Developmental Validation of the VersaPlex® 27PY System

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Abstract

The VersaPlex® 27PY System is a short tandem repeat (STR) multiplex from Promega that co-amplifies 27 loci. This six-dye multiplex was designed for casework samples and includes loci that meet database requirements for the United States (CODIS) and Europe (ESS). The 23 autosomal loci amplified by the VersaPlex® 27PY System have high powers of discrimination and include the D6S1043 locus, which is especially informative for Asian populations. The multiplex also includes two rapidly mutating Y-STRs that can provide useful genetic information for forensic samples. In this article we describe the developmental validation experiments using the VersaPlex® 27PY System to meet SWGDAM requirements. We document how reaction variables including PCR inhibitors, reaction volume, cycle number, annealing temperature and magnesium concentration can affect multiplex performance. We also evaluate the sensitivity, specificity, precision and reproducibility of the VersaPlex® 27PY System. The results of the experiments demonstrate the capabilities of the VersaPlex® 27PY System as a tool for forensic laboratories.

Introduction

The VersaPlex® 27PY System (Cat.# DC7020) is designed for laboratories interested in a highly discriminatory STR chemistry that is optimized for forensic casework. The VersaPlex® 27PY amplification reaction simultaneously targets 27 STR loci in the human genome and uses six fluorescent dyes. These loci meet the needs of the global forensic community and include the expanded European Standard Set (ESS) loci and Combined DNA Index System (CODIS) 20 loci (1,2) (CSF1PO, FGA, TH01, TPOX, vWA, 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. For additional discrimination power, the Penta D, Penta E and D6S1043 loci are also included. The D6S1043 locus is known to be highly variable in all population groups, particularly in Asian populations (3). The VersaPlex® 27PY system also targets two rapidly mutating Y-STR loci, DYS570 and DYS576, to help differentiate between related male individuals and assist in complex mixture interpretation.

To demonstrate the robustness of the VersaPlex® 27PY System to common deviations from the optimal protocol, we demonstrate how amplification reactions are impacted by changes to magnesium, polymerase, master mix and primer concentration. We also show the impact of alterations to the annealing temperature, reaction volume and cycle number. Given that many forensic samples are contaminated by environmental factors or multiple human DNA sources, we subjected the VersaPlex® 27PY System to a variety of non-human DNA sources to test the specificity of our primer mix as well as its ability to generate minor donor profiles from two- and three-person mixtures. We tested the ability of the VersaPlex® 27PY System to generate complete profiles with decreasing amounts of DNA and in the presence of five separate PCR inhibitors, which reflect conditions commonly encountered in forensic samples. Finally, we demonstrate that the allele calls generated with the VersaPlex® 27PY System are concordant with allele calls generated with other chemistries.

Experiments were performed using the VersaPlex® 27PY System to satisfy the Scientific Working Group on DNA Analysis Methods (SWGDAM) requirements for a developmental validation (4). Scientists at Promega Corporation and the National Institute of Standards and Technology (NIST) collaborated to produce the data summarized in this article. The data presented here demonstrate the advantages and limitations of the VersaPlex® 27PY System to aid forensic scientists with implementation and interpretation of this STR chemistry in their laboratories.

Materials and Methods

Samples

Human DNA from two males and one female was purified from blood using the Maxwell® CSC Blood DNA Kit (Cat.# AS1321) protocol on a Maxwell® CSC instrument and quantified using the PowerQuant® System (Cat.# PQ5002). Individuals providing blood or semen samples for this study did so anonymously under the informed consent procedure approved by Promega's Human Subjects Board for providing samples for research use. Single-source human DNA samples purified from blood were combined to achieve the mixture ratios tested for two-person mixtures of the following male (M) to female (F) ratios: 1:0, 19:1, 9:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:9, 1:19 and 0:1. Three-person mixtures were made with a major female contributor and two minor male contributors at the following ratios: 1:1:10 (M:M:F) and 1:1:20 (M:M:F). All mixture samples were amplified in quadruplicate with 1ng of DNA per reaction.

For the nonhuman specificity experiments, purified DNA was purchased from several vendors. Primate DNA was obtained from a private collection or purchased from Coriell Institute for Medical Research (Camden, NJ). All other DNAs were purchased from ATCC (Manassas, VA), Novagen/EMD Millipore (Burlington, MA) or Zyagen (San Diego, CA). The species tested included: chimpanzee (male and female), gorilla, macaque, cat (male and female), chicken (male and female), cow, deer, dog, horse, mouse, pig, rabbit, rat, *Candida albicans, Enterococcus faecalis, Escherichia coli, Fusobacterium nucleatum, Lactobacillus acidophilus, Micrococcus luteus, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Staphylococcus epidermidis, Streptococcus mitis, Streptococcus mutans and Streptococcus salivarius*. Primate DNA samples were amplified using 1ng of template DNA per reaction, while the remaining DNA samples were amplified using 10ng of template DNA per reaction.

Concordance testing was performed at NIST using a subset (n = 763) of the NIST 1036 US population samples (5,6), an additional set of 99 DNA extracts (7) and the six components present in the Standard Reference Material® (SRM) 2391d for a total of 868 samples. A subset (n = 664) of the 1036 US population samples were used to examine stutter. All work presented in this paper has been reviewed and approved by the NIST Research Protections Office.

DNA Amplification

Amplification took place using an Applied Biosystems ProFlex™ PCR System thermal cycler. The recommended amplification setup and cycling parameters described in the VersaPlex® 27PY Technical Manual (8) were followed unless otherwise noted. This included 25µl reactions with 5µl of VersaPlex® 27PY 5X Master Mix, 5µl of VersaPlex® 27PY 5X Primer Pair Mix and up to 15µl of DNA input. The target for DNA input was 1ng per reaction. The standard thermal cycling protocol was followed: one cycle of 96°C for 5 minutes; 29 cycles of 96°C for 5 seconds then 60°C for 1 minute; 60°C final extension for 10 minutes and a 4°C infinite hold.

In the sensitivity experiment four replicates of each of two extracted male DNA samples were amplified at the following final DNA amounts per 25µl amplification reaction: 2ng, 1ng, 500pg, 250pg, 125pg, 62.5pg, 31.25pg and 15.6pg.

To evaluate the effect of changes to cycle number, annealing temperature, reaction volume, primer pair mix and master mix concentrations, polymerase concentration and magnesium concentration (as specified by the SWGDAM 2016 guidelines [Section 3.9]), we intentionally deviated from the recommended protocol as described below. For almost all these experiments, extracted DNA samples from two male sources were amplified in two replicates of four, targeting 1ng and 200pg of template per reaction. In the cycle number experiments amplifications were performed using 27, 29 or 31 cycles. In the annealing temperature experiments, the temperature gradient feature of the ProFlex® thermal cycler was used to amplify reactions with 58°C, 59°C, 60°C, 61°C or 62°C annealing temperatures. In the reaction volume experiments, three reaction volumes of 25µl, 12.5µl and 6.25µl were used each targeting 40pg/µl and 8pg/µl of template per reaction. In the primer pair mix, master mix, polymerase and magnesium concentration experiments, each individual component was tested at final concentrations of 0.75X, 0.9X, 1X, 1.1X and 1.25X in reactions with 1ng and 200pg of template DNA. In addition, the primer pair mix and master mix were both simultaneously varied, with final concentrations of 0.9X, 1X and 1.1X in reactions with 1ng and 200pg of template DNA.

