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Research paper

Development and validation of a novel multiplexed DNA analysis system, InnoTyper® 21

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ABSTRACT

We report here a novel multiplexed DNA analysis system consisting of 20 *Alu* markers and Amelogenin for analysis of highly degraded forensic biological samples. The key to the success of the system in obtaining results from degraded samples is the primer design yielding small amplicon size (60–125 bp) for all 20 markers. The markers included in the InnoTyper® 21 system are bi-allelic, having two possible allelic states (insertion or null) and thus termed INNULs. The markers are short interspersed nuclear elements (SINEs), a category of retrotransposable elements (REs) which are non-coding genomic DNA repeat sequences, or "mobile insertion elements," comprising approximately 40% of the human genome. *Alu* elements are primate specific SINEs that have reached a copy number in excess of one million in the human genome, which makes these markers highly sensitive and desirable for forensic samples with extremely degraded DNA. Until now however, due to the inherent size difference associated with insertion and no insertion alleles, the use of *Alu* REs has not been practical for forensic applications. The novel primer design described herein has allowed the development of a multiplexed *Alu* system yielding fragment sizes amenable to degraded DNA samples, as frequently encountered in missing persons cases or forensic samples such as hair shafts. Although use of *Alu*s in human identity has been studied using single marker amplification and reported before, we report for the first time development and validation of a system with multiplexed RE markers. Studies performed include PCR optimization, species specificity, sensitivity, degradation and inhibition, precision and accuracy, nonprobative samples, mixture, and population database studies. A population study using 592 samples including five populations was performed using InnoTyper 21. The data indicated the random match probability for the combination of these 20 *Alu* markers was greater than 1 in 3.8 million for the populations studied, indicating the greater statistical power of these autosomal nuclear DNA markers over haplotype systems typically used in such degraded samples. Results demonstrate the system is successful in obtaining results from highly degraded DNA. A sensitivity study performed demonstrated at least 95% recovery of alleles from as low as 50 pg of total input DNA, and partial profiles from as low as 25 pg. This study has demonstrated that the bi-allelic INNULs in the InnoTyper 21 system provide a sensitivity of detection and a power of discrimination that makes them useful for human identification of extremely degraded samples.

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1. Introduction

Forensic DNA testing methodology has seen several improvements and innovation in recent years, mainly increased number of loci in a single multiplex for high discrimination power and utilization of massively parallel sequencing methodology [1–3]. Short tandem repeat (STR) loci are the primary genetic markers used in human identity testing. These markers are highly polymorphic and afford a high degree of sensitivity of detection such that relatively low quantities (e.g., 100 picograms) of template DNA can be analyzed [4–8]. However, one of the limitations of STR markers is that the amplicon size of some of these markers can be greater than 200 base pairs (bp), even up to 400–600 bp, which can make it difficult to type highly degraded samples. In recent years several STR multiplexes have been designed with reduced amplicon sizes for such applications (i.e., the so-called mini-STRs) [9–11]. However, only a few STR markers can be simultaneously designed with amplicon sizes less than 150 bp to be more suitable for typing highly degraded samples, and run together on current capillary electrophoresis instruments. For highly degraded samples, mitochondrial DNA (mtDNA) is typically used [12,13]. However, current mtDNA analysis is laborious and expensive, few forensic laboratories currently have mtDNA typing capabilities, and, due to a lack of recombination, its power of discrimination is low.

Single nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms (InDels) have been investigated as an alternative to STRs to analyze highly degraded DNA [14,34–37]. These markers can be detected in shorter amplicons and have low mutation rates. A type of marker similar in nature to InDels is retrotransposable element (such as *Alu*) insertion polymorphisms, or INNULs (for insertion/null) [15]. INNULs are highly abundant in the human genome and extremely stable once inserted. These markers can be useful for human identity testing [16–22].

