 s. The 3500xL Genetic Analyzer used 1.2 kV and either 12 or 24 s for injection. Any deviations from these injection conditions are noted in the results. The analysis utilized a minimum threshold of 50 RFU for the 3130*xl* Genetic Analyzer, 175 RFU for the 3500xL Genetic Analyzer, and 150 RFU for the 3730 DNA Analyzer except for the concordance study analysis, which used individual laboratory guidelines with thresholds ranging from 50 to 150 RFU. Lastly, a 20% global stutter filter was applied for all studies with the exception of the sensitivity and species studies.

3. Results

3.1. Sensitivity studies

Because the PowerPlex® 18D System can accommodate purified template DNA and direct amplification materials, sensitivity studies were performed using both extracted DNA and FTA® materials. Extracted DNA was tested with two DNA dilution series with final amounts of 0.05 ng, 0.1 ng, 0.2 ng, 1 ng, and 5 ng per reaction. The DNA titrations were characterized at multiple cycle numbers: 26, 27, 28, and 30 cycles, and analyzed with no global filter. Sensitivity was appropriate for a typical direct amplification sample and the lower cycle number listed in the technical manual (27 cycles), while increased cycle number improved sensitivity. Complete expected profiles (all expected alleles at each locus were present) were observed with ≥1 ng extracted DNA using 27 or 28 cycles, and ≥0.1 ng using 30 cycles (Table 1). Average locus peak height ratios at ≥1 ng were ≥90% (Supplemental Fig. 2). Allelic imbalances (peak height ratios of <70%) due to stochastic effects [3–5] were observed with 0.05 ng, 0.1 ng, and occasionally 0.2 ng of template DNA (Supplemental Table 1).

Sample distribution can be variable across direct amplification materials [6]. In contrast to extracted samples in which the entire sample is used to create a homogenous extracted solution, direct amplification uses a small portion from a heterogeneous, dispersed sample. With this distribution, sampling contributes to peak height variation. Although a single punch contains adequate material for reliable genotyping, a laboratory may chose to include

Table 1
Percentage of complete expected profiles observed (complete profiles observed/replicate number) at each extracted DNA quantity.

	26 cycles	27 and 28 cycles	30 cycles
50 pg	0%	0%	11%
0.1 ng	0%	0%	83%
0.2 ng	0%	0%	94%
1 ng	100%	100%	100%
5 ng	100%	100%	100%
n=	6	18	18

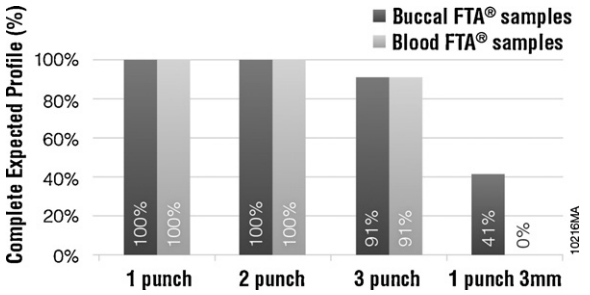

Fig. 1. Percentage of completeprofiles varying buccal or blood FTA® punch number and size (buccal FTA® samples $n = 22$, blood FTA® samples $n = 11$).

multiple punches within a reaction to overcome variation or potential failures due to sampling. Varied punch number and size was evaluated using buccal and blood FTA® samples to determine the impact of increased FTA® amounts on the PowerPlex® 18D System. Reactions included one, two, or three 1.2 mm buccal FTA® punches or a single 3.0 mm buccal FTA® punch. Samples containing one or two 1.2 mm punches reliably gave complete profiles (alleles at all loci) (Fig. 1). With the punch number increased to three 1.2 mm punches, an occasional failure was observed. Reactions containing a single 3.0 mm punch size showed minimal amplification. Failures with three 1.2 mm punches and one 3.0 mm punch are likely due to the increased amount of inhibiting substances carried in the FTA® paper overwhelming the reaction.