Analysis of Amplified Products

The VersaPlex® 27PY System uses six fluorescent dyes to detect amplification products and size standard by capillary electrophoresis. The VersaPlex® 6C Matrix Standard was used to establish spectral resolution on the capillary electrophoresis instruments (9). For fragment sizing and allele designation, the WEN Internal Lane Standard 500 (WEN ILS 500) and VersaPlex® 27PY Allelic Ladder Mix, both included in the VersaPlex® 27PY System, were used. Prior to capillary electrophoresis, 1µl of amplified sample or allelic ladder was combined with 9.5µl of Hi-Di™ formamide (Applied Biosystems) and 0.5µl of WEN ILS 500. Samples were denatured at 95°C for 3 minutes and then snap chilled in an ice bath. Amplification products were separated using an Applied Biosystems® 3500xL Genetic Analyzer equipped with a 36cm capillary array and POP-4™ polymer (Applied Biosystems) using a 1.2kV 24-second injection with a 13kV run voltage. Fragment sizes and allele calls were assigned using GeneMapper® ID-X v1.4 software (Applied Biosystems). For consistency across experiments, a conservative 175RFU calling threshold was used based on manufacturer recommendations (10). For the precision study, three full injections (72 wells) of VersaPlex® 27PY Allelic Ladder Mix were injected on a 3500xL Genetic Analyzer.

Casework Samples

Sample lysates were prepared from a large set of mock casework samples, including 10µl of blood or semen spotted onto several fabric types and multiple differently sourced touch samples that were treated as close to real forensic samples as possible (commonly used surfaces were swabbed with a moist cotton swab), using the Casework Extraction Kit (Cat.# DC6745) and DNA was purified using the Maxwell® 16 Instrument and Maxwell® FSC DNA IQ™ Casework Kit (Cat.# AS1550) following the recommended protocols. Individuals providing blood or semen samples for this study did so anonymously under the informed consent procedure approved by Promega's Human Subjects Board for providing samples for research use. Blood was stored at 4°C and semen was stored frozen at –20°C. Both sample types were brought to room temperature before spotting on clean denim, khaki or

cotton t-shirts (black- and teal-colored). After purification, all DNA samples were quantified using the PowerQuant® System (Cat.# PQ5002). For samples of sufficient concentration, 1ng of DNA was amplified in a 25µl reaction using the standard amplification protocol. In a separate experiment, all DNA samples, regardless of concentration, were amplified using 2µl of each sample in a 25µl reaction. For both experiments, the amplified products were analyzed by capillary electrophoresis using a 1.2kV 24-second injection with a 13kV run voltage.

Concordance

A large concordance study (n = 868 samples) was performed at NIST using the DNA extracts described above. Genotypes generated using the VersaPlex® 27PY System were compared with the results comprised from multiple STR chemistries (5,6). All amplifications were performed at NIST following the recommended VersaPlex® 27PY protocol, and genotyping was done using GeneMapper® ID-X v1.5 software (Applied Biosystems).

Stutter

DNA extracts from 664 samples (described above) were amplified at NIST according to the recommended protocol and amplification products were separated on an Applied Biosystems® 3500xL Genetic Analyzer. Reverse and forward stutter peaks were determined to be ±3 bases from the peaks in D22S1045 (a trinucleotide repeat locus), ±4 bases from the peaks in the tetranucleotide repeat loci and ±5 bases from the peaks in Penta E and Penta D (pentanucleotide repeat loci). In addition, data from n–2 stutter peaks were reported for D1S1656, D19S433 and FGA. Peaks were excluded from the stutter calculations if the peaks were observed in a shared stutter position where the reverse stutter from one allele and the forward stutter from another allele overlapped. To be included in the stutter calculations, the stutter peak had to belong to a true allele peak that was over 500RFU but below 15,000RFU. Percent stutter was calculated by dividing the peak height of the stutter peak by the peak height of the true allele and multiplying by 100.

Inhibitor Stocks

The inhibitors hematin, humic acid, tannic acid and EDTA were all purchased in powder form from Sigma-Aldrich (St. Louis, MO). These powders were dissolved to make liquid inhibitor stocks as previously described (11). We evaluated the following final inhibitor concentrations: hematin (500µmol/L, 750µmol/L, 1,000µmol/L), humic acid (100ng/µl, 200ng/µl, 300ng/µl), tannic acid (500ng/µl, 750ng/µl, 1,000ng/µl) and EDTA (500µmol/L, 750µmol/L, 1,000µmol/L). We also evaluated carryover of residual chemicals in the DNA IQ™ Lysis Buffer (Cat.# A8261) as a potential inhibitor. We treated undiluted DNA IQ™ Lysis Buffer as a 1x stock and evaluated final concentrations of 0.012X, 0.018X and 0.024X. Each inhibitor stock was diluted in water to a concentration that was two times higher than the final concentration, and 12.5µl of each 2X inhibitor was added to a 25µl VersaPlex® 27PY amplification reaction containing 1ng of DNA. We amplified two different male DNAs to evaluate the effect of inhibitors with four replicates of each male DNA at each inhibitor concentration.

Results and Discussion

Specificity

The primers included in the VersaPlex® 27PY System were designed to be specific for human DNA, and the PCR conditions were optimized to minimize potential cross-reactivity with other sources of DNA. Amplification from nonhuman DNA may still occasionally be seen, depending on the sample due to much higher copy numbers of nonhuman (e.g., microbial DNA) to human DNA in environmental samples. Such higher template amounts can allow for amplification even with mismatches between primers and template. DNA samples from a panel of twelve microorganisms, twelve vertebrates and four primates were amplified with the VersaPlex® 27PY System to characterize nonspecific amplification products that were observed. Amplification products were detected with 10ng of DNA from the following species: *F. nucleatum*, chicken, cow, mouse and pig (Figure 1, Panel A). The sizes of the artifacts observed for each species are listed in Table 1. This list includes artifacts that were observed with a

175RFU threshold as well as those observed with a 50RFU threshold. The height and presence of some of these artifacts over 50RFU were inconsistent among replicate amplifications, as can be expected from nonspecific amplification of nonhuman DNA. Numerous amplification products were also detected with 1ng of all four primate DNA samples tested. In general, the profiles obtained from primates can be distinguished from human DNA profiles because primates have multiple off-ladder alleles, some loci with no allele peaks and an overall imbalanced appearance (Figure 1, Panel B).

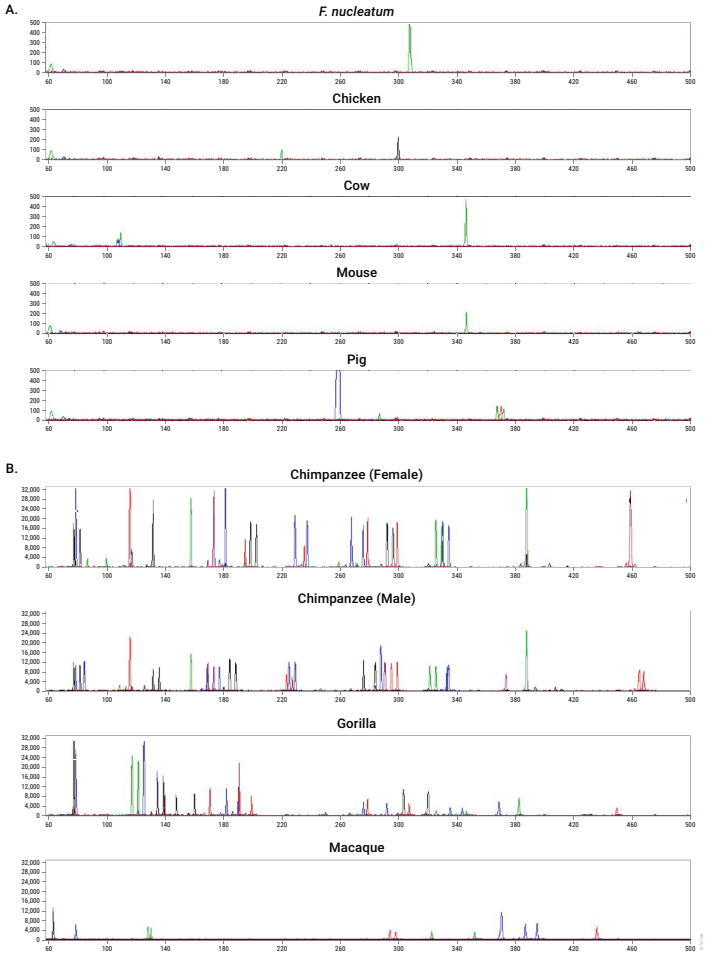

Figure 1. Specificity of the VersaPlex® 27PY System. Panel A. Representative electropherograms of nonhuman DNA samples that produced artifact peaks with the VersaPlex® 27PY System. The peak height scale for all samples is 500RFU. **Panel B**. Representative electropherograms of the four primate samples amplified with the VersaPlex® 27PY System. The peak height scale for all samples is 32,000RFU.

Table 1. Artifacts observed from nonprimate DNA samples amplified with the VersaPlex® 27PY System. Outside marker range is abbreviated as OMR, and off-ladder is noted as OL. Artifacts marked with an asterisk (*) required a 50RFU threshold to be called by GeneMapper® ID-X v1.4. All other artifacts were called using a 175RFU threshold.

DNA Source	Artifact Size	Allele Call
F. nucleatum	~308bp (JOE)	OMR
Chicken	~221bp (JOE)* ~300bp (TMR)	OMR 12.3 in D7S820
Cow	~109-110bp (FL)* ~111bp (JOE)* ~346bp (JOE)	OL in D3S1358 OL in D16S539 12 in CSF1PO
Mouse	~347bp (JOE)	OL in CSF1PO
Pig	~259-260bp (FL) ~287bp (JOE)* ~368-372bp (JOE)* ~369-370bp (CXR)*	OL and 9 in D10S1248 26 in D2S1338 OMR OMR

Sensitivity

Two male DNA samples (DNA01, DNA02) were amplified using 2ng, 1ng, 500pg, 250pg, 125pg, 62.5pg, 31.25pg and 15.6pg of DNA per reaction. Full profiles were generated for both DNA sources from 2ng down to 125pg (Figure 2, Panel A). At 62.5pg, allele dropout was observed to some degree with both DNA samples. Across both DNAs, 367/380 alleles were called for all eight replicates. Similar allele dropout rates were observed for both DNAs at lower concentrations as well. The average peak height across four replicates for each DNA at each input amount was consistent for each locus (Figure 2, Panel B). The VersaPlex® 27PY System can generate full profiles at 62.5pg and at 15.6pg can call alleles for about a quarter of the loci in the multiplex. The distribution of peak height ratios at 1ng and 125pg is shown in Figure 2, Panel C. The median peak height ratios at each condition for the two DNAs were very similar as was the distribution of the peak height ratios. For both DNAs, the lower DNA amount amplified (125pg) displayed a lower peak height ratio than the 1ng amplification reactions, reflecting the increased levels of stochastic variation seen at lower template amounts. In addition, no change in the sloping behavior of peak heights was detected when the DNA input amount was increased or decreased from the optimal 1ng input.

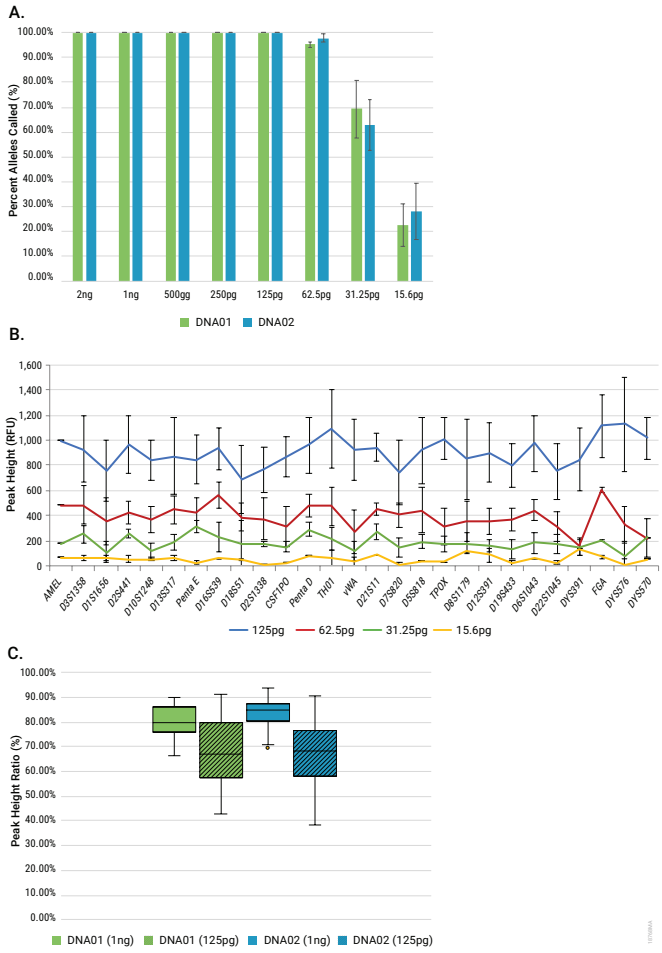

Figure 2. Sensitivity of the VersaPlex® 27PY System. Panel A. Four replicates of 2ng, 1ng, 500pg, 250pg, 125pg, 62.5pg, 31.25pg and 15.6pg of each male DNA were amplified following the recommended VersaPlex® 27PY System protocol. Data were analyzed with a 175RFU threshold. The percentage of alleles called is displayed with the error bars representing the standard deviation of the four replicate reactions for each condition. **Panel B.** The average peak heights for each locus from the 125pg, 62.5pg, 31.25pg and 15.6pg amplification reactions. The error bars represent the standard deviation of the four replicate reactions for each condition. **Panel C.** Distribution of observed peak height ratios. Data from the 1ng and 125pg amplification reactions are shown. The central lines indicate the median peak height ratio for each condition. The error bars represent the standard deviation of the four replicate reactions.

Inhibitors

In this study we selected five PCR inhibitors to determine the level of inhibitors that the VersaPlex® 27PY System can tolerate. Hematin, humic acid and tannic acid represent inhibitors present in many forensic samples. EDTA and DNA IQ™ Lysis Buffer were selected to test the impact of carryover of DNA purification reagents into a purified DNA sample.

The results shown in Figure 3, Panel A, demonstrate that the VersaPlex® 27PY System provides robust amplification in the presence of significant concentrations of inhibitors. Under ideal conditions without exogenous inhibitors, the VersaPlex® 27PY System yields a well-balanced allele profile, with 100% of alleles called at the 175RFU threshold (Figure 3, Panel B). Our findings showed that 100% of alleles were called with the lowest concentration of humic acid and EDTA tested. In the presence of 500µmol/L hematin, 365/380 (96.1%) of alleles were called (Figure 3, Panels A and C). Tannic acid saw a similar degree of allele dropout at 500ng/µl, with 362/380 alleles called across both DNAs and eight replicates. With hematin, as the concentration increased to 750µmol/L and 1,000µmol/L the percent of alleles called dropped to 21% and 0%, respectively. At 200ng/µl humic acid the percent of alleles called fell to 68.8% on average across the two DNAs tested. No alleles were called in the presence of 300ng/µl of humic acid. While almost all alleles were present at 500ng/µl of tannic acid, 79.5% and 48.5% of alleles were called with the addition of 750ng/µl or 1,000ng/µl of tannic acid, respectively. All alleles were called at 500µmol/L EDTA, with a small decrease at 750µmol/L (94.8%). However, the number of alleles called sharply decreased when the EDTA concentration was increased to 1,000µmol/L (15.2%). The DNA IQ™ Lysis buffer was the most potent inhibitor, with only 67.5% of alleles called even at a concentration of 0.012X (Figure 3, Panels A and D). Only 3.4% of alleles were called at 0.018X, and no alleles were called at 0.024X. The concentrations of DNA IQ™ Lysis Buffer tested were equivalent to 0.3µl (0.012X), 0.45µl (0.018X) and 0.6µl (0.024X) in a 25µl reaction. Hematin, humic acid, tannic acid and EDTA

showed similar patterns in the electropherograms as the inhibitor concentration increased, with the Amelogenin, Penta E, D2S1338, Penta D, D8S1179, FGA and DYS570 loci being the first to drop in peak height or drop out (Figure 3, Panel C). The pattern of dropout was unique with DNA IQ™ Lysis Buffer, with a distinct "ski-slope" effect where the largest loci in all dye channels dropped out first and there was preferential amplification of the smallest loci (Figure 3, Panel D).