The INNUL polymorphism is based on the presence and absence of retrotransposable elements (REs) which consist of long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs). SINEs were originally defined by their interspersed nature and length (75–500 bp). LINE full-length elements are ~6 kb in length, contain an internal promoter for polymerase II, two open reading frames (ORFs), and end in a polyA-tail [15]. Ustyugova et al. [23] demonstrated that REs could be used for cell line identification. Mamedov et al. [24] and Novick et al. [25] described a set of *Alu*s (a type of SINE) for paternity testing. Both of these studies intimated that the systems could be applied to forensic analyses. The lack of, or very minimal, mutation rate in *Alu*s [26] compared to STR systems [27] makes an *Alu* based genotyping system appealing for kinship analyses compared with the less stable STRs. In addition, *Alu* based systems do not yield stutter artifacts due to slippage during the PCR as seen with STRs [1–4]. Although REs make up over 40% of the human genome [28] and present myriad potential targets for human identity testing, these INNULS have received limited attention for use in forensic human identity testing.

The most likely reason for a lack of interest is that the insertion alleles are quite different in size from the null state and thus the alleles are susceptible to preferential amplification and effects of DNA degradation. Forensic samples are often compromised in quality and quantity. Degraded samples may contain fragments of DNA that are less than 165 bp in length and the quantities may be limited to sub-nanogram levels of recoverable DNA [29–32]. REs can range in size from hundreds (SINEs) to several thousand (LINEs) bp in length [16–22]. Previous attempts to use *Alu* sequences for identity testing capitalized on the size difference between insertion and null alleles by amplifying the entire region with the same forward and reverse primers [24]. The insertion allele would be 200–400 bp larger than the null allele and could be detected by electrophoresis based on size differences. While useful for paternity testing and some population studies where DNA is not limited or compromised, the large size difference between amplicons of the no-insertion (null) and insertion alleles will impact amplification efficiency and increase allele dropout during the PCR, which is a limitation for forensic samples (i.e., preferential amplification favoring the smaller allele amplicon and degradation possibly causing drop-out of the insertion allele). Thus, the use of REs as a multiplexed marker system has not been embraced for the analysis of forensic samples [24].

To overcome the multiplexing difficulties due to allele size differences, the unique sequences of the *Alu* insertion were exploited to detect the presence or absence of *Alu* insertion at a given genomic position [17,18]. One unique property of these REs is a direct repeat sequence at the beginning of the *Alu* insertion and at the end of the *Alu* element called target site duplication (TSD) [16,17]. Fig. 1 shows the primer design strategies employed to design a highly efficient multiplexed amplification system of 20 retrotransposon insertion polymorphisms and Amelogenin. Utilizing this strategy, one can design primers to create very small amplicons to type highly degraded DNA samples regardless of the size of *Alu* insertion [17,18].

The design, performance, and developmental validation of InnoTyper® 21 system are described below. The results show that the INNUL system can enhance capabilities for typing biological evidence and, in particular, highly compromised samples.

2. Materials and methods

2.1. Fluorophore selection and matrix standard

The InnoTyper 21 Human Identification kit uses a five-dye chemistry for amplicon detection which is compatible with Applied Biosystems® Genetic Analyzers. Proper spectral calibration is critical to evaluate multicolor systems with the Applied Biosystems Genetic Analyzers. The IGT 5-Dye Matrix Standard (InnoGenomics Technologies) consists of DNA fragments labeled with the fluorescent dyes: FAM™, JOE, TMR, ROX, and TGI-ORANGE. These matrix fragments are used on the Applied Biosystems 310, 3130, 3130xL, 3500 or 3500xL Genetic Analyzers to perform a spectral calibration on dye set G5. Once generated, this file is applied during sample detection to calculate the spectral overlap between the five different dyes and separate the raw fluorescent signals into individual dye signals. The IGT 5-Dye Matrix Standard was developed for use with the 5-dye InnoTyper 21 System.

2.2. ILS-155 Internal Lane Standard

ILS-155 (InnoGenomics Technologies) contains eleven single stranded DNAs with the TGI-Orange fluorophore attached. The

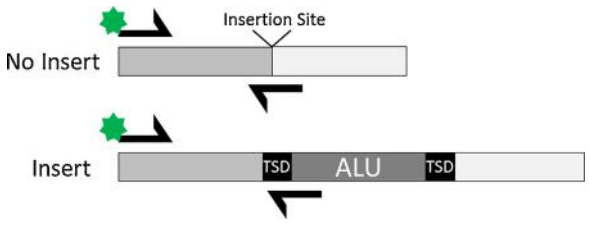

Fig. 1. Primer design strategy to type insert and native (or null) alleles with similar but not exact amplicon size differences between the two allelic states. The strategy employs a common forward primer with fluorescent label at the 5′ end and two specific reverse primers to amplify either insertion allele or null allele, or both in the case of a heterozygote individual.