3.2. FTA® sample stability and performance

Stable long-term sample storage affords the opportunity for retesting after several years. Five buccal FTA® and five blood FTA® samples were assayed to confirm complete and accurate profiles after storage on FTA® for more than eight years. An additional five blood FTA® samples were tested which were stored as liquid blood for approximately six years, then on FTA® paper for two years (eight years total). All samples gave full profiles which were concordant with previous results at the loci included in the PowerPlex® 16 System (Fig. 2).

3.3. Reproducibility

Sample panels from two donors consisting of buccal FTA® cards, blood FTA® cards, and DNA extracts were distributed to five independent laboratories. Expected genotypes were obtained from all replicates of each sample type at each of the five independent sites (Supplemental Fig. 3). Sample analysis performed with multiple instruments, 3130, 3130xl, or 3500xL Genetic Analyzers, and GeneMapper® *ID* 3.2 and GeneMapper® *ID-X* (Life Technologies/Applied Biosystems) analysis software versions gave matching genotypes.

3.4. Accuracy

Commercially available PCR standards, Standard Reference Material® 2391b PCR-based DNA Profiling Standards (NIST), were evaluated to confirm genotype accuracy. Extracted DNA samples 1 through 10 were amplified using 1 ng and 30 cycles or 5 ng and 27 and 28 cycles. Samples 11 and 12 arrived as cells on filter paper, and were amplified both as extracted DNA (5 ng) and as a direct amplification (1.2 mm punch). Full concordance with the genotypes listed in the Certificate of Analysis for Standard Reference Material® 2391b was obtained for all samples at two independent sites after analysis on 3130*xl* and 3500xL Genetic Analyzer capillary electrophoresis platforms.

3.5. Precision

To demonstrate sizing precision, allelic ladder was run on multiple capillary electrophoresis instruments across multiple capillaries. Data from a total of 87 allelic ladder samples was collected consisting of 15 samples on the 3130 Genetic Analyzer, 32 samples on the 3130*xl* Genetic Analyzer, 32 samples on the 3500xL Genetic Analyzer, and 8 samples on the 3730 DNA Analyzer. Size variability was determined by calculating the standard deviation for each allele on each instrument.

Very little variation was seen in the sizing and migration of the allelic ladder at each locus across the capillary electrophoresis instruments tested (Fig. 3, Supplemental Figs. 4–6). Three average standard deviations were appreciably less than 0.2 bases for each

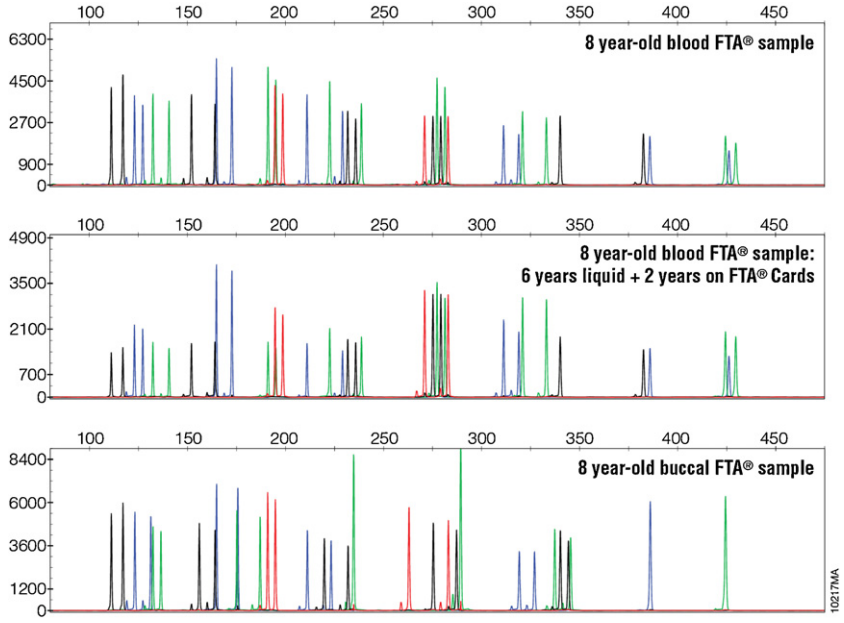

Fig. 2. Electropherograms from 8 year-old blood FTA® sample (top), blood FTA® sample from aged liquid blood (center), and 8 year-old buccal FTA® (bottom). Samples were analyzed on a 3130*xl* Genetic Analyzer with a 3 kV 5 s injection ($n = 5$).

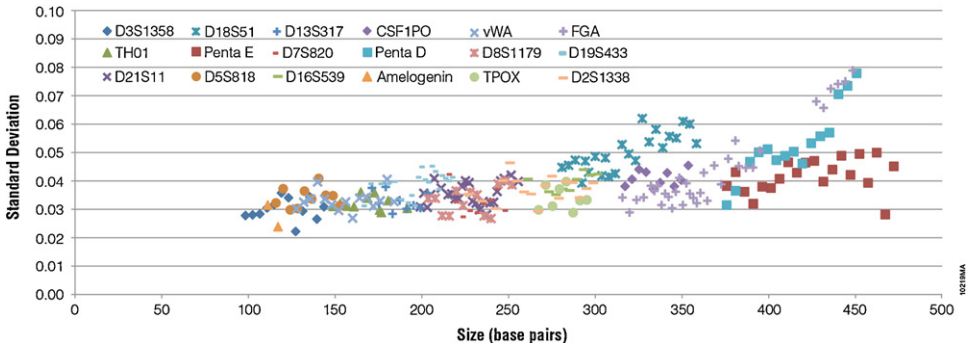

Fig. 3. Sizing variation for all allelic ladder alleles on the 3130*xl* Genetic Analyzer using a 3 kV 5 s injection (n = 32). Similar plots for the 3130 and 3500xL Genetic Analyzers, and 3730 DNA Analyzer can be viewed in Supplemental Figs. 2–4.

locus on the 3130, 3130*xl*, and 3500xL instruments (Supplemental Table 2). The 3730 DNA Analyzer produced more variation than the other instruments. However, three average standard deviations were less than 0.35 bases for each locus on this instrument. Variation from all instruments was significantly less than the ±0.5 bases allowed by the allele-calling bin window provided in the PowerPlex® 18D System bin file for GeneMapper® *ID* 3.2 and *ID-X* software. The median locus standard deviation was 0.036, 0.035, 0.042, and 0.065 bases on the 3130, 3130xl, 3500xl, and 3730 respectively. Not surprisingly, the highest locus variation seen was at the largest loci, Penta D and Penta E.

3.6. Stutter

Because stutter peaks [7,8] can complicate interpretation, a locus-specific stutter filter can be applied during analysis to remove labels from stutter-position peaks with relative peak intensities equal to or lower than those previously observed. Stutter filter ratios were calculated by averaging ratios from 187 extracted DNA samples and 50 blood FTA® samples at alleles with peak heights less than 6000 RFU. The average stutter plus three standard deviations (Table 2) was used as the stutter filter in the PowerPlex® 18D Panel, Bins, and Stutter files for GeneMapper® *ID* 3.2 and *ID-X*. The highest ratios (stutter peak height/allele peak height) were seen at D3S1358, D21S11, and D2S1338 with none greater than 8.8%. The loci with the lowest stutter ratios were TH01, Penta D, and TPOX. Raw data for the stutter calculations can be reviewed in greater detail in Supplemental Table 3.

Table 2
Average stutter ratio (stutter peak height/allele peak height). The stutter filter has been set as the average stutter ratio plus three standard deviations.

	n=	Average	Mean + 3 std dev
D3S1358	195	0.088	0.13
TH01	77	0.023	0.04
D21S11	262	0.084	0.13
D18S51	271	0.072	0.13
Penta E	128	0.038	0.08
D5S818	111	0.074	0.13
D13S317	179	0.061	0.11
D7S820	126	0.051	0.11
D16S539	233	0.072	0.12
CSF1PO	197	0.058	0.10
Penta D	86	0.023	0.05
Amelo	–	–	–
vWA	86	0.076	0.13
D8S1179	66	0.066	0.12
TPOX	93	0.035	0.07
FGA	209	0.071	0.12
D19S433	56	0.062	0.11
D2S1338	190	0.081	0.14

3.7. Allele frequency

The loci included in the PowerPlex® 18D System have been tested extensively in previous multiplex products including the PowerPlex® 16 System and the AmpFℓSTR® Identifiler® PCR Amplification Kit. No additional population studies were performed in the present developmental validation. Population studies to determine allele frequencies have been published for the CODIS core loci plus D2S1338 and D19S433 [9,10] along with the CODIS core loci plus Penta D and Penta E [11,12]. Additionally, a list of 365 global references on subsets of the 18 loci included in the PowerPlex® 18D System is available [13].

3.8. Concordance

The design of the PowerPlex® 18D System conserves the proven primer-binding sites from previously released Promega STR systems, amelogenin, Penta D, Penta E, and the 13 CODIS loci from the PowerPlex® 16 System and D2S1338 and D19S433 from the PowerPlex® ESI System. High confidence was placed in the PowerPlex® 18D System because of existing concordance data [14,15]. Nevertheless, the new combination of loci and improved master mix for direct amplification warranted verification.

Concordance was confirmed by comparing a total of 418 PowerPlex® 18D genotypes generated from diverse sample materials to profiles from existing, NDIS-approved multiplexes including the PowerPlex® 16 System and the AmpFℓSTR® Identifiler® PCR Amplification Kit. The variety of sample types included FTA® (blood and buccal), S&S 903 (blood), Bode Buccal DNA Collectors™, Omni Swabs™, and extracted DNA. Direct amplifications were performed using 1.2 mm disks punched from FTA® and S&S 903 added directly to reactions or 1.2 mm disks from Bode Buccal DNA Collectors™ pretreated with Bode PunchPrep™. Alternatively, reactions were performed using 2 μl of an Omni Swab™ extract generated with prototype SwabSolution™ (Promega) or 1 μl of extracted DNA. Profiles were generated on 3130 and 3130*xl* Genetic Analyzers as well as a 3730 DNA analyzer.

Of 418 full profile samples, 415 samples were concordant with prior laboratory results (Table 3). Three discordant calls were detected in the 6688 loci compared (0.04%). In one individual, a D2S1338 23, 23 genotype with the Identifiler® Kit presented as a 17, 23 with the PowerPlex® 18D System. For a second individual, the PowerPlex® 16 System produced a 12, 12 at CSF1PO, while the PowerPlex® 18D System and the Identifiler® Kit produced a 9, 12. Sequencing performed on the D2S1338 and CSF1PO samples uncovered single nucleotide mutations in both cases and confirmed the PowerPlex® 18D System genotype. In the case where the PowerPlex® 16 System and the PowerPlex® 18D System differed, formulation differences between the two systems and the large template amount used with the PowerPlex® 18D System

Table 3
Number of full profile samples compared for concordance across four independent laboratories using various collection and storage materials. NJSP, New Jersey State Police; AZ DPS, Arizona Department of Public Safety; NIST, National Institute of Standards and Technology; TX DPS, Texas Department of Public Safety.

Sample	NJSP	AZ DPS	NIST	TX DPS	Total	System used for comparison:
Buccal FTA® samples	47				47	Identifiler® Direct
Bode Buccal DNA Collector™ (PunchPrep™ pretreatment)		77			77	Identifiler®
Blood FTA® samples			50		50	Identifiler® and PowerPlex® 16
Blood on S&S 903 paper			50			(Same donors as blood FTA®)
Extracted DNA			187		187	Identifiler® and PowerPlex® 16
Omni Swab™ (prototype SwabSolution pretreatment)				57	57	Identifiler®
					418	

likely allowed the PowerPlex® 18D System to overcome a primer binding site mutation better than the PowerPlex® 16 System. A third individual genotyped 13, 13 at D19S433 with the Identifiler® Kit while the PowerPlex® 18D System generated a 13, 15 genotype. Sequencing of the D19S433 discordant sample exposed a four-nucleotide deletion flanking an allele containing 16 repeats. The PowerPlex® 18D System successfully amplified the allele, though the allele was called as a 15 allele due to the four-nucleotide deletion. All three discordant calls were due to a null allele in the system used for comparison, not with the PowerPlex® 18D System.

3.9. Cycle number

Direct amplification samples, either on storage cards or swabs, contain variable amounts of material due to donor shedding levels, collection techniques, and sample type. This variability can be observed directly in STR analysis as a wide range of sample signal strengths within a typical sample. To improve rerun rates due to signal strength, cycle number optimization can identify conditions which either fit common sample intensities or allow detection of the weakest samples. The cycle number evaluation included amplifications at 25, 27, 30, and 32 cycles using two donors each for blood FTA® samples, buccal FTA® samples, and extracted DNA (1 and 5 ng). The technical manual recommends using 27 cycles with 5 ng of DNA. As expected, peak heights rose with increasing cycle number (Fig. 4). Full profiles were reliably seen with buccal FTA®, blood FTA®, and 5 ng of extracted DNA with 27 cycles or more. At 30 or more cycles buccal and blood FTA® samples gave several off-scale peaks. Injection conditions may also be altered to increase or decrease signal strength.

3.10. Final extension

A final 60 °C extension step in PCR is known to improve adenylation of the amplified fragments [16,17]. Without the final extension step fragments would often have $n-1$ peaks (minus A) or shoulders, which can complicate data interpretation. Excess-template samples common with direct amplification can also affect adenylation. A final extension study included a final extension step of 0, 10, or 20 min at 60 °C using blood FTA® samples, buccal FTA® samples, and 5 ng of extracted 2800 M Control DNA. Minus A shoulders and peaks were manually counted. Blood FTA® samples yielded the most minus A, followed by buccal FTA® and extracted DNA sample types. A final extension step of 10 min removed the majority of the minus A peaks. Increasing the final extension from 0 to 10 min reduced minus A from 17% to 4% in blood FTA® samples (Supplemental Fig. 7). Lengthening the extension step to 20 min was not detrimental to the results, and could further reduce minus A peaks in heavy samples or with lower quality PCR plates.

3.11. Annealing temperature

Large deviations from the recommended annealing temperature have the potential to alter the performance of an STR system. The optimal annealing temperature was evaluated by testing FTA® sample and extracted DNA (5 ng) amplifications with annealing temperatures ±2 °C and ±4 °C from the recommended annealing temperature of 60 °C (56 °C, 58 °C, 60 °C, 62 °C and 64 °C). No allelic dropout was seen for all sample types at temperatures from 56 °C to 64 °C (Supplemental Fig. 8). The optimal annealing temperature of 60 °C was verified since artifacts appeared at 56 °C and peak heights decreased at particular loci at 64 °C. At an annealing temperature of 56 °C observed artifacts included: 119, 127, 198, 237, 275, 277, 346, 354, and 405 bases in FAM, 129 and 349 bases in JOE, and 135 bases in ET-CXR. Artifacts were generally higher with extracted DNA than buccal FTA®. At 64 °C reduced peak heights were observed at amelogenin, D8S1179, and D2S1338. Though optimal results are seen at the recommended annealing temperature, the PowerPlex® 18D System was tolerant to some annealing temperature variation.

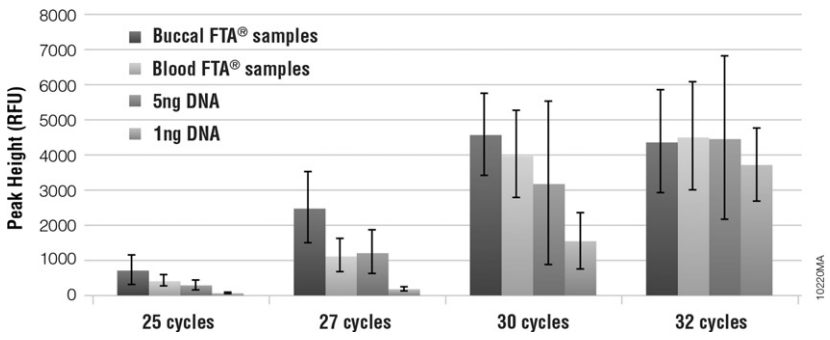

Fig. 4. Average peak heights with increasing cycle numbers across multiple sample types.

Table 4
Highest concentrations of PCR inhibitor where full profiles were observed with both replicates. All concentrations were final concentrations in the 25 μl reaction ($n = 2$, EDTA $n = 3$) NA = not tested.

	Hematin	Humic	Tannic	EDTA	Ethanol	SDS
DNA	800 μM	200 ng/μl	>500 ng/μl	0.5 mM	8%	0.8 mg/ml
Buccal 2 punches	500 μM	150 ng/μl	150 ng/μl	1.0 mM	2.5%	0.4 mg/ml
Blood 1 punch	NA	NA	NA	0.5 mM	NA	NA

3.12. Reduced reaction volumes

Direct amplification materials are typically cruder than purified samples and often include inhibitors from FTA® paper or whole blood. Adding the same amount of direct amplification material recommended for a full reaction into a reduced reaction volume can negatively impact performance. Reduced reaction volumes were tested using one or two punches buccal FTA® sample, blood FTA® sample, and 5 ng of extracted DNA at 25 μl, 18.75 μl, 12.5 μl, and 6.25 μl reaction volumes. Examination showed reaction volumes ≥12.5 μl produced reliable full profiles with one punch buccal FTA® sample (Supplemental Fig. 9), one punch blood FTA® sample, or DNA from 1 to 5 ng. Two punches of buccal FTA® sample with reaction volumes less than 25 μl demonstrated variable success. Reliable performance from reduced reaction volume is achievable; however adjustments in sample input may be necessary.

3.13. Reaction components

Primer, DNA polymerase, and magnesium concentrations in large multiplex systems are essential [18,19] and highly optimized for consistent and robust results. Component concentrations ±25% from the manufactured formulation were evaluated using multiple sample types, including two donors each for buccal FTA® samples, blood FTA® samples, and extracted DNA (5 ng). The PowerPlex® 18D System was sufficiently robust to handle component concentration changes 25% above or below the recommended concentrations with minor impact on results with all the sample types evaluated. Full profiles were seen at all primer, DNA polymerase, and magnesium concentrations. Further, a corresponding 25% change in peak heights was seen with changing primer concentrations (Supplemental Fig. 10). With altered magnesium concentrations, an equivalent 20–25% change in peak heights was observed (Supplemental Fig. 11). Changes in polymerase concentration, however, produced little change in peak height (Supplemental Fig. 12).

3.14. Species specificity

Although the PowerPlex® 18D System is intended for human single source samples, biological materials from other species may contaminate samples. Microorganism species cross-reactivity is especially relevant since typical samples originate from microbe-rich environments such as the oral cavity. Microbial as well as primate and domestic animal DNAs were characterized using 30 cycles, three more cycles than recommended for a typical sample. One nanogram was tested for primates and bacteria, 10 ng for domestic animals, and 2 ng for yeast. The primate species DNA yielded many peaks both on and off-ladder, similar to results with other STR multiplexes. Multiple off ladder peaks distinguish the resulting profile from a human profile (Supplemental Fig. 13). Sporadic peaks were observed with domestic animal DNA. Prominent peaks included 107 bp in TMR for cow, horse, dog, pig, and deer (horse, dog, pig, and deer not shown), 440 bp in JOE (atypical peak shape) for chicken, and 111 bp in FAM for rabbit. The microorganisms gave minimal cross reactivity. The only detectable peaks were 440 bp in JOE for *E. coli*, and 134 bp and 387 bp in JOE for *A. lwoffii*.

3.15. Inhibiting substances

With a system capable of direct amplification from unwashed FTA® punches, the selection of usable samples widens significantly to encompass simple cell lysates and FTA® punches as well as purified DNA. A diverse collection of PCR-inhibiting substances at high concentrations may be encountered with unpurified or poorly purified samples originating from either the sample or the purification method. Several common forensic inhibitors were titrated into reactions to assess tolerance to multiple contaminating compounds. The evaluation included environmental substances such as hematin [20], humic acid [21], tannic acid [21], chocolate, tobacco and coffee, as well as substances from typical purification methods including EDTA [22], ethanol [22], and SDS [23]. All concentrations listed were final reaction concentrations. Inhibitor concentration ranges included 0–1 mM hematin, 0–350 ng/μl humic acid, and 0–500 ng/μl tannic acid. Concentrations of EDTA at 0–1 mM, ethanol at 0–12%, and SDS at 0–1 mg/ml were evaluated as well. Inhibitor titrations were performed using both buccal FTA® and 5 ng of extracted DNA. EDTA testing additionally included blood FTA® samples. Lastly, chocolate, sugar, tobacco, and coffee (10 μl) evaluations were performed singly with buccal FTA® reactions.

The PowerPlex® 18D System tolerated a broad range of inhibitors, giving full profiles with both extracted DNA and buccal FTA® samples at substantial inhibitor concentrations (Table 4). For all inhibitors, peak heights dropped with increasing concentrations of inhibitor (Supplemental Figs. 14–19). Typically, the largest loci were the first to drop out. Extracted DNA generally showed higher tolerance to inhibiting substances than FTA® samples. More specifically, full profiles were generated with hematin concentrations ≤800 μM using 5 ng of extracted DNA and ≤500 μM using buccal FTA® samples (Supplemental Fig. 20). With humic acid, full profiles were seen with ≤200 ng/μl using 5 ng of extracted DNA and ≤150 ng/μl using buccal FTA®. Tannic acid inhibited buccal FTA® reactions at lower concentrations than with extracted DNA, ≤150 ng/μl and >500 ng/μl respectively. Large amounts of chocolate and tobacco were severely inhibiting. However, lower amounts of tobacco were overcome. Coffee and sugar allowed full profiles to be collected but with reduced overall peak heights (Supplemental Fig. 21).

The PowerPlex® 18D System tolerated considerable concentrations of purification-related inhibitors as well. Full profiles were observed with ≤1 mM EDTA with buccal FTA® samples and ≤0.5 mM EDTA with blood FTA® samples and 5 ng of extracted DNA. Ethanol contamination of ≤8% generated full profiles with 5 ng of extracted DNA. Buccal FTA® samples gave full profiles with ≤2.5% ethanol. Contamination with SDS was tolerated at ≤0.8 mg/ml with 5 ng of extracted DNA and ≤0.4 mg/ml with buccal FTA® samples.

4. Conclusion

Direct amplification with STR multiplex systems presents opportunities for process simplification. However, inherent variation brings new challenges and considerations for optimization. Although direct amplification offers the benefits of faster and

easier processing, forensic and database samples are not uniform or pristine. Sample distribution across the material varies, as does the amount of material collected. When sampling the sample distribution and density variability can compound, and therefore, larger peak height variations can be observed sample-to-sample compared with extracted DNA samples. Additionally, typical samples are crude introducing various combinations and levels of PCR inhibitors directly into a reaction. Reagent improvements made with the PowerPlex® 18D System have been specifically targeted to address direct amplification challenges, and quality genotypes can be obtained with significantly less labor and resources than previously. Nonetheless, optimization of individual laboratory conditions and processes is recommended.

SWGDAM and NDIS requirements have been established to ensure new STR multiplexes are sufficiently robust and reliable for human identification purposes. The data reported demonstrates the PowerPlex® 18D System produces high quality data that can be reliably generated under sub-optimal conditions and challenging samples. The PowerPlex® 18D System has been shown to be sensitive to low amounts of DNA, robust in handling changes in component concentrations, annealing temperature and PCR inhibitors, and is sufficiently precise to make accurate genotype calls. Most importantly, multiple laboratories found concordance with existing STR multiplexes and high full-profile success rates with their current laboratory samples, covering a wide variety of materials. Given these results, the PowerPlex® 18D System can confidently be used for forensic and identification testing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2012.07.008.

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