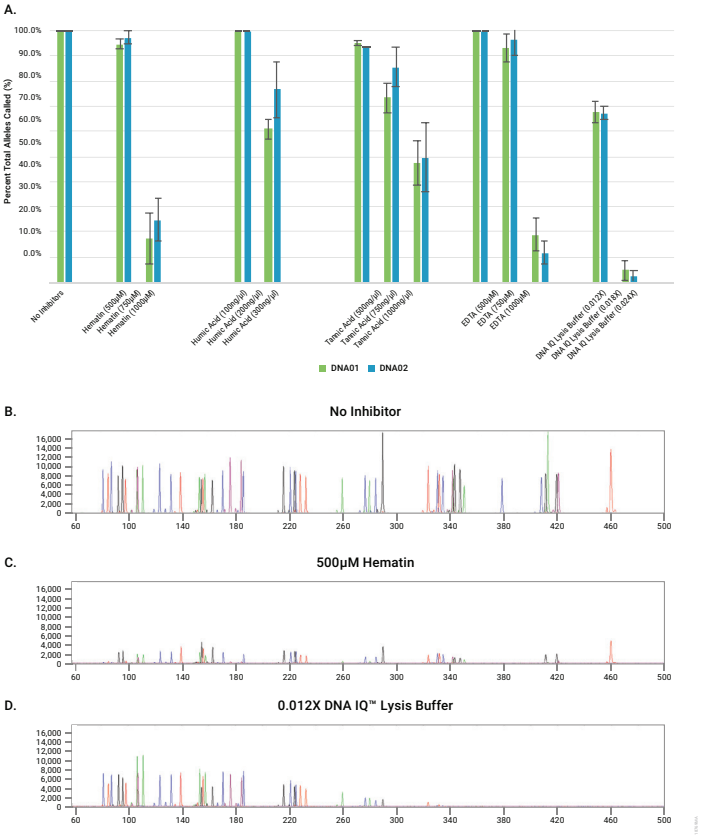

Figure 3. Inhibitor titration in VersaPlex® 27PY amplification reactions with two male DNA sources. Amplification reactions used the recommended protocol, with 1ng of each DNA without inhibitors or with increasing concentrations of hematin, humic acid, tannic acid, EDTA or DNA IQ™ Lysis Buffer **(Panel A)**. Error bars represent the standard deviation of the percent alleles called for four replicate amplification reactions for each condition. Representative electropherograms of the inhibitor titrations: no inhibitors **(Panel B)**, 500µM hematin **(Panel C)** or 0.012X DNA IQ Lysis Buffer **(Panel D)**. Peak height scale is set to 18,000RFU.

Sizing Precision

Sizing precision is a critical component of the genotyping process. To assess sizing precision of the VersaPlex® 27PY System amplification products, we used the supplied internal lane standard (WEN ILS 500) to determine individual allele size in the VersaPlex® 27PY Allelic Ladder Mix in multiple capillaries and over multiple injections. The average base pairs of each allele in the allelic ladder (418 fragments) was calculated along with the standard deviation and graphed as shown in Figure 4. In order to be within three standard deviations of a 0.5-base binning window for allele calling, the maximum standard deviation is 0.16 bases. The highest standard deviation observed for an allele in the VersaPlex® 27PY Allelic Ladder Mix was 0.072 bases.

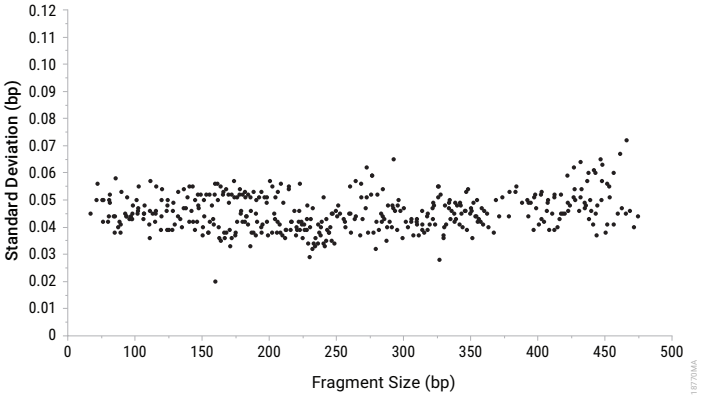

Figure 4. Precision and reproducibility of the VersaPlex® 27PY System. A total of 72 allelic ladders (1µl per well, three injections) were analyzed on an Applied Biosystems® 3500xL Genetic Analyzer using a 1.2kV 24-second injection and 13kV run voltage. The average fragment size (in base pairs) of each allele in the ladder was plotted against the observed standard deviation of the mean size for that allele.

Reproducibility

To demonstrate reproducibility of the VersaPlex® 27PY System, we used the genomic samples (Components A–D) of SRM 2391d from NIST (12) and the 2800M Control DNA provided with the system. Full and concordant profiles were obtained from all four components of SRM 2391d (Table 2).

Table 2. Reproducibility of the VersaPlex® 27PY System. Genotypes obtained for NIST SRM 2391d amplified with the VersaPlex® 27PY System. Amplification reactions were performed following the recommended protocol, and 1µl of each amplification reaction was analyzed on an Applied Biosystems® 3500xL Genetic Analyzer using a 1.2kV 24-second injection.

	NIST SRM 2391d			
	Component A	Component B	Component C	Component D
Amelogenin	X	X, Y	X, Y	X, Y
D3S1358	17, 17	15, 17	14, 18	14, 17, 18
D1S1656	15.3, 18.3	13, 15.3	15, 16	15, 15.3, 16, 18.3
D2S441	11, 11	11, 11	11, 14	11, 14
D10S1248	14, 15	12, 15	12, 16	12, 14, 15, 16
D13S317	9, 12	11, 11	12, 14	9, 12, 14
Penta E	13, 14	5, 7	12, 14	12, 13, 14
D16S539	12, 13	9, 11	9, 12	9, 12, 13
D18S51	14, 15	17, 18	16, 18	14, 15, 16, 18
D2S1338	25, 25	17, 23	23, 24	23, 24, 25
CSF1PO	12, 14	12, 12	10, 11	10, 11, 12, 14
Penta D	8, 9	11, 13	9, 13	8, 9, 13
TH01	7, 9.3	7, 7	8, 9.3	7, 8, 9.3
vWA	17, 19	15, 17	14, 17	14, 17, 19
D21S11	29, 30	28, 29	29, 31	29, 30, 31
D7S820	8, 10	10, 10	9, 10	8, 9, 10
D5S818	10, 11	12, 12	13, 15	10, 11, 13, 15
TPOX	8, 9	8, 12	8, 10	8, 9, 10
D8S1179	12, 13	12, 15	12, 15	12, 13, 15
D12S391	21, 24	19, 20	17, 18	17, 18, 21, 24
D19S433	13, 15	11, 16.2	13, 15	13, 15
D6S1043	12, 19	13, 18	11, 18	12, 12, 18, 19
D22S1045	14, 16	12, 15	14, 15	14, 15, 16
DYS391	—	11	10	10
FGA	21, 24	24, 26	22, 23	21, 22, 23, 24
DYS576	—	15	17	17
DYS570	—	20	18	18

Casework-Type Samples

The VersaPlex® 27PY System was used to genotype challenging samples commonly encountered by forensic laboratories. The sample types that were tested are described in Table 3 along with the number of alleles above the 175RFU threshold. For most samples, what appeared to be a full, single-source profile was obtained, but because of the lack of a known reference DNA profile for these samples it was impossible to know the expected number of alleles. Representative electropherograms are shown for DNA recovered from a plastic fork (Figure 5, Panel A) and 10µl of semen spotted on denim fabric (Figure 5, Panel B). Unsurprisingly, touch samples recovered the smallest amount of DNA. To better understand the profiles that can be recovered from casework samples, a wide variety of DNA input amounts was amplified using the VersaPlex® 27PY System. Two microliters of each sample, including those samples that did not appear to be single-source and those whose concentrations resulted in less than 1ng per amplification, were added to a 25µl reaction and amplified using the recommended protocol. When the average number of alleles called across four replicates is plotted against input DNA amount (Figure 5, Panel C), a sharp increase in the number of called alleles occurs around 50–75pg, echoing the previously observed sensitivity data (Figure 2, Panel A). It is likely that data for more loci could be recovered with a lower allele-calling threshold.

Table 3. A summary of the number of alleles called from various mock casework samples. The number of alleles called from various mock casework samples averaged across four replicate amplification reactions. Based on the sample types and the resulting electropherograms, these are presumed to be single-source DNA samples. DNA was extracted following the Casework Extraction Kit protocol followed by purification using the Maxwell® FSC DNA IQ™ Casework Kit. 1ng of DNA was amplified using the standard VersaPlex® 27PY protocol.

Sample	Average No. Alleles Called
2800M Control DNA	47
DNA01	48
DNA02	47
Aluminum Bottle 2	44
Aluminum Can 2	45
Aluminum Can 3	47
Aluminum Can 4	46
Blood on Denim: Donor 1; Replicate 1	47
Blood on Denim: Donor 1; Replicate 2	47

Table 3 cont.

Sample	Average No. Alleles Called
Blood on Denim: Donor 2; Replicate 1	45
Blood on Denim: Donor 2; Replicate 2	45
Blood on Khaki: Donor 1; Replicate 1	47
Blood on Khaki: Donor 1; Replicate 2	47
Blood on Khaki: Donor 2; Replicate 1	45
Blood on Teal T-Shirt: Donor 1; Replicate 1	47
Blood on Teal T-Shirt: Donor 1; Replicate 2	47
Blood on Teal T-Shirt: Donor 2; Replicate 1	45
Blood on Teal T-Shirt: Donor 2; Replicate 2	45
Plastic Fork 2	45
Semen on Denim: Replicate 1	47
Semen on Denim: Replicate 2	47
Semen on Khaki: Replicate 1	47
Semen on Khaki: Replicate 2	47
Semen on Black T-Shirt: Donor 1; Replicate 1	47
Semen on Black T-Shirt: Donor 1; Replicate 2	47
Semen on Black T-Shirt: Donor 2; Replicate 1	47
Semen on Black T-Shirt: Donor 2; Replicate 2	47

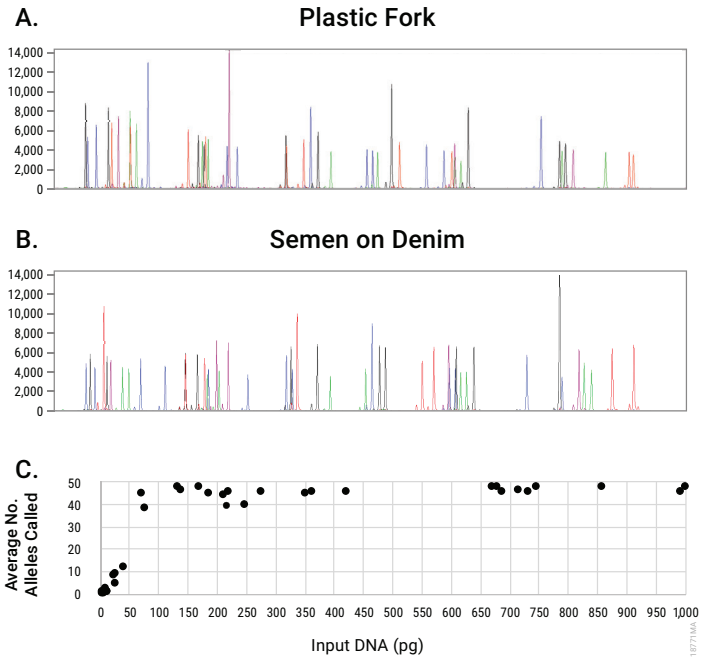

Figure 5. Representative results from mock casework samples. Panel A. A representative electropherogram of the amplification of DNA recovered from a plastic fork. **Panel B.** A representative electropherogram of the amplification of DNA recovered from semen spotted on denim fabric. **Panel C.** A plot of the average number of alleles called for a range of DNA input amounts. Each data point is the average of four replicate amplification reactions.

Concordance and Characterization of Genetic Markers

The loci included in the VersaPlex® 27PY System are included in many previous STR chemistries, most recently in the PowerPlex® 21 (13) and PowerPlex® Fusion 6C Systems (11). The chromosomal mapping of these loci was previously documented (14–19), and multiple studies on the allele frequency distribution are published (3,20–24). In collaboration with NIST, a concordance study was carried out. In this study, a total of 44,268 allele calls (from the 868 samples) generated using the VersaPlex® 27PY System were compared with an existing database, and only two discordant alleles were found for a final concordance rate of 99.995%. The two discordant alleles were a null X in Amelogenin and a discordant call in D7S820, both of which are described in detail in the PowerPlex® Fusion 6C System developmental validation (11).

Stutter

Stutter is a common occurrence during STR amplification, and the intensity of the stutter peaks varies depending on the repeat structure and the number of repeat units (25,26,27). For this reason, it is important to determine the expected stutter percentage for each locus to aid in data interpretation. Stutter data for the VersaPlex® 27PY System are summarized in Table 4. The VersaPlex® 27PY stutter file filters out stutter peaks with peak heights that exceed the average stutter percentage plus three times the standard deviation. This file is available for download from the Promega web site. Based on the data set used to calculate stutter, these values encompass 99% of observed stutter peaks. For every locus a minus-stutter and a plus-stutter filter is applied to one repeat below and one repeat above, respectively, a given allele. In addition, n–2 filters are included for specific loci where this type of stutter is most common (D1S1656, D19S433 and FGA).

Table 4. Observed stutter for the VersaPlex® 27PY System.

	Minus Stutter					Plus Stutter				
	Count	Average (%)	SD (%)	Average + 3SD (%)	Max (%)	Count	Average (%)	SD (%)	Average + 3SD (%)	Max (%)
D3S1358	425	8.20	1.36	12.3	13.1	527	0.78	0.30	1.7	2.6
D1S1656	766	8.10	1.87	13.7	16.3	792	1.12	0.45	2.5	6.6
D2S441	564	4.50	1.29	8.4	9.7	553	0.92	0.41	2.2	3.4
D10S1248	372	7.50	1.59	12.3	12.5	260	0.40	0.38	1.5	3.7
D13S317	363	4.55	1.86	10.1	10.2	415	0.77	0.49	2.2	5.1
Penta E	826	2.70	1.56	7.4	7.8	425	0.41	0.33	1.4	3.2
D16S539	469	5.58	1.73	10.8	10.2	474	1.14	0.64	3.1	7.7
D18S51	714	7.93	2.02	14.0	15.8	640	0.93	0.47	2.3	3.6
D2S1338	832	7.83	1.50	12.3	12.5	164	0.53	0.48	2.0	3.9
CSF1PO	357	5.09	1.87	10.7	10.6	483	1.24	0.65	3.2	9.1
Penta D	635	1.58	0.83	4.1	6.1	315	0.55	0.48	2.0	3.5
TH01	622	1.97	0.82	4.4	7.5	179	0.37	0.37	1.5	3.0
vWA	450	6.61	2.41	13.8	11.9	449	0.62	0.66	2.6	10.9
D21S11	565	7.68	1.35	11.7	14.1	656	1.22	0.45	2.6	5.6
D7S820	423	4.33	1.70	9.4	10.3	507	0.68	0.27	1.5	3.7
D5S818	328	5.39	1.74	10.6	15.2	470	1.08	0.38	2.2	4.2
TPOX	503	2.44	1.07	5.7	7.1	174	0.39	0.58	2.1	4.2
D8S1179	453	6.82	1.38	11.0	11.1	451	0.89	0.74	3.1	12.8
D12S391	645	8.70	2.65	16.6	18.1	200	0.78	0.79	3.1	8.3
D19S433	456	6.69	1.77	12.0	11.3	146	1.15	0.73	3.3	4.0
D6S1043	550	6.87	1.31	10.8	11.3	494	0.79	0.60	2.6	9.8
D22S1045	473	7.19	3.18	16.7	15.0	617	4.68	1.74	9.9	8.6
DYS391	526	6.21	0.81	8.6	9.5	402	0.71	0.51	2.2	4.0
FGA	633	7.14	1.65	12.1	12.5	621	0.90	0.39	2.1	4.4
DYS576	489	8.20	1.12	11.5	14.1	482	1.11	0.49	2.6	3.9
DYS570	535	8.39	1.03	11.5	12.8	520	0.95	0.34	2.0	4.7

	N-2 Stutter				
	Count	Average (%)	SD (%)	Average + 3SD (%)	Max (%)
D1S1656	909	1.48	0.45	2.8	2.4
D19S433	220	0.48	0.45	1.8	4.3
FGA	54	0.45	0.16	0.9	2.2

Mixtures

Samples processed in a forensic laboratory are frequently mixtures of genetic material from more than one individual. To simulate these sample types, extracted DNA samples from one male and one female were combined at eleven different ratios and amplified with the VersaPlex® 27PY System to evaluate the system's ability to detect contributor alleles (Figure 6, Panel A). All unique alleles were recovered with 1:1 M:F and 2:1 M:F mixtures. We observed a nearly full profile of unique alleles in the 5:1 M:F mixture, with a single allele dropping out in one replicate (291/292 alleles recovered). Similarly, in the 9:1 M:F mixture, 289/292 unique alleles were recovered across four replicates. Even in the 1:19 M:F mixture, we recovered 90.1% of total unique alleles. Furthermore, in the three-person mixtures, 97.3% and 84.3% of total alleles were recovered in the 1:1:10 and 1:1:20 M:M:F mixtures, respectively.

Similar allele recovery rates were observed when analysis was limited only to unique alleles from minor contributors (Figure 7, Panel B). Full minor contributor profiles are recovered from the 1:2, 1:5 and 1:9 M:F mixtures. Even when the female contributor DNA is in far excess (1:19 M:F, or 50pg male DNA:950pg female DNA), 77.3% of the unique male alleles are recovered. In the three-person mixtures, 95.3% and 72.2% of unique male alleles were recovered in the 1:1:10 and 1:1:20 mixtures. However, 99% and 97% of the unique female alleles were recovered in the 5:1 and 9:1 M:F mixtures respectively, suggesting that re-amplification or re-injection with a longer injection time could recover those alleles that either dropped out or fell below the 175RFU allele calling threshold. The results from both the two-person and three-person mixtures confirm VersaPlex® 27PY System's ability to consistently detect male DNA in a mixture when there is as little as ~50pg of male DNA in the reaction.

Figure 6. Amplification of two- or three-person mixture samples with the VersaPlex® 27PY System. Each reaction contained 1ng of total DNA. Error bars represent the standard deviation from four replicate amplification reactions. **Panel A.** The total percentage of unique alleles called for each mixture. **Panel B.** The percentage of unique minor alleles called for each mixture.

PCR-Based Procedures: Cycle Number

The recommended number of PCR cycles for the VersaPlex® 27PY System is 29 cycles. However, we tested 27, 29 and 31 cycles to demonstrate the impact of changing the cycle number (Figure 7, Panel A). Full allele profiles were observed for both male DNAs at 1ng and 200pg using 29 and 31 cycles. Full profiles also were observed with 1ng of DNA at 27 cycles, but 4/380 alleles dropped out when 200pg was used. The average peak height using 29 cycles was close to 6,000RFU for the 1ng reactions and close to 1,200RFU for the 200pg reactions (Figure 7, Panels B,C). Increasing the cycle number to 31 cycles resulted in overall higher average peak heights (~20,000RFU for 1ng and ~5,000RFU for 200pg), a noisier baseline and occasional signal saturation (i.e., peaks greater than 32,000RFU) for homozygous loci in the 1ng reactions. The saturation and noisy baseline with higher cycle number and dropout with reduced cycle number should be considered when interpreting data generated using the VersaPlex® 27PY System.

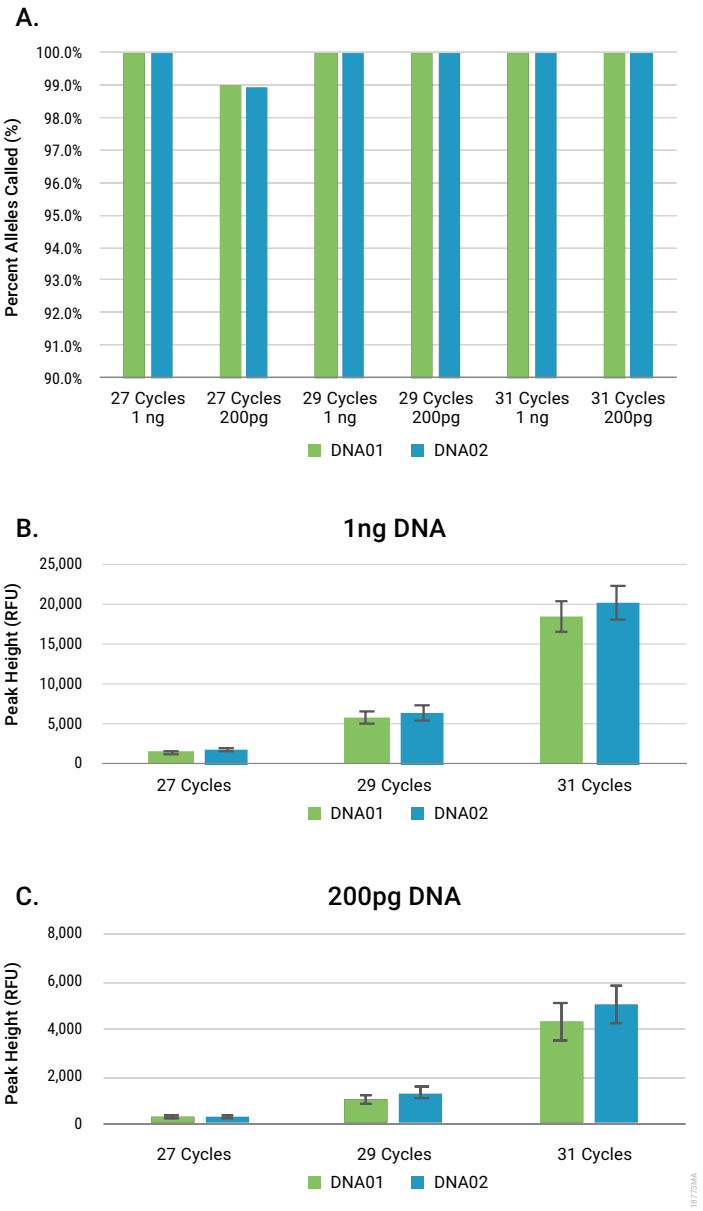

Figure 7. The effect of varying the number of PCR cycles. Panel A. The average percent of alleles called for two male DNAs across 27, 29 or 31 cycles. 1ng or 200pg of extracted male DNA was amplified for each condition. **Panel B.** The average peak heights for 1ng of two male DNAs amplified for 27, 29 or 31 cycles. **Panel C.** The average peak heights for 200pg of two male DNAs amplified for 27, 29 or 31 cycles. Error bars represent the standard deviation from four replicate reactions for each condition.

PCR-Based Procedures: Annealing Temperature

While developing the VersaPlex® 27PY System we determined that a 60°C annealing temperature was optimal. The use of alternate annealing temperatures is not recommended, but programming errors and temperature drifts can occur in thermal cyclers without any external warning. For this reason, we tested a range of annealing temperatures from 58°C to 62°C to evaluate how the VersaPlex® 27PY System tolerates these different conditions. In general, lowering the annealing temperature to 59°C or 58°C did not have a significant impact on the amplification profile except we observed an increase in peak height of the Amelogenin locus (Figure 8, Panel A). Increasing the annealing temperature to 61°C resulted in an overall decrease in peak heights, while increasing the temperature to 62°C resulted in the largest changes to the profile appearance. The five loci that were the most affected by changes in annealing temperature were Amelogenin, FGA, Penta E, D8S1179 and DYS391 (Figure 8, Panels A and B). The D8S1179 and D16S539 loci were also impacted. Despite changes in peak height, we observed 100% of expected alleles with both DNA sources and both 1ng and 200pg of template DNA at all five annealing temperatures. In addition, no new artifacts were observed as the annealing temperature was increased or decreased. These results support the importance of using the recommended annealing temperature while demonstrating the robustness of the VersaPlex® 27PY System.

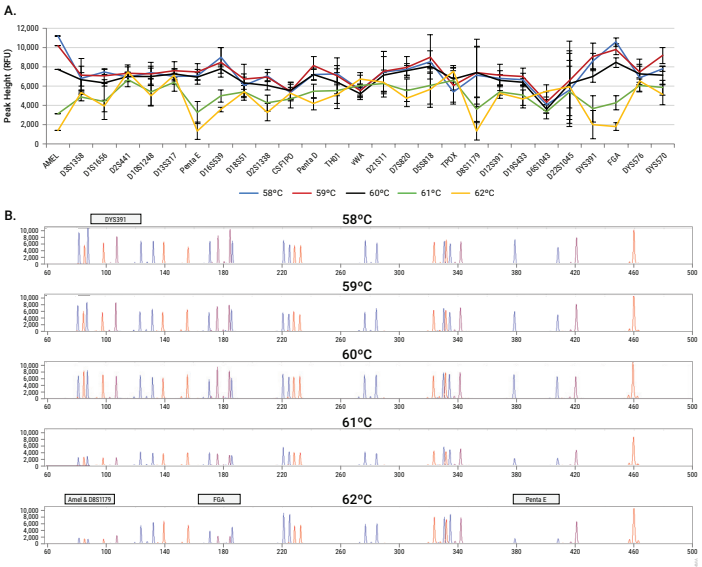

Figure 8. Varying the annealing temperature with the VersaPlex® 27PY System. Panel A. The average peak heights for each locus amplified from 1ng of male DNA using the VersaPlex® 27PY System at 58°C, 59°C, 60°C, 61°C and 62°C. Error bars represent the standard deviation from four amplifications at each annealing temperature. **Panel B.** Representative electropherograms of a single 1ng amplification reaction at each annealing temperature. Five loci, Amelogenin, D8S1179, DYS391, FGA and Penta E are more reactive to higher annealing temperature than the other loci in the VersaPlex® 27PY System.

PCR-Based Procedures: Reaction Volume

In the VersaPlex® 27PY System Technical Manual, the recommended reaction volume is 25µl, with a target input of 1ng template DNA. Reduced-volume reactions including half-volume (12.5µl) and even quarter-volume (6.25µl) reactions are used by forensic laboratories for various reasons including cost savings and preservation of sample. In this experiment we compared the results from these three reaction volumes with two fixed template concentrations. Full profiles were obtained with all three reaction volumes at a 40pg/µl (i.e., 1ng at 25µl) or 8pg/µl (i.e., 200pg at 25µl) concentration for DNA02. Nearly full profiles were obtained for DNA01 at all three reaction volumes and concentrations with one allele not being called at both the 40pg/µl and 8pg/µl concentrations with the 6.25µl reaction volume. The average peak height dropped slightly as the reaction volume increased for DNA01 amplified at 40pg/µl (DNA02 did not show a significant peak height change across reaction volumes for either concentration tested) but was consistent for the 8pg/µl reaction (Figure 9, Panel A). The average peak height ratio for the different reaction volumes stayed consistent at the 40pg/µl concentration (Figure 9, Panel B). However, the average peak height ratio dropped more and had more variability at 8pg/µl as the reaction volume was lowered (Figure 9, Panel B). This lower average peak height ratio is likely due to stochastic effects because at 8pg/µl the 6.25µl reaction contained 50pg of template DNA compared to the 25µl reaction, which contained 200pg of DNA. This decrease in peak height ratio may make data interpretation more challenging from reduced-volume reactions with low template input.

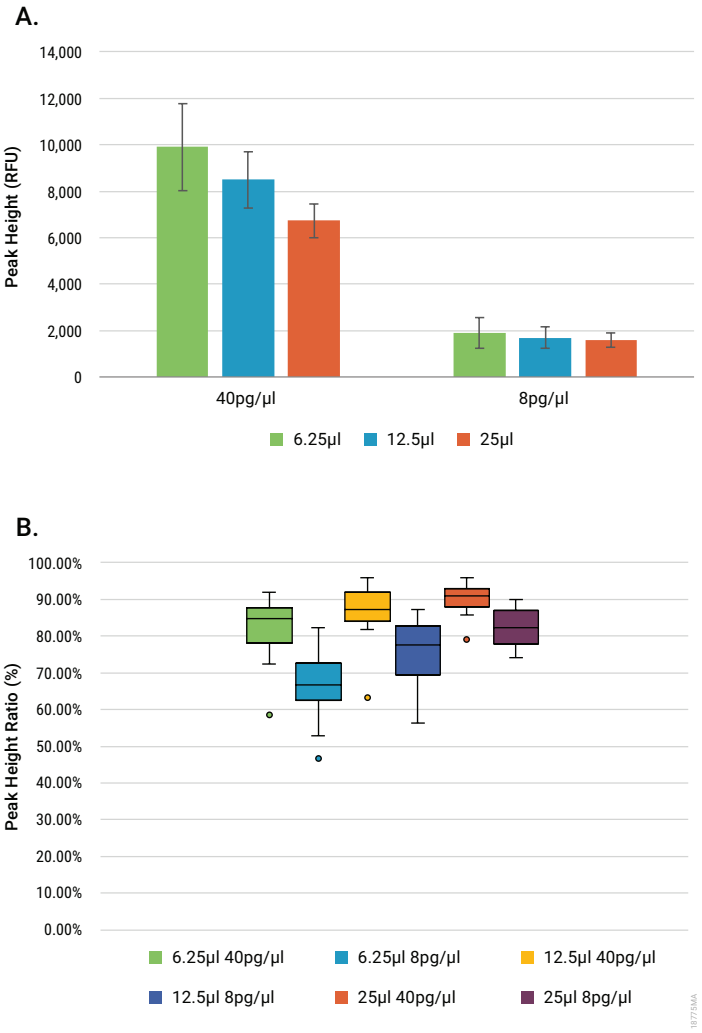

Figure 9. The effect of varying reaction volume. Panel A. The average peak heights for each reaction condition for a male DNA. Error bars represent the standard deviation of four replicate amplification reactions. **Panel B.** The peak height ratios for an amplified male DNA sample using the VersaPlex® 27PY System. The same relative concentration of DNA (40pg/µl or 8pg/µl) was amplified in 6.25µl, 12.5µl or 25µl reactions. The central lines represent the median peak height ratio for each condition. Error bars represent the standard deviation of four replicate amplification reactions.

PCR-Based Procedures: Primer Pair Mix Concentration and Master Mix Concentration

The VersaPlex® 27PY System primer pair mix and master mix are both at 5X concentrations and are added at a final 1X concentration. However, pipetting errors can occur and inadvertent deviations in input to a PCR reaction are possible. To determine the effects of changing the primer pair mix or master mix, reactions were assembled with ±25% and ±10% volumes of primer pair mix or master mix and with ±10% volumes of both primer pair mix and master mix simultaneously. Overall, increasing the primer pair concentration (Figure 10, Panel A) or master mix concentration (Figure 10, Panel B) resulted in an increase in peak heights, while decreasing either one resulted in lower peak heights. When primer concentration or master mix concentration were independently changed to 0.9X or 1.1X the impact was minimal, but when both were simultaneously changed the impact was more noticeable (Figure 10, Panel C). Increasing the primer pair mix or master mix concentration individually or simultaneously resulted in a modest increase in peak heights. Critically, decreasing any component individually or together sees a comparably larger decrease in average peak height across all loci. No allele dropout was observed with 1ng or 200pg of template DNA at any concentration of primer pair mix or master mix or combination of the two, and no new artifacts were observed. Despite substantial changes in the final concentrations of these two components that affected several loci, the VersaPlex® 27PY System provided well balanced and robust amplification.

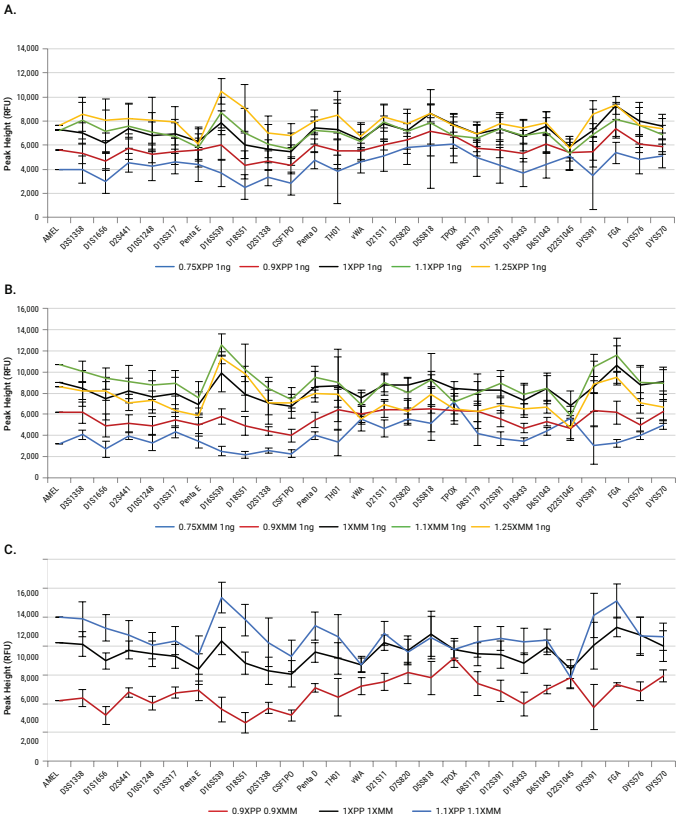

Figure 10. The effect of varying primer pair and master mix concentrations. Average peak heights for all loci in the VersaPlex® 27PY System after amplifying 1ng of male DNA using altered amounts of primer pair mix (**Panel A**), master mix (**Panel B**) or primer pair and master mix (**Panel C**). Error bars represent the standard deviation of four replicate amplification reactions.

PCR-Based Procedures: Magnesium Concentration

Another critical component of the VersaPlex® 27PY 5X Master Mix is magnesium chloride. The presence of magnesium is essential for PCR as it acts as both a cofactor for the polymerase and it binds to the primers and dNTPs (28). However, DNA extracts from forensic samples may contain magnesium chelators that can change the final magnesium concentration available in a reaction. To evaluate the performance of the VersaPlex® 27PY System under varying magnesium conditions, a range of final magnesium concentrations including ±25% and ±10% were tested. Changing the magnesium concentration did not cause any new artifacts or allele dropout even with the lower template amount of 200pg. Overall, increasing magnesium concentration led to an increase in peak heights, while decreasing magnesium concentration led to a decrease in peak heights (Figure 11, Panel A). Lowering magnesium concentration had a larger impact on peak heights than increasing magnesium concentration. The impact on observed peak heights was minimal with a 10% change and larger with a 25% change in concentration. Most loci responded similarly to varying the magnesium concentration, with Amelogenin and D16S539 being the largest exceptions, indicating the VersaPlex® 27PY System remained well balanced when under these conditions.

PCR-Based Procedures: Polymerase Concentration

To determine the impact of changes in polymerase concentration, amplifications were set up with ±25% and ±10% polymerase. No allelic dropout was observed, and no novel artifacts were discovered under any condition tested. Across all polymerase concentrations tested, we did not observe a consistent difference in overall average peak height in the 1ng (Figure 11, Panel B) or 200pg (data not shown) reactions. There also was no significant change to overall profile balance at the different polymerase concentrations, indicating robust performance of the VersaPlex® 27PY System.

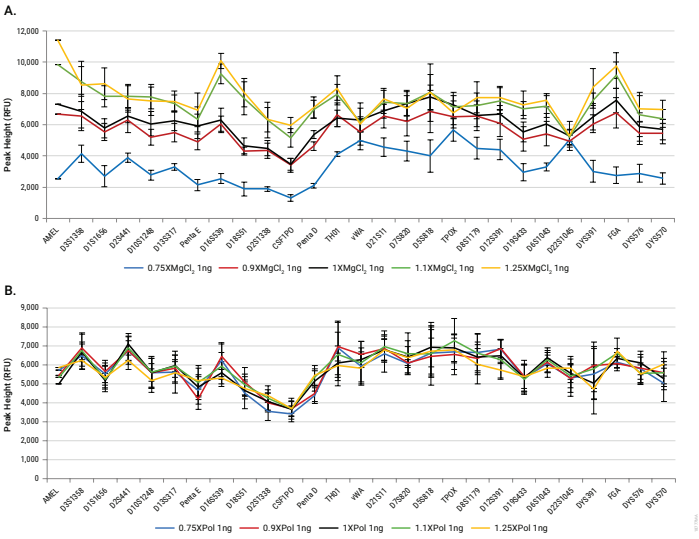

Figure 11. The effect of varying magnesium and polymerase concentrations. The average peak height of every locus for 1ng of male DNA amplified with the VersaPlex® 27PY System and various amounts of $MgCl_2$ (**Panel A**) or Taq polymerase (**Panel B**). Error bars represent the standard deviation of four replicate amplification reactions for each condition.

Conclusion

The VersaPlex® 27PY System provides a 6-color STR genotyping solution to laboratories using an extracted DNA workflow that are also interested in using the D6S1043 locus. In this study we evaluated the ability of the VersaPlex® 27PY System to withstand variations from the optimized protocol. We demonstrated that the VersaPlex® 27PY System can generate full profiles with as little as 125pg of template DNA and nearly full profiles at 62.5pg. The system can provide useful information with mixture samples and provides robust amplification in the presence of inhibitors. In addition, we demonstrated the system's ability to tolerate changes in reaction conditions, including annealing temperature, cycle number, reaction volume and PCR component concentrations. These experiments were performed as part of a collaboration and followed the guidelines set forth by SWGDAM, allowing this data to support internal validations performed by individual laboratories. The results from these experiments demonstrate that the VersaPlex® 27PY System is reliable for genotyping casework samples.

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All work performed at NIST (to include the concordance and stutter evaluations) has been reviewed and approved by the US National Institute of Standards and Technology 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 US 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 Review Board.

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