fragments are 55, 60, 70, 85, 95, 105, 115, 125, 135, 145, and 155 base pairs in size. There are two fragments smaller and three fragments larger in size than the smallest and largest InnoTyper 21 alleles, respectively, which allow using the Local Southern method for size calling with GeneMapper®, GeneMapper® ID and GeneMapper® *ID-X* software (Applied Biosystems).

2.3. Primer design

Primers were designed using Primer3 (input version 0.4.0, http://frodo.wi.mit.edu/primer3/). A set of three primers was designed for each marker: one forward primer and two reverse primers, one for the insertion and one for the null allele. The primer design strategies to obtain small amplicons were reported earlier [16,17]. The sex determining marker, Amelogenin, consists of one labeled forward primer and one unlabeled reverse primer. All the designed primers have T_m values in the range of 55–65 °C [33]. To obtain reverse complement information, the program Reverse Complement was used from the Harvard Medical Technology Group and Lipper Center for Computational Genomics (arep.med.harvard.edu/). Subsequently, the primers were screened against the GenBank non-redundant database to determine if they were unique DNA sequences.

2.4. Marker selection

Markers were selected from existing literature and through BLAST sequence analysis [15–25,38–41]. After initial selection, the potential loci were assessed for suitability for primer design using Primer 3 software [42]. Utilizing the above described primer design strategy, the markers were first evaluated by gel electrophoresis for amplification efficiency, formation of single amplicon and also allele frequency on a set of 25 genomic DNA samples. Chromosomal location and distance were also considered for marker selection. The selected markers were distributed throughout the genome at different chromosomes or a different arm of the same chromosome. Only three sets of markers AC2265/AC2305, MLS09/TARBP and AC1141/NBC51 are on the same arm of the same chromosome, but they are far apart (see Table 1 for chromosomal location). After extensive evaluation [16], twenty *Alu* markers plus Amelogenin were selected for the InnoTyper 21 multiplex (Table 1).

2.5. Primer set optimization

The allele sizes of InnoTyper 21 range from approximately 63 bp to 123 bp. Twenty-one markers are distributed into four dye channels (blue, green, yellow, and red) in a way that no marker overlaps another within one channel and that the two sister alleles sit adjacent to each other (Fig. 2). Initially, single-plex reactions composed of one forward and two reverse primers for each selected marker were carried out to ensure locus-specific amplification. Then multiplex reactions were optimized through primer melting temperatures, primer concentration adjustment and empirical performance testing. Twenty-two DNA samples were amplified at 500 pg to evaluate peak heights and peak height ratios of heterozygous alleles. Non-template controls were amplified in triplicate to evaluate formation of primer-dimer peaks in the range of 60–130 bp.

2.6. Creation of allelic ladder

In order to create a consistent source of target DNA for manufacturing of the allelic ladder, each individual allele was amplified and cloned using Thermo Scientific CloneJet PCR Cloning Kit as per manufacturer's recommended procedure. The PCR products obtained by amplifying genomic DNA from homozygote individuals were used to set up the ligation reaction along with the cloning vector, pJET1.2/blunt, T4 DNA Ligase, reaction buffer and water. The reaction mixture was used directly for transformation. Transformation of the circular plasmid DNA into chemically competent *Escherichia coli* cells was performed, and the LB agar plate containing ampicillin was incubated overnight at 37 °C. Four to six clones were selected for screening. Sequencing primers included with the CloneJet PCR Cloning Kit were used to set up PCR amplification, and the amplified products were analyzed on a 2% agarose gel for the presence of an insert. Using the Zyppy Plasmid Miniprep Kit from Zymo Research Corp, Irvine, CA 92614, U.S.A., plasmid DNA was isolated. The purified plasmid DNA containing cloned portions of the allelic ladder were verified by DNA sequencing. Amplified products from these plasmid DNAs were mixed in appropriate amounts to create the allelic ladder for InnoTyper 21.

The allelic ladder consists of 40 different INNUL alleles as well as Amelogenin X and Y. Fig. 2 shows the InnoTyper 21 allelic ladder.

Table 1
Selected twenty *Alu* markers and Amelogenin in the InnoTyper 21 kit. Marker AC4027 is from hg16 human genome; remaining markers are from hg18 human genome.

Marker	Florescence Dye	Chromosome	Band	Location	Gene ID
AC4027	FAM	7	7q21.11	chr7:82559246-82559572	AC004027.1; 997_1332del
MLS26	FAM	3	3p22.1	chr3:40216628-40216628	Ya5-MLS26; RIP_Alu_chr3_040_01
ALU79712	FAM	20	20p12.2	chr20:11465280-11465588	79712; RIP_Alu_chr20_011_01
NBC216	FAM	7	7p14.1	chr7:38474999-38475312	Ya5NBC216; 4601; Ya5505; RIP_Alu_chr7_038_01
NBC106	FAM	21	21q22.2	chr21:40508751-40509060	Yb8NBC106; RIP_Alu_chr21_040_01
RG148	JOE	2	2q23.3	chr2:150467557-150467867	Yc1RG148; RIP_Alu_chr2_150_03
NBC13	JOE	16	16p12.1	chr16:26515540-26515866	pAlu16-26535378; Yb8NBC13; RIP_Alu_chr16_026_02
AC2265	JOE	13	13q33.1	chr13:102807866-102808174	pAlu13-102846400; 79718; Ya5ac2265; RIP_Alu_chr13_102_01
MLS09	JOE	1	1q25.3	chr1:179124190-179124190	Ya5-MLS09; RIP_Alu_chr1_177_01
AC1141	TAMRA	3	3q11.2	chr3:96598900-96599212	Yb8AC1141; pAlu3-96397335; RIP_Alu_chr3_096_01
TARBP	TAMRA	1	1q42.2	chr1:234,527,060-234,614,849	AL136124.10; 33110_33420Sdel
AMEL	TAMRA	X, Y	Xp22.1-22.3 Yp11.2		AL137015.9; 46641_46955del
AC2305	TAMRA	13	13q13.3	chr13:38926483-38926791	Ya5ac2305; RIP_Alu_chr13_038_01
HS4.69	TAMRA	5	5q34	chr5:164366293-164366709	NT_023133
NBC51	TAMRA	3	3q28	chr3:191773344-191773631	Ya5NBC51; Ya5NBC345; RIP_Alu_chr3_191_01
ACA1766	ROX	8	8q12.1	chr8:61367553-61367857	Ya5ACA1766; RIP_Alu_chr8_061_01
NBC120	ROX	22	22q11.21	chr22:16427377-16427718	Yb8NBC120; RIP_Alu_chr22_016_04
NBC10	ROX	4	4q31.21	chr4:144792753-144793064	Yb9NBC10; RIP_Alu_chr4_144_01
NBC102	ROX	17	17q23.3	chr17:58919634-58919634	Ya5NBC102; Ya5ACE; RIP_Alu_chr17_058_01
SB19.12	ROX	19	19q13.43	chr19:61803374-61803676	Sb19.12; RIP_Alu_chr19_061_01
NBC148	ROX	14	14q31.1	chr14:80666808-80667112	Yb8NBC148; RIP_Alu_chr14_080_02

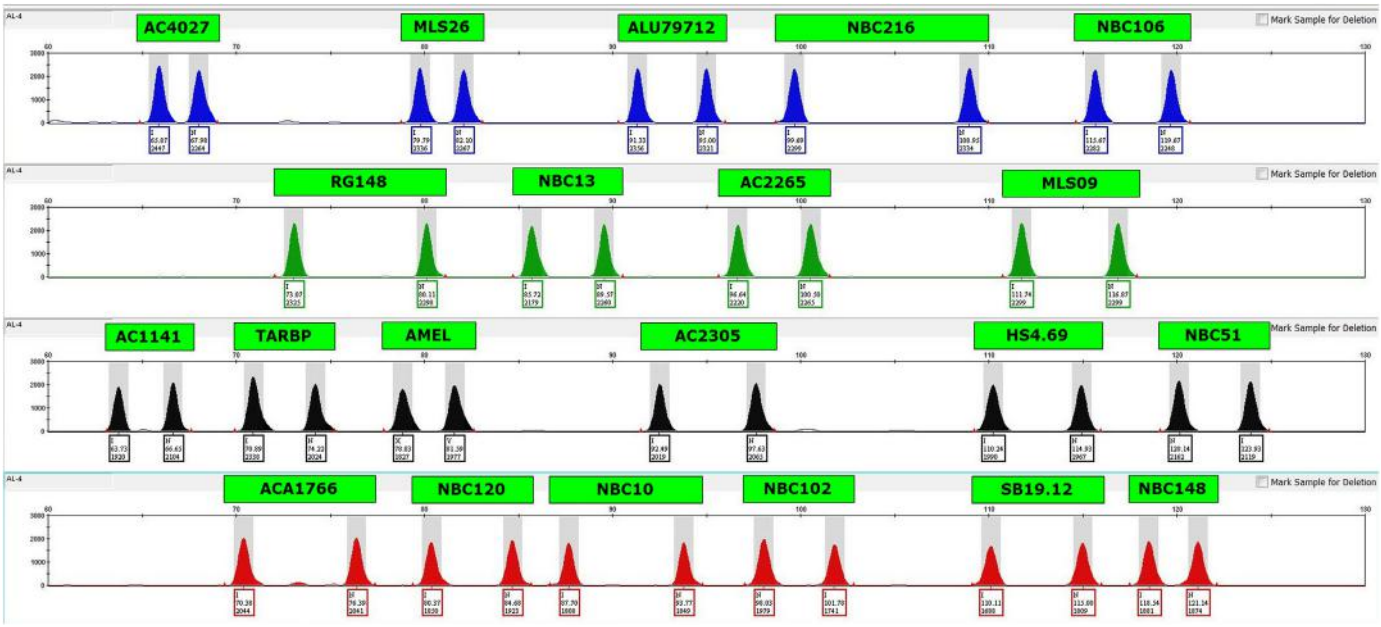

Fig. 2. InnoTyper 21 Allelic Ladder showing locus and allele configuration.

The peaks labeled "I" are the insertion alleles and "N" are the null (i.e., no insertion) alleles. In all instances, primer design optimization resulted in the insertion allele having a smaller size than the no-insertion allele, except for markers NBC51 and NBC102, where optimal primer design resulted in the no-insertion allele being smaller than the insertion allele.

2.7. Optimization of PCR components

The components of InnoTyper 21 PCRs are dNTPs, monovalent salt, Mg^{2+}, carrier proteins, hot start DNA polymerase, sodium azide, Tween 20, and DMSO [33]. Each of these components was tested at a series of concentrations individually to determine the optimal concentrations of each. Pristine DNA samples were amplified in triplicate at 100 pg, 50 pg, and 25 pg (data not shown) and evaluated for peak heights and profile recoveries, and three non-template controls were amplified in each condition for the evaluation of primer-dimer formation within the marker region (60 bp to 130 bp).

2.8. Optimization of thermal cycling conditions

Optimal thermal cycling conditions should generate sufficient sample peak heights while minimizing the occurrence of off-scale allele peaks or allelic dropout events [33]. The thermal cycling parameters of InnoTyper 21 were examined to determine the optimal performance conditions on a GeneAmp® PCR system 9700 with a gold plated silver block using the 9600 emulation mode (Applied Biosystems). The tested parameters were cycle number (31, 32, 33, and 34 cycles), annealing temperature (58 °C, 59 °C, and 60 °C), and elongation temperature (60 °C, 65 °C, and 72 °C). At least three non-template controls were amplified in each condition for the evaluation of baseline, artifacts, and primer-dimer formation within the marker region (60–130 bp). The optimal conditions were selected by amplifying pristine DNA samples in triplicate at 50 pg and evaluated for peak heights and degrees of profile recoveries. From this study, the standard thermal cycling conditions for InnoTyper 21 was determined to be 95 °C for 15 min (enzyme activation), followed by 31 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 60 s, and then the final extension at 60 °C for 60 min with a 4 °C temperature hold. These parameters are set to yield high sensitivity of detection from low level samples while minimizing the production of PCR artifacts.

2.9. DNA samples and DNA extraction

Anonymous samples used for the validation experiments and control DNA were obtained from The Blood Center (New Orleans), DNA Diagnostic Center (Ohio), Tulane Health Sciences Center (New Orleans), Coriell Institute for Medical Research (New Jersey), and the National Institute of Standards and Technology (Maryland). Extraction methods used include PCIA organic extraction method and ChargeSwitch® Nucleic Acid Purification Technology (Invitrogen). DNA obtained from ATCC (Manassas, VA) was used for the mixture study. Non-probative sample types included mock sexual assault kit swabs, hair, bone, tooth, blood, semen and saliva samples, as well as degraded and inhibited DNA samples. All extractions were run with a reagent blank. Samples were stored at −20 °C until amplification.

2.10. Quantification of DNA using real-time PCR amplification

All human DNA samples were quantified using the InnoQuant® Human DNA Quantification & Degradation Assessment Kit (InnoGenomics Technologies) [43] on the 7500 Real-Time PCR System (Applied Biosystems). The data were analyzed using the HID Real-Time PCR Analysis Software v1.1/1.2 (Applied Biosystems) as described in the InnoQuant® Human DNA Quantification & Degradation Assessment Kit User Guide. Non-human DNA samples were quantified using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific).

2.11. PCR amplification

Unless noted otherwise, InnoTyper 21 amplifications were performed in a reaction volume of 25 μL using the standard thermal cycling conditions as described. The final optimized

reactions contain 3.5 μL of InnoTyper 21 Primer Mix, 5 μL of InnoTyper 21 Master Mix, 0.5 μL of IGT DNA Polymerase, and a maximum volume of 16 μL of target DNA template. VWR® PCR 8-Well Tube Strips (VWR) were used to amplify DNA samples in the GeneAmp PCR system 9700 with a gold plated silver block (Applied Biosystems). Samples also were typed with the Identifiler® Plus (Thermo Fisher Scientific) [7] and MiniFiler® (Thermo Fisher Scientific) [9] kits following their user manuals. The Identifiler Plus PCR reactions were performed using 29 cycles and the MiniFiler reactions were performed using 30 cycles.

2.12. Capillary electrophoresis

PCR amplified products were separated and detected on the Applied Biosystems 3130 Genetic Analyzer with POP-4™ polymer (Applied Biosystems) using the specified G5 variable binning module as described above in the Fluorophore Selection and Matrix Standard section for InnoTyper 21, as well as the corresponding Identifiler Plus and MiniFiler user guides. For InnoTyper 21, 1 μL of the amplified product or allelic ladder and 0.2 μL of ILS-155 Internal Lane Standard were added to 10.8 μL of deionized Hi-Di™ Formamide (Applied Biosystems), denatured at 95 °C for 3 min, and chilled on ice for 3 min. Samples were injected and separated on the Applied Biosystems 3130 using the default "FragmentAnalysis36_POP4_1" Run Module Settings for the 4-capillary 3130 instrument. The data were collected using the Applied Biosystems 3130 Data Collection Software application v3.0. The procedures of sample preparations of Identifiler Plus and MiniFiler for capillary electrophoresis were followed as described in their respective user guides.

2.13. Data analysis

Electrophoresis results were analyzed with GeneMapper® *ID-X* software v1.1/1.4 or GeneMapper® 4.0 by setting the analytical threshold at 50 relative fluorescence units (RFU) for InnoTyper 21, Identifiler Plus, and MiniFiler. The Local Southern size calling method was used to analyze CE results of InnoTyper 21 (the Precision Study data was analyzed with Local Southern, 2nd order, and 3rd order least squares sizing methods), with a baseline window of 30 points. InnoTyper 21 consists of twenty-one bi-allelic markers including Amelogenin. Each *Alu* marker will produce either a single peak (homozygous insertion or no insertion) or two peaks (heterozygous sister allele peaks). Amelogenin will produce one X homozygous peak for female samples and X and Y peaks for male samples. The maximum number of peaks that could be possible to obtain with InnoTyper 21 is forty two. In this study a profile recovery expressed in percent (%) is calculated as: a homozygous peak is counted as 2 and each heterozygous sister peak is counted as 1. The sum of the peaks at 21 markers is then taken and this peak count is divided by the maximum count of 42 and multiplied by 100. Regardless of whether there is a difference in sample genotypes, the total peak count is 42 with a full profile. Partial profiles give a peak count less than 42. A mean profile recovery is obtained by taking a mean of profile recoveries of three amplification results, and a standard deviation (SD) of the mean also is calculated. A mean peak height of triplicate results was calculated as follows: first, the average peak height of each replicate was calculated individually. The mean peak height of triplicate results was then calculated by taking a mean of each replicate's average peak height, and SD calculated. Mean profile recoveries with SDs and mean peak heights with SDs of the Identifiler Plus and MiniFiler results were calculated in a similar manner as InnoTyper 21 results.

2.14. Accuracy, precision, and reproducibility

Allelic ladder sizing precision was evaluated from six injections of the ladder run on the Applied Biosystems 3130 Genetic Analyzer. The standard deviation of the mean size of each ladder allele was calculated. Twenty-five DNA samples from twelve Caucasians, twelve African Americans, and the kit control DNA were amplified at 0.5 ng total DNA input in triplicate with the InnoTyper 21 kit and subjected to electrophoresis on the Applied Biosystems 3130 Genetic Analyzer. The genotypes of the obtained profiles from twenty-five DNA samples were compared to their replicate results for reproducibility of the InnoTyper 21 kit. Sample allele sizing was evaluated from the twenty-five DNA sample amplifications in triplicate, which produced a total of 2301 alleles. The results were used to calculate the deviation of the sample allele size from the mean of the allelic ladder allele size.

2.15. Species specificity

DNA samples from three primates (1 ng each from green monkey, orangutan, and chimpanzee) were subjected to PCR amplification four times each using the InnoTyper 21 kit. Five non-primate mammals (5 ng and 10 ng each from cat, dog, deer, rat, and mouse), three non-mammalian species (5 ng and 10 ng each from chicken, fish, and mosquito), and 5 micro-organisms (5 ng and/or 10 ng each from *E. coli*, *Saccharomyces cerevisiae*, and *Staphylococcus aureus* and 10 ng each from *Ralstonia eutropha* and *Rhodococcus rubber*) were subjected to PCR amplification using the InnoTyper21 kit in duplicate. Species DNA samples were obtained from Coriell (Camden, New Jersey) (orangutan and chimpanzee), AMRESCO (Solon, OH) (fish sperm), the Microbiology Department of Tulane University (New Orleans, LA) (*S. cerevisiae*, *S. aureus*, green monkey, mouse, mosquito,), and other DNA sources were obtained internally (*R. eutropha, R. rubber, E. coli*, cat, dog, deer, chicken, and rat).

2.16. Sensitivity study

Two DNA samples, NIST SRM 2372 Component A and control DNA 1212, were serially diluted and amplified in triplicate with the InnoTyper 21 kit. The NIST Human DNA Quantitation Standard (SRM 2372A) was used in the sensitivity study as a means of obtaining accurate sample concentrations. Both DNA templates were amplified at 400, 200, 100, 50, 25, 12.5, and 6.25 pg. The triplicate results were analyzed for profile recoveries, peak heights, and peak height ratios (PHR). PHR was calculated by dividing the smaller peak height by the larger peak height of heterozygous alleles.

2.17. Degradation study

A TruSonik TS-2.5L ultrasonic cleaning device was used to mechanically shear DNA samples. The device, which emits ultrasonic waves at a frequency of 40 KHz, was filled with distilled water and set at 60 °C. 110 μL of extracted DNA (sample 285) from blood were sonicated in time increments for up to 20 hours. 10 μL of the DNA solution was removed at 0, 2, 4, 6, 8, 11, 14, 16, and 20 hours of sonication. The degraded DNA samples were quantified and the extent of degradation was assessed using the InnoQuant kit (InnoGenomics Technologies). The InnoQuant kit is a multiplex qPCR system targeting two independent retrotransposon genomic targets to obtain quantification of an 80 bp "short" DNA fragment and a 207 bp "long" DNA fragment in a DNA sample. The ratio of the two quantitation values (short/long) provides a "Degradation Index" (DI), or a semi-quantitative measure of a sample's extent of degradation. The more degraded a DNA sample is, the higher the DI

value becomes. The InnoQuant DI values were used as a measure of a sample's extent of degradation. Nine different levels of the degraded DNA (sample 285) with DI values of 0.89, 2.16, 2.48, 13.14, 15.16, 62.42, 66.16, 75.16, and 241.69 were obtained by sonication. 200 pg of each level of the degraded DNA was amplified in triplicate with the InnoTyper 21, Identifiler Plus, and MiniFiler kits. The triplicate results were analyzed for degree of profile recovery and mean peak heights including their standard deviations. The DNA sample with DI of 241.69 was serially diluted and amplified in triplicate with the InnoTyper 21 kit and MiniFiler kits. The triplicate results were analyzed for profile recovery and peak heights.

2.18. Inhibition study

An inhibition study was performed with hematin (Sigma-Aldrich), humic acid (Alfa Aesar, Ward Hill, MA), and melanin (Sigma-Aldrich) to evaluate performance of the InnoTyper 21 kit in the presence of PCR inhibitors. Hematin is a heme-containing known inhibitor, humic acid is the major organic constituent found in soil, and melanin is a dark pigment occurring in hair and skin. The kit control DNA 1212 (400 pg) was amplified in triplicate in the presence of various concentrations of the inhibitors. Hematin and melanin were prepared at 1 mM and 1 mg/mL, respectively, in 0.1 N NaOH. Humic acid was prepared at 500 ng/μL in TE^{-4} buffer (10 mM Tris at pH 8.0, 0.1 mM EDTA). Each inhibitor was added to the PCR reaction separately to obtain final concentrations ranging 0 to 10 ng/μL of melanin, 0 to 105 μM of hematin, and 0 to 30 ng/μL of humic acid.

2.19. Mixture study

Although mixtures of human DNA are not normally analyzed in a capillary electrophoresis platform with bi-allelic systems such as InnoTyper 21, DNA profiles originating from more than one individual will sometimes be observed in the analysis. Mixtures may result from contamination, either at a laboratory or prior to the sample arriving at a laboratory, or when samples anticipated to contain mixtures (e.g., touch DNA samples) are processed with InnoTyper 21. Therefore, experiments were designed to test a variety of mixture ratios with InnoTyper 21 to determine at what ratio the minor component begins to drop out of the profile observed.

A mixture of two pristine DNA samples was amplified in duplicate at various ratios while holding the total amount of DNA input constant at 500 pg. The ratios were 1:0 (500 pg:0 pg), 15:1 (469 pg:31 pg), 7:1 (438 pg:62 pg), 3:1 (375 pg:125 pg), 1:1 (250 pg:250 pg), and 0:1 (0 pg:500 pg). The major DNA component is female, and the minor component is male. PHR of the mixtures and PHR of the single source samples (data from accuracy, precision, and reproducibility section) were compared.

2.20. Case-type samples study

Casework-type samples typically encountered in a forensic laboratory were examined to determine if reliable results could be obtained with InnoTyper 21. Samples included male and female samples, samples with known low quantities of DNA, and samples that may be compromised in quantity and/or quality. A total of 39 samples of various types were tested with InnoTyper 21, including blood, hair shafts (i.e. lacking root material), saliva, semen, bone, and teeth. The non-probative casework samples had been genotyped previously with the Identifiler Plus and Yfiler® systems (Thermo Fisher). Degradation index values obtained from the InnoQuant quantitation kit were evaluated and correlated with profile recovery. Concordance in conclusions between InnoTyper 21 and previously typed systems was evaluated, as well as peak heights and PHRs.

2.21. Analysis of highly degraded non-probative human remains

Highly degraded human remains were analyzed with InnoTyper 21 by performing a sensitivity study with skeletal remains previously tested with STRs and mtDNA, and by testing a set of challenging human remains with no or minimal previous profile data using autosomal STRs and mtDNA. DNA extraction for the remains was performed via a demineralization protocol followed by PCIA organic extraction method and quantified using the Quantifiler® Duo DNA Quantification Kit (Thermo Fisher). The samples were amplified with InnoTyper 21 using an earlier version of the kit that employed a different size standard (CC5 from Promega), used a maximum DNA input volume of 10 μL, and did not include an allelic ladder for fