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Research paper

Evaluation of the Illumina® Beta Version ForenSeq™ DNA Signature Prep Kit for use in genetic profiling

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ABSTRACT

While capillary electrophoresis-based technologies have been the mainstay for human identity typing applications, there are limitations with this methodology's resolution, scalability, and throughput. Massively parallel sequencing (MPS) offers the capability to multiplex multiple types of forensically-relevant markers and multiple samples together in one run all at an overall lower cost per nucleotide than traditional capillary electrophoresis-based methods; thus, addressing some of these limitations. MPS also is poised to expand forensic typing capabilities by providing new strategies for mixture deconvolution with the identification of intra-STR allele sequence variants and the potential to generate new types of investigative leads with an increase in the overall number and types of genetic markers being analyzed. The beta version of the Illumina ForenSeq DNA Signature Prep Kit is a MPS library preparation method with a streamlined workflow that allows for targeted amplification and sequencing of 63 STRs and 95 identity SNPs, with the option to include an additional 56 ancestry SNPs and 22 phenotypic SNPs depending on the primer mix chosen for amplification, on the MiSeq desktop sequencer (Illumina). This study was divided into a series of experiments that evaluated reliability, sensitivity of detection, mixture analysis, concordance, and the ability to analyze challenged samples. Genotype accuracy, depth of coverage, and allele balance were used as informative metrics for the quality of the data produced. The ForenSeq DNA Signature Prep Kit produced reliable, reproducible results and obtained full profiles with DNA input amounts of 1 ng. Data were found to be concordant with current capillary electrophoresis methods, and mixtures at a 1:19 ratio were resolved accurately. Data from the challenged samples showed concordant results with current DNA typing methods with markers in common and minimal allele drop out from the large number of markers typed on these samples. This set of experiments indicates the beta version of the ForenSeq DNA Signature Prep Kit is a valid tool for forensic DNA typing and warrants full validation studies of this MPS technology.

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1. Introduction

The current capabilities of forensic DNA typing allow forensic analysts to reliably characterize biological evidence to support criminal investigations. DNA typing methods are used to provide identifications or exculpations, build databases that help find associations and link cases, and generate investigative leads. For years, capillary electrophoresis (CE)-based technologies have been the standard method for DNA typing applications. However, with CE, there are limits to the number of markers that can be multiplexed together as similarly sized amplicons must be labeled with different fluorescent markers in order to accurately assign alleles to the appropriate locus. CE methods also tend to focus on only one marker type at a time, due to a limit on the number of markers that can be multiplexed or different analytical streams.

Massively parallel sequencing (MPS), also known as next-generation sequencing, systems are capable of overcoming the limitations mentioned above and expanding forensic analysts' capabilities to characterize forensic biological evidence. MPS technologies have the potential to sequence at a greater capacity and speed with an overall reduced cost per nucleotide. MPS with the ForenSeq DNA Signature Prep Kit (Illumina, San Diego, CA, USA) costs approximately 84 US dollars (USD)/45 USD a sample with 32/96 samples sequenced per run, and the user generates data on over 200 markers. The manufacturer's protocols recommend up to 32

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casework samples or up to 96 database samples per run with the ForenSeq DNA Signature Prep Kit. Meanwhile, for example, CE kits such as GlobalFiler® Express (Thermo Fisher Scientific, Waltham, MA USA) and Yfiler® Plus (Thermo Fisher Scientific) cost 20 USD and 32 USD a sample, respectively, and the user generates data on 24 markers with GlobalFiler® Express and 27 markers with Yfiler® Plus kits. With MPS, a large number of markers and samples can be sequenced simultaneously. Different types of forensically relevant genetic markers can be multiplexed, and sequence variants within shared STR alleles (or allele and stutter product), undetectable by CE, can be identified. Targeted amplicons in the ForenSeq DNA Signature Prep Kit are clonally amplified and sequenced all at once generating a read depth for each target and allowing for a consensus in sequence calls that produces a greater resolution in sequence accuracy.

The ability to sequence a larger number of markers and multiple types of markers at once leads to many benefits for the DNA typing field. While short tandem repeats (STRs) have been the primary marker used for DNA typing applications due to their highly polymorphic and informative nature, new markers offer additional types of information. Single nucleotide polymorphisms (SNPs) comprise approximately 85% of human genetic variation and can provide identity, ancestry, phenotypic, and pharmacogenetic information [1–10]. This information can help produce investigative leads that were not possible previously or in cases that had reached dead ends. As SNPs reflect only a single base change, they can be analyzed with smaller amplicons and, therefore, are considered more attractive markers for degraded samples [4,5,9,10].

Because a large number of markers can be sequenced simultaneously, more robust associations can be generated from database searches and more thorough kinship analyses can be completed, such as facilitating familial searching and missing person identifications. With MPS, the traditional repeat length of STRs can be determined along with elucidation of any sequence variants present within the alleles [11–16]. Maintaining the ability to determine the traditional repeat length of STRs promotes backward compatibility with current DNA databases. Identification of intra-allelic sequence variants provides greater discrimination power and opportunities for better mixture deconvolution [11–16]. For example, the presence of an intra-allelic sequence variant could help an analyst discriminate between a stutter peak and a minor contributor in a mixture or even detect a minor contributor peak masked by the major contributor.

The beta version of the ForenSeq DNA Signature Prep Kit in conjunction with the MiSeq desktop sequencer (Illumina) and the beta build of ForenSeq Universal Analysis Software (UAS; Illumina) were evaluated for high throughput genotyping of reference and challenged samples. This study was divided into five sets of experiments: reliability, sensitivity of detection, mixture analysis, concordance, and the ability to evaluate challenged samples. Additionally, informative metrics, including depth of coverage (DoC), allele coverage ratios (ACRs), and sequence coverage ratios (SCRs), were used to evaluate the quality and reliability of the data produced.

2. Materials and methods

2.1. Samples

Policies and procedures approved by the Institutional Review Board for the University of North Texas Health Science Center (UNTHSC) in Fort Worth, TX were followed for the collection and use of samples in this study. Samples for each experiment were prepared and extracted as outlined below. All DNA was quantified using the Quantifiler® Human DNA Quantification Kit (Thermo Fisher Scientific) and a 7500 Real Time PCR System (Thermo Fisher Scientific), following the manufacturer's recommended protocol [17].

Experiment 1. Reliability—Three replicates of the 2800M control DNA, supplied with the kit and each at one ng of total input DNA, were sequenced.

Experiment 2. Sensitivity—Dilution series of the 2800M and 9947A control DNAs were prepared at 1000 pg, 500 pg, 250 pg, 100 pg, and 50 pg of total input DNA and sequenced.

Experiment 3. Mixture detection—Seven mixtures were prepared, each consisting of two individuals at a total of one ng of input DNA. One mixture was prepared at a 1:1 male/female ratio. Four mixtures were prepared at a 1:9 ratio as follows: 1:9 male/female; 1:9 female/male; 1:9 male/male; and 1:9 female/female. Two mixtures were prepared at a 1:19 ratio as follows: 1:19 male/female; 1:19 female/male. The reference samples used in the mixtures were obtained from anonymous donors at UNTHSC and were extracted with the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols [18].

Experiment 4. Concordance—Ten reference samples, each of which were unrelated and included one Central Caribbean, two Asian, and seven Caucasian individuals, were sequenced and typed for autosomal STRs and Y-STRs using both MPS and CE methods. These reference samples were sequenced previously on the Ion Torrent Personal Genome Machine® (Ion PGM™) (Thermo Fisher Scientific) and typed for identity informative SNPs on both MPS platforms. The samples used to evaluate concordance were obtained from anonymous donors at UNTHSC and were extracted with a Qiagen DNA Investigator kit or a QIAamp DNA Blood Mini Kit (Qiagen) [18,19].

Experiment 5. Challenged samples—Five aged buccal swabs that were collected over six years prior to DNA extraction and five human bone samples, with previously generated STR data and available from the University of North Texas Center for Human Identification missing person identification laboratory, were sequenced. Traditional STR typing using CE was completed as well for comparison purposes. The aged buccal swabs were extracted with the QIAamp DNA Blood Mini Kit (Qiagen) following the manufacturer's protocols [18]. The human bone samples were extracted according to the protocols described in Marshall et al. [20].

2.2. Capillary electrophoresis concordance data

Conventional STR typing was completed using the AmpFlSTR® Identifiler® Plus PCR Amplification Kit (Thermo Fisher Scientific) and the AmpFlSTR® Yfiler® PCR Amplification Kit (Thermo Fisher Scientific) with one ng of DNA for each reaction per the recommended manufacturer's protocols [21,22]. The GeneAmp® PCR System 9700 thermal cycler (Thermo Fisher Scientific) was used for PCR amplification, and electrophoresis was carried out on an ABI Prism® 3130xl Genetic Analyzer (Thermo Fisher Scientific). Raw data were analyzed with GeneMapper® ID software v3.2.1 (Thermo Fisher Scientific).

2.2.1. Ion PGM™ concordance data

SNP typing was completed on the Ion PGM™ using the HID-Ion AmpliSeq™ Identiy Panel (Thermo Fisher Scientific) as described in Churchill et al. [23].

2.2.2. ForenSeq DNA Signature Prep Kit (Beta version)

The beta version of the ForenSeq DNA Signature Prep Kit provides PCR primer mixes for the targeted amplification of 63 STRs (i.e., 29 autosomal STRs, 25 Y-STRs, and 9 X-STRs) and 95 identity informative SNPs (iSNPs) with the option to include 56 ancestry informative SNPs (aSNPs) and 22 phenotypic informative SNPs (pSNPs) depending on the primer mix used [24]. However, the ancestry informative and phenotype informative SNPs were not analyzed in this study. The sizes of the targeted amplicons for SNPs range from 64 to 231 base-pairs (bps), and the sizes of the targeted amplicons for STRs range from 61 to 430 bps [24]. Additional information about the ForenSeq DNA Signature Prep Kit can be found at http://support.illumina.com/forensics-support/forensics-kits/forenseq-dna-signature-kit.html. The beta version of the ForenSeq DNA Signature Prep Kit's primer mixes may be obtained upon request from Illumina [personal communication from Illumina].

2.2.3. Library preparation

The ForenSeq DNA Signature Prep Kit was used to prepare libraries. Genomic targets were amplified and tagged using ForenSeq oligonucleotide primer mix B and a GeneAmp® PCR System 9700 thermal cycler following the manufacturer's recommended beta protocol [24]. Next, the targets were enriched by amplifying the tagged amplicons with primers that add index sequences which allow for sample multiplexing and adapters necessary for cluster generation. This enrichment was completed with a GeneAmp® PCR System 9700 thermal cycler following the manufacturer's protocol [24]. The libraries then underwent a bead-based purification with reagents included in the ForenSeq DNA Signature Prep Kit, and recommended protocols were followed to purify the reaction components from the PCR products. Finally, a bead-based normalization was completed using reagents included in the ForenSeq DNA Signature Prep Kit following recommended protocols [24]. The normalized libraries for each sample were pooled in equal volume amounts. A total of 32 samples were pooled per run. Seven μl and ten μl in the first and second run, respectively, of the pooled libraries were diluted in hybridization buffer and denatured according to the manufacturer's protocols [24].

2.2.4. MPS sequencing and data analysis

Sequencing was performed on the MiSeq desktop sequencer with a MiSeq ForenSeq Sequencing Kit (351 × 31 bp) (Illumina) according to the manufacturer's protocol [24]. Data analysis was performed using the beta build of ForenSeq UAS. Additionally, the ForenSeqRunStatistics XML file was analyzed further with an in-house Excel-based workbook to verify genotyping calls and chart marker performance within a run as output by the ForenSeq UAS. STR genotypes generated in the ForenSeq UAS were verified with STRait Razor where possible [11].

2.2.5. Final statistical analysis

DoC, also known as read depth, and ACRs, i.e., heterozygote balance, were calculated for each autosomal and *X* (for females) STR locus and each identity informative SNP locus. SCRs were calculated for each STR locus. ACRs were calculated by dividing the lower coverage allele by the higher coverage allele at that locus (e.g. 400*X*/450*X* = 0.89; 1.0 indicating equal coverage). SCRs used the bp sequence of the STR alleles versus the repeat structure of the alleles. SCRs were calculated by dividing the number of reads used to make nominal repeat length allele calls and the number of reads

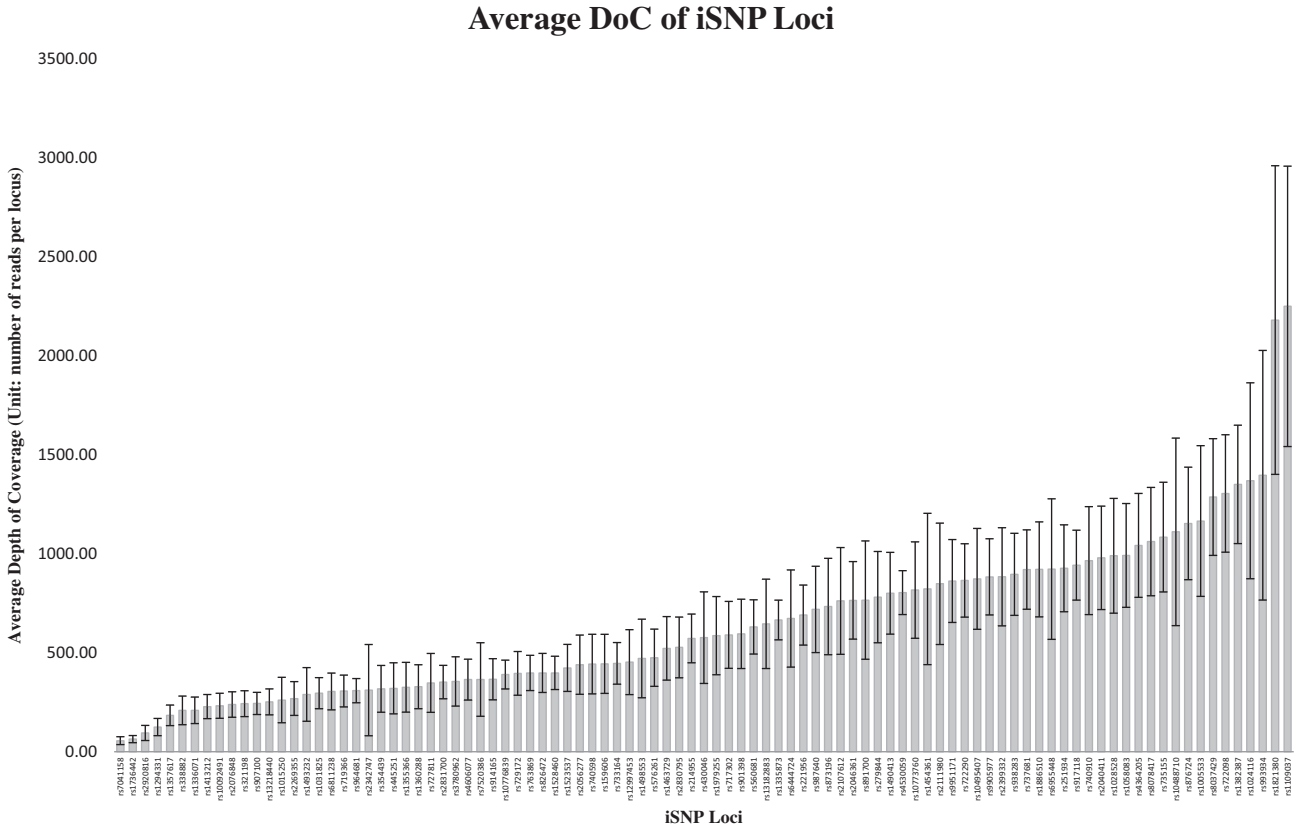

Fig. 1. Average DoC for the identity informative SNPs in the ForenSeq DNA Signature Prep Kit for the 10 reference samples and 2800M control DNA amplified with one ng of DNA. Error bars represent standard deviation.

attributed to PCR artifacts (i.e. stutter) by the total number of reads at that marker (e.g., 450*X*/500*X* = 0.90; 0.9 equates to 90% of reads on target without nucleotide misincorporation).

3. Results

3.1. MiSeq run information

The MiSeq sequencing runs in this study had a cluster density of 716 K/mm2 and 1295 K/mm^2, respectively. A total of 94.12% of clusters generated in the first run passed filters, and 86.31% of clusters generated in the second run passed filters. This value refers to the chastity filter, which helps remove unreliable data from the analysis pipeline often corresponding to clusters with too much signal intensity for bases other than the called base. The signal from each cluster is evaluated in the first 25 cycles to calculate the chastity [25]. The chastity of a base call is defined as the "ratio of the highest intensity divided by the sum of the highest and second highest intensities" [25]. Clusters pass this filter if only one or fewer cycles have a chastity below the threshold of 0.6 [25]. There were 0.188% (first run) and 0.149% (second run) of molecules within a cluster phasing (falling behind) and 0.093% (first run) and 0.080% (second run) of molecules within a cluster pre-phasing (going ahead). A total of 8,647,049 reads were generated in the first run, and 14,890,283 reads were generated in the second run. When examining samples with a uniform DNA input of one ng (i.e., reference and control samples), an average of 3.32% of the total reads (±0.48%) were identified with each sample's unique barcodes, thus indicating a successful bead-based normalization. A more detailed description of the intensity (number of reads) and percent of reads passing filter for each sample can be found in Supplementary Table 1.

3.2. Analysis of data quality

A series of informative metrics were calculated for the data produced from the 2800M control DNA used in experiment one (an evaluation of reliability) and the ten reference samples used in experiment four (an evaluation of concordance with CE). These metrics included DoC, ACRs, and SCRs. Performance metrics of the 63 STRs and 95 identity informative SNPs were analyzed separately.

The average DoC across the SNPs was 644 reads per locus (X) (±405X). The average DoC for each marker fell within the range of 56X–2,248X (Fig. 1). Eighty-eight of the 91 heterozygous SNP loci analyzed in this study had an average ACR of 0.6–1.0 (Fig. 2). The remaining three SNPs were rs7520386, rs338882, and rs6955448. These three SNPs had an average DoC of 365X (±186X), 209X (±72X), 922X (±355X) and an average ACR of 0.3 (±0.09), 0.5 (±0.17), and 0.5 (±0.10), respectively. The four SNPs seen with ACRs of zero in Fig. 2 were homozygous for all individuals typed in this study, and therefore, ACRs could not be calculated for these markers.

The average DoC across the STRs was 2,104X (±2,045X). The average DoC for each marker fell within the range of 68X–13,014X (Fig. 3). Thirty-nine of the 40 STR markers for which ACRs were calculated (autosomal and X-markers (on females); ACRs were not calculated for Y-markers as they have only one allele, except for two Y-markers with known duplicates) had an average ACR of 0.6–1.0 (Fig. 4). The remaining STR was D22S1045. This STR had an average DoC of 2,730X (±2,126X) and an average ACR of 0.3 (±0.17).

Sequence variation beyond the nominal repeat-length based allele calls traditionally made for STR markers also was analyzed. SCRs were calculated as they allow for a comparison of the number of unique sequence reads used to make allele calls versus the

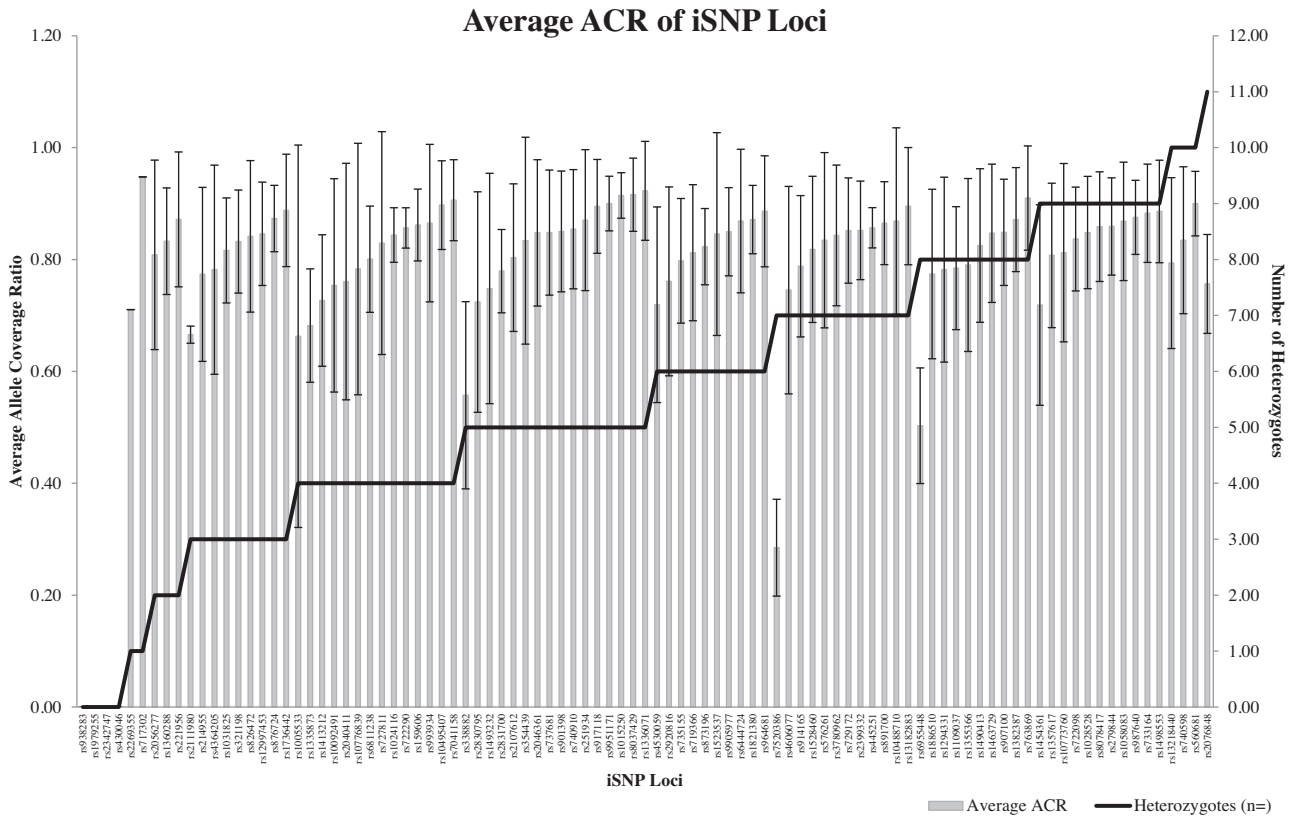

Fig. 2. Average ACRs for the identity informative SNPs in the ForenSeq DNA Signature Prep Kit for the 10 reference samples and 2800M control DNA amplified with one ng of DNA. ACRs were calculated by dividing the lower coverage allele by the higher coverage allele at that locus, with 1.0 indicating equal coverage. Horizontal black line indicates the number of heterozygotes for each calculated ACR. Error bars represent standard deviation.

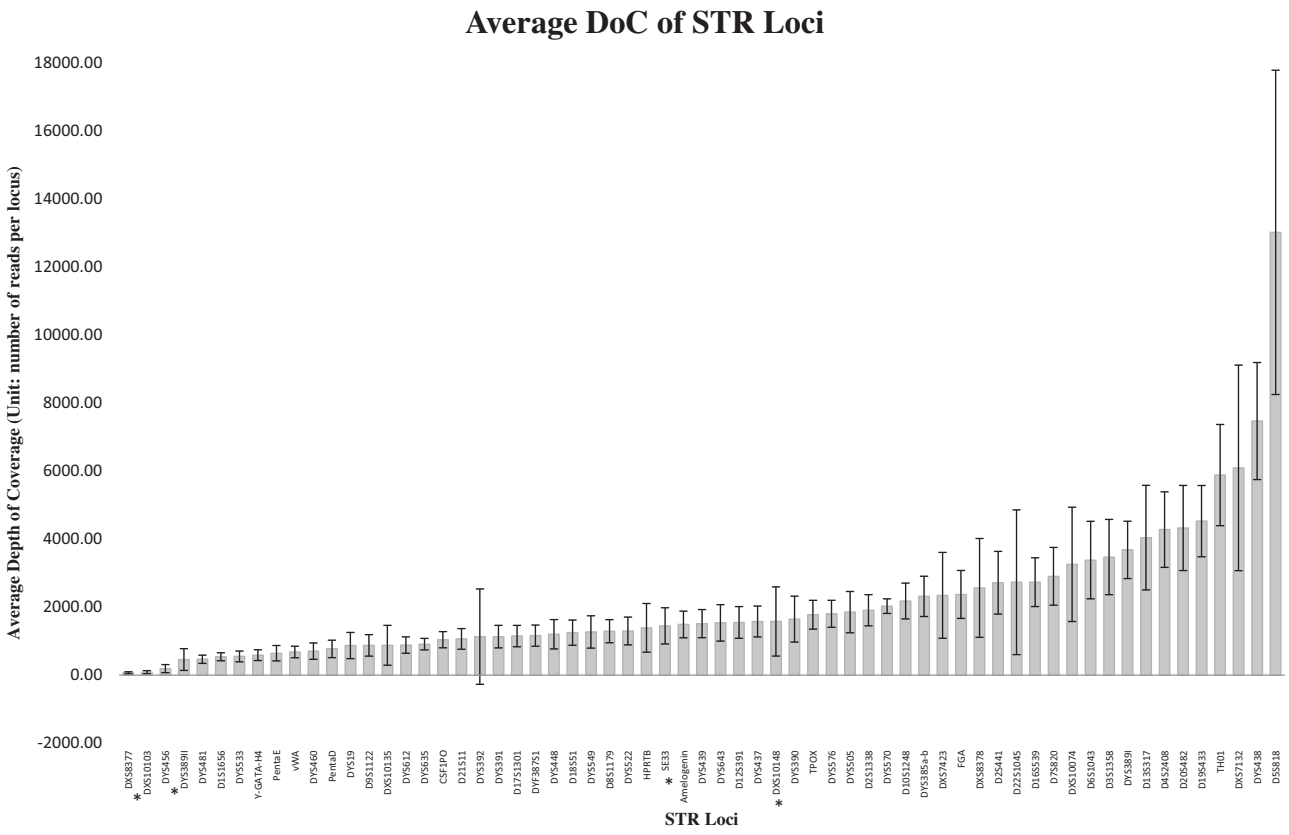

Fig. 3. Average DoC for the STRs in the ForenSeq DNA Signature Prep Kit for the 10 reference samples and 2800M control DNA amplified with one ng of DNA. Error bars represent standard deviation. *Some of the illustrated loci have been removed from the current version of the ForenSeq DNA Signature Prep Kit.

number of unique sequence reads that can be attributed to noise (i.e., sequencing/PCR errors). SCRs for the STRs ranged from 0.54–0.98 (Fig. 5). Only the STRs DYS456 and DXS8377 had SCRs that fell below 0.60, with SCRs of 0.54 and 0.58, respectively. In addition to the calculation of SCRs, the STR alleles were examined for the presence of sequence variants (i.e. intra-allelic SNPs or repeat-motif variations). Sequence variants were identified for a total of 22 loci with several of these loci having sequence variants present in more than one allele (Supplementary Table 2).

3.3. Reliability

Overall, reproducible results were obtained across all three replicates of the 2800M control DNA. Genotypes were evaluated for all typable alleles. Alleles were called when a DoC of ten reads or higher was observed. Full and reproducible profiles were generated for all 95 identity informative SNPs and 62 of the 63 STRs. The DYS392 locus was marked as inconclusive for two of the three replicates due to the low number of reads generated for these two replicates at this marker.

3.4. Sensitivity

The ForenSeq DNA Signature Prep Kit's level of sensitivity of detection was evaluated by varying the amount of input DNA from 1000 pg to 50 pg of two control samples. Alleles were called when a DoC of ten reads or higher was observed. In counting the number of alleles observed for each profile (Table 1), heterozygotes were counted as two alleles whereas homozygotes were only counted as one allele since it was not possible to check for allele dropout in homozygotes. Full and concordant profiles were seen for the one ng DNA input amounts. Most alleles (i.e., 99.5%, 98.3%, and 94.4%) were observed in the sequence data from the 500 pg, 250 pg, and 100 pg samples, respectively (Table 1). More substantial dropout was seen in the 50 pg samples with only 69.4% of the alleles being detected in the sequence data (Table 1). DoC for the dilution series in both samples decreased linearly overall with decreasing amounts of input DNA, and allelic imbalance (loci with ACRs of 0.4 or lower) was observed starting at DNA input amounts of 250 pg and lower.

3.5. Mixtures

A total of seven mixtures were analyzed in this study. One male/female mixture was prepared at a 1:1 ratio. Four mixtures (i.e., male/male, female/female, male/female, female/male) were prepared at a 1:9 ratio. Two mixtures (i.e., male/female and female/male) were prepared at a 1:19 ratio. The ForenSeq DNA Signature Prep Kit's ability to assess mixtures was evaluated by the identification of STR alleles consistent with the minor contributor's type within each mixture. Within the 1:1 mixture, 52 potential alleles were identified. Within the mixtures prepared at a 1:9 ratio, 12, 13, 42 (Y alleles were counted as part of the minor contributor's type for this male/female mixture), and 14 potential alleles were identified, respectively. Within the mixtures prepared at a 1:19 ratio, 40 and 12 potential alleles were identified, respectively. STR alleles lying within the minus stutter position ($n-4$) or the plus stutter position ($n+4$) were not considered for minor contributor assessment. For the 1:1 mixture, all the 1:9 mixtures, and one of the 1:19 mixtures, all minor contributor alleles were identified in addition to the major contributor alleles and stutter products. In the female/male mixture prepared at a 1:19 ratio, the minor contributor alleles at the D12S391, D13S317, DXS10135, and DXS7423 loci were not called due to a DoC below ten reads. These

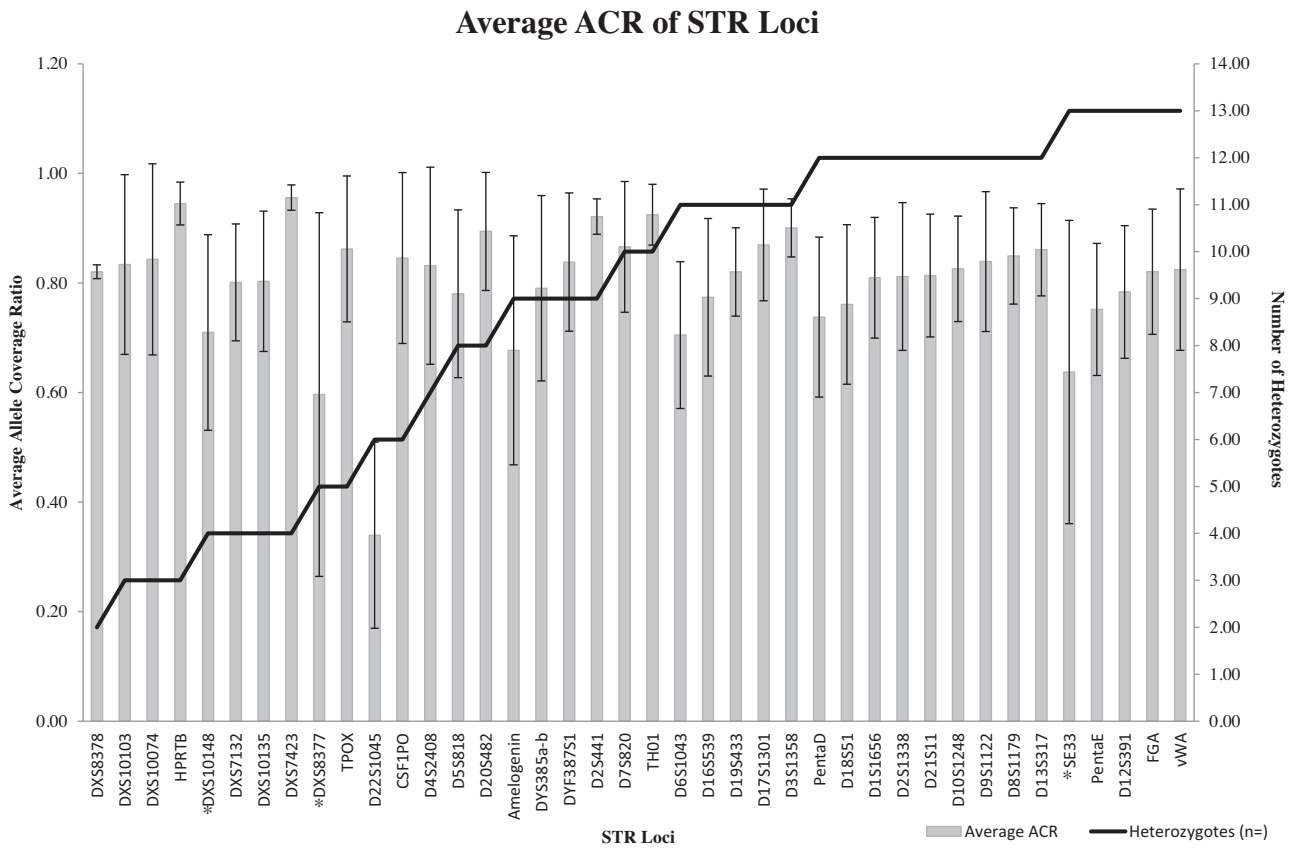

Fig. 4. Average ACRs for the STRs in the ForenSeq DNA Signature Prep Kit for the 10 reference samples and 2800M control DNA amplified with one ng of DNA. ACRs were calculated by dividing the lower coverage allele by the higher coverage allele at that locus, with 1.0 indicating equal coverage. Horizontal black line indicates the number of heterozygotes for each calculated ACR. Error bars represent standard deviation. *Some of the illustrated loci have been removed from the current version of the ForenSeq DNA Signature Prep Kit.

markers exhibited an average DoC of 1,549X (±466), 4,041 (±1,537), 878 (±586), and 2,346 (±1,262) in the reference and control samples, respectively. Furthermore, intra-allelic sequence variants within the repeats of some loci (Supplementary Table 2) allowed for assignment of both true alleles and stutter products of the minor contributor. Fig. 6 provides an example of how these intra-allelic sequence variants aided in the interpretation of the 1:9 male/male mixture.

As done with the STR alleles, the ForenSeq DNA Signature Prep Kit's ability to assess mixtures was evaluated by the identification of SNP alleles consistent with the minor contributor's type within each mixture. SNP loci with non-overlapping alleles (e.g., major genotype AA; minor genotype GG) were considered for evaluating mixtures. Within the 1:1 mixture, 18 potential SNP alleles were identified. Within the mixtures prepared at a 1:9 ratio, 8, 13, 9, and 9 potential SNP alleles were identified, respectively. Within the mixtures prepared at a 1:19 ratio, 9 potential SNP alleles were identified for each mixture. The minor contributor's type was identified at all SNP loci except for rs2342747 in the second 1:9 mixture, rs7041158 in the third and fourth 1:9 mixture and first 1:19 mixture, and rs727811 in the first 1:19 mixture. Alleles were not called when DoC was below ten reads. Repeated allele and marker dropout was observed with rs7041158 when evaluating challenged samples, and this locus also was the lowest performing SNP marker, in terms of average DoC, in the reference and control DNA samples as well with an average DoC of 56X (±20X). The SNP rs727811 exhibited an average DoC of 347X (±149X) in the reference and control DNA samples. The SNP rs2342747 exhibited an average DoC of 311X (±230X) in the reference and control DNA samples. However, SNP rs2342747 typed homozygous for all the reference and control DNA samples. This observation prompted further investigation of the results for this locus, which lead to the identification of a SNP 29 bps 3′ of rs2342747. This SNP may be located in a primer binding site and be the cause of the observed dropout. In addition, similar allele dropout was observed at locus rs430046. These allele dropouts are not observed when using primers from the commercial version of the ForenSeq DNA Signature Prep Kit, indicating this issue has likely been addressed.

3.6. Concordance

A control sample (run in triplicate) and ten reference samples were used to evaluate the concordance of data generated with the ForenSeq DNA Signature Prep Kit. With MPS, alleles were called when a DoC of ten reads or higher was observed. With CE, alleles were called when a RFU signal of 50 or higher was observed.

As a control sample, the 2800M's genotypes were known for each marker included in the ForenSeq DNA Signature Prep Kit [24]. Therefore, concordance was evaluated for each marker. The genotypes generated for each replicate of the control sample were concordant for all typable alleles with the profile provided for 2800M in the ForenSeq DNA Signature Prep Guide [24]. The DYS392 locus was not called for two of the three replicates due to the low number of reads generated for these two replicates at this marker.

To evaluate concordance between MPS and traditional CE methods, the ten reference samples were typed with the ForenSeq DNA Signature Prep Kit and the Identifiler® Plus and Yfiler® kits. While locus dropout was seen in two loci with CE (DYS392 and DYS635) and one locus with MPS (DYS392), results were

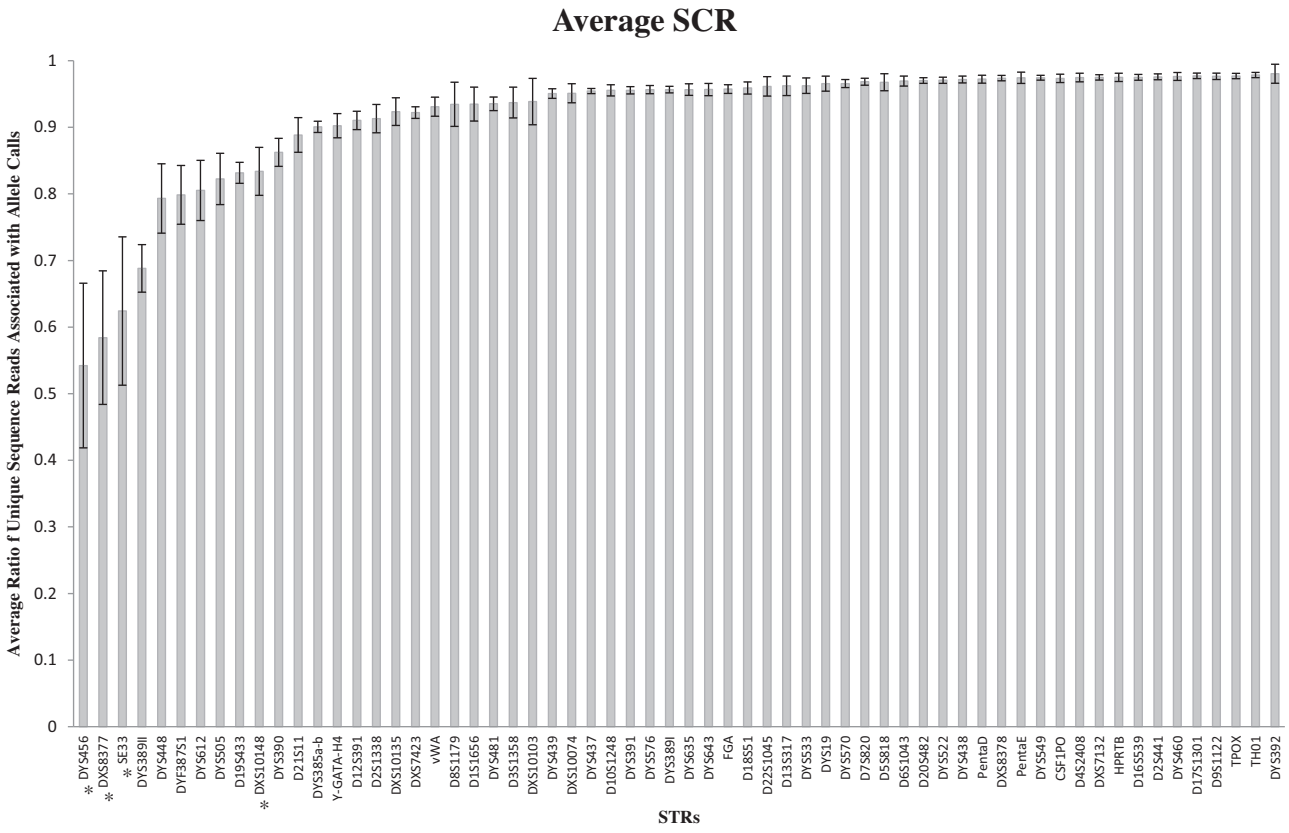

Fig. 5. Average SCRs for the STRs in the ForenSeq DNA Signature Prep Kit for the 10 reference samples and 2800M control DNA amplified with one ng of DNA. SCRs were calculated by dividing the number of reads attributed to nominal repeat length allele calls and stutter by the total number of reads at that marker (e.g., 0.9 equates to 90% of reads on target without nucleotide misincorporation). Error bars represent standard deviation. *Some of the illustrated loci have been removed from the current version of the ForenSeq DNA Signature Prep Kit.

concordant between the two methods for all typable alleles. STR genotypes generated in the ForenSeq UAS were verified with STRait Razor where possible, and all overlapping loci between the two bioinformatic methods (55 STR loci) were found to produce concordant genotypes [11].

To include additional concordance data for the identity informative SNP loci, the ten reference samples were typed with the ForenSeq DNA Signature Prep Kit on the MiSeq and the HID-Ion AmpliSeq™ Identity Panel on the PGM. Concordance was evaluated on the 84 overlapping loci in these two panels. Results were concordant between the two panels for all typable alleles, except at the loci rs10488710, rs430046, and rs2342747. Allele dropout was observed in one sample for rs10488710 when typed with the ForenSeq kit. Allele and locus dropout was observed in three samples for rs430046 when typed with the ForenSeq kit. Allele and locus dropout was observed in two samples for rs2342747 when typed with the ForenSeq kit. The latter two loci were discussed previously.

Table 1
Number of alleles observed for STR and SNP markers for dilution series of two control DNAs.

	Alleles 0bserved	
	SNPs	STRs
2800M_1000pg	129 (100%)	90 (100%)
2800M_500pg	129 (100%)	90 (100%)
2800M_250pg	128 (99.2%)	88 (97.8%)
2800M_100pg	121 (93.8%)	88 (97.8%)
2800M_50pg	101 (78.3%)	84 (93.3%)
9947A_1000pg	130 (100%)	59 (100%)
9947A_500pg	128 (98.5%)	59 (100%)
9947A_250pg	126 (96.9%)	59 (100%)
9947A_100pg	118 (90.8%)	58 (98.3%)
9947A_50pg	52 (40.0%)	46 (78.0%)

The percentage of alleles observed out of the total number of possible alleles is included in parentheses.

3.7. Challenged samples

Ten challenged samples were chosen based on data generated for these samples with standard CE methodology. These samples included DNA from aged swabs that yielded poor STR results and five bone DNA samples that yielded a varying number of typable alleles with CE technology. Each sample produced concordant results between the two methods for the typable markers with the exception of one marker (DYS392) for one bone sample. With CE, a '15 allele' was observed at the DYS392 locus for this bone sample whereas an '11 allele' was observed with MPS. While STR allele dropout was seen in both CE and MPS results, MPS methods were able to consistently detect equal or more STR alleles than standard CE methods (Table 2). Results also were obtained for at least 89 of the 95 identity informative SNPs in the ForenSeq panel in nine of the ten samples analyzed (Table 2). Only one locus, rs7041158, exhibited complete marker dropout in at least half of the samples. This SNP locus was the lowest performing marker, in terms of average DoC, in the reference and control DNA samples with an average DoC of 56X (±20X).

4. Discussion

A beta level evaluation of the ForenSeq DNA Signature Prep Kit, used in conjunction with the MiSeq desktop sequencer and the

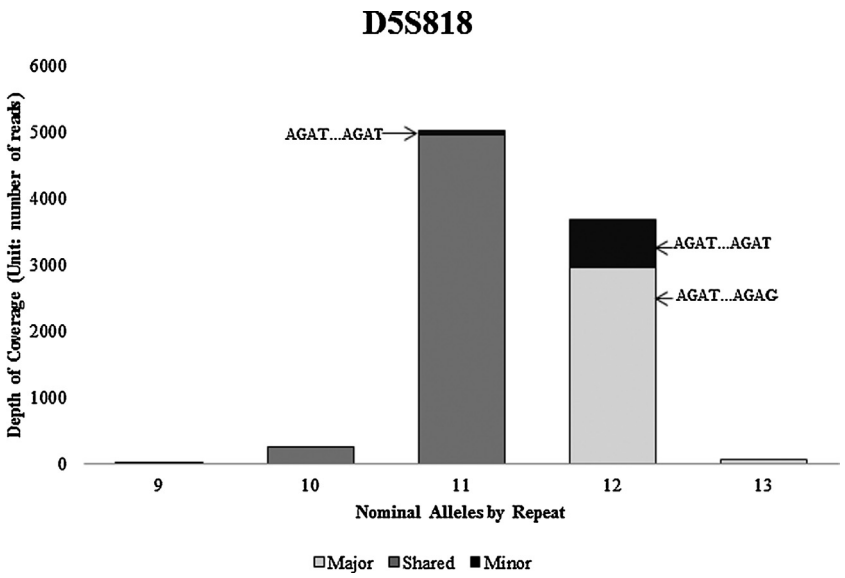

Fig. 6. Minor (11,12) and major (11,12) contributor alleles at locus D5S818 from the male/male mixture prepared at a 1:9 ratio are illustrated. An intra-allelic sequence variant within the 12 allele distinguishes the minor contributor's 12 allele from the major contributor's 12 allele and distinguishes the minor contributor's associated stutter from the 11 allele. Because of the quantitation of contributors at the 12 allele, strong inferences can be made that the minor contributor also has an 11 allele, depending on ACR threshold, (marked as "Shared" in the figure). In addition, minus stutter can be detected at positions 9 and 10, and plus stutter can be detected at position 13.

Table 2
The total number of STR and SNP loci observed with CE and MPS for the ten challenged samples.

Sample ID	STR Loci Observed		SNP Loci Observed
	CE	MPS[a]	MPS[a]
31	29 (100%)	63 (100%)	93 (97.9%)
12	24 (82.8%)	57 (90.5%)	95 (100%)
13	1 (3.4%)	23 (36.5%)	90 (94.7%)
14	29 (100%)	63 (100%)	94 (98.9%)
15	29 (100%)	63 (100%)	95 (100%)
16	29 (100%)	63 (100%)	94 (98.9%)
17	17 (58.6%)	56 (88.9%)	92 (96.8%)
18	28 (96.6%)	62 (98.4%)	95 (100%)
19	1 (3.4%)	11 (17.5%)	25 (26.3%)
20	15 (51.7%)	49 (77.8%)	89 (93.7%)

The percentage of loci observed out of the total number of possible loci is included in parentheses.
[a] MPS has more markers in its multiplex.

ForenSeq UAS, was completed to determine utility in analyzing a large panel of forensically relevant genetic markers on reference and challenged samples. This evaluation was divided into five sets of experiments in order to examine the ForenSeq system's reliability, sensitivity, ability to interpret mixtures, concordance with CE, and success with challenged samples. While limited to only a small collection of samples, this study demonstrated the feasibility of typing a large multiplex of forensically relevant genetic markers on the MiSeq platform. The results from this study lend support that the ForenSeq system can produce reliable results.

The quality and reliability of the raw data produced with the ForenSeq DNA Signature Prep Kit were evaluated by examining the DoC, ACRs, SCRs, reliability (Experiment 1), and concordance (Experiment 4). Considerable variation in DoC was observed among the STR and SNP markers (Figs. 1 and 3). These differences in coverage from marker-to-marker likely stem from differences in PCR amplification efficiency. The accuracy and efficiency of the library preparation procedure, such as an incomplete purification process or an unsuccessful bead-based normalization, could also affect DoC. The broad range of performance seen with these markers while running reference samples could be exacerbated further, potentially, if the sample quality were to be decreased (e.g., forensic samples), the amount of input DNA were to be lowered, or the number of markers in the multiplex were to be increased (e.g., using the ForenSeq primer mix that includes aSNPs and pSNPs as well). MPS systems are capable of tolerating multiplexes with both high and low performing markers without the impact of saturated signal output that may be observed in CE systems. However, while the two or more magnitudes range in DoC across the markers in the MPS multiplex should not affect the reliability of the results seen in this study, this variation in coverage can impact sample throughput. The minimum performing markers will drive the throughput requirements and the highest performing markers will limit the throughput.

A minimum analytical coverage threshold has not been set (and likely will be laboratory specific). A number of considerations for determining DoC thresholds must be addressed prior to implementation. These include, but are not limited to, a more thorough validation and assessment of overall kit performance and the possible inclusion of controls to assess the DoC in a more dynamic fashion. A dynamic threshold based on internal controls within each run has been demonstrated previously [4,15]. A high DoC can be generated easily by a number of factors (e.g., low-throughput multiplexing of samples [4], number of markers assayed [15], library normalization, etc.). Other factors (e.g., ACR and SCR) must be taken into account to assess profiles. Regardless, care must be taken to monitor the DoC with MPS data. Determining a sufficient read depth for each marker will provide guidance for potential allele dropout and when allele sequence reads can be distinguished from stutter or noise (i.e., PCR/sequence errors). The ACRs calculated for both the STRs and SNPs on the reference samples in this study (Figs. 2 and 4) indicated well-balanced data had been generated. Only three SNPs (rs7520386, rs338882, and rs6955448) and one STR (D22S1045) had an ACR below 0.6. Illumina has addressed the low ACR for D22S1045 in the current version of the ForenSeq DNA Signature Prep Kit manual by noting to interpret D22S1045 with caution as "more imbalance in read counts may be seen between alleles of a heterozygote than observed at other loci" [24].

The reads for the STR alleles were readily distinguishable from the reads attributed to noise (e.g., PCR/sequencing errors or low level somatic variation). The noise should be described thoroughly

during validation processes as the SCR varied among the markers, and the level could impact interpretation of mixed samples. Likely a per-marker basis account for the noise will be needed for interpretation. A more comprehensive validation will be detailed in a future manuscript.

Finally, genotype accuracy was evaluated in two separate manners: examining concordance of the genotype calls made with MPS and CE technologies and reproducibility of genotype calls on reference samples. A total of ten reference samples were typed with both MPS and CE, and results were concordant. Concordant and reproducible results also were obtained across the replicates of the 2800M control DNA typed in the reliability study. In experiment one, the DYS392 locus demonstrated low depth of coverage and was marked as inconclusive for two of the three replicates due to the low number of reads. As mentioned above, making genotype calls with too low a read depth could allow for missed alleles or inclusion of sequencing errors as real variants. Stutter evaluation is another important part of STR typing interpretation. Increased stutter across a few Y-STRs (including DYS392) in this small sample set emphasizes the need for further validation work to understand the limits of the system. Stutter analyses were not performed and presented in this study because of the limited sample size.

While one ng of input DNA is the manufacturer's recommended amount of starting material for the ForenSeq DNA Signature Prep Kit, the limits of the kit's sensitivity of detection (Experiment 2) were tested with lower DNA quantities. Dilutions of two control DNAs from 1000 pg to 50 pg were prepared and sequenced. The ForenSeq DNA Signature Prep Kit achieved full profiles with DNA input amounts of one ng. Most alleles (94.4%) were observed in the sequence data with DNA input amounts as low as 100 pg indicating the potential to reach sensitivity levels comparable with current DNA typing technologies. The ForenSeq DNA Signature Prep Kit's success with challenged samples also was evaluated in this study (Experiment 5). The ten challenged samples used in this study produced concordant results for all typable alleles between MPS and CE technologies with the exception of one marker for one of the bone samples. A '15 allele' was observed at the DYS392 locus for this bone sample using CE technologies whereas an '11 allele' was observed with MPS. Similar discordance has been observed between CE-based STR kits and will be observed with MPS and CE kit comparisons as well. The likely cause is due to forward primer binding site position in the CE format which is 5′ of the repeat than in the MPS format. The former construct likely contains an insertion (more work will be necessary to fully resolve this discrepancy). Overall, MPS out-performed CE in terms of the number of typable alleles produced (Table 2). MPS provided more detectable STR alleles for seven of the ten challenged samples (the same number of STR alleles was detected for the other three samples) at shared loci, and there was the ancillary benefit of SNP genotypes provided by the MPS marker multiplex (Table 2).

One of the notable advantages to the way STR data are analyzed stems from the ability of MPS to distinguish intra-STR allele sequence variants that were undetectable by traditional CE approaches. Twenty-two of the STRs analyzed in this relatively small sample size study exhibited sequence variants among some of their alleles. Some markers, such as D12S391, had variants present in multiple alleles. This variation offers additional information to assist in profile interpretation. These variants can provide a greater discrimination power when distinguishing individuals and searching through databases, can aid in kinship analyses, and may help deconvolute some mixtures. Experiment three provides an example of the utility of the intra-STR allele sequence variants. In instances where individuals have overlapping alleles (or overlapping allele and stutter) of the same repeat-length size, differences in the actual sequence of the allele can aid in discrimination between the individuals and allow for quantitation of the contribution of those alleles. Fig. 6 illustrates a specific example where an intra-allelic sequence variant in the 12 allele of the D5S818 locus assisted in the interpretation of a mixture and helped identify the alleles from the minor and major contributors.

5. Conclusion

This study described an evaluation of the beta level format of the ForenSeq DNA Signature Prep Kit. Such evaluations are important because they indicate the strengths and limitations of MPS systems designed for forensic analyses as they continue to advance and improve. The evaluation illustrated the ForenSeq kit is capable of producing reliable and accurate MPS data. This study also supports that the MiSeq and its related chemistries and workflow are a viable MPS system for forensic genetic analysis and has the potential for utility in forensic genetic laboratories. The large number of genetic markers in the ForenSeq multiplex offers forensic analysts a number of analytical benefits, such as more markers from the same amount of input DNA, and, essentially, eliminates the need to choose or prioritize between relevant genetic markers applicable to specific cases. The large set of STRs allows for compatibility to be maintained with current DNA databases while increasing the potential for typing success and discrimination power. Grouping autosomal STRs, X-STRs, and Y-STRs into the same amplification provides identity and lineage information simultaneously, and including SNPs for simultaneous analysis expands the scope of available information. The smaller amplicon sizes afforded with SNP analysis increase the potential for successful typing of degraded or low quality samples. The ForenSeq kit also offers a straight-forward and streamlined workflow with relatively fast turn-around time for data generation. Starting with genomic DNA, one analyst was able to obtain raw data on over 200 markers in three to four days with only one of those days spent at the lab bench. The promising nature of the results from this study warrants full validation studies of the ForenSeq DNA Signature Prep Kit.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2015.09.009.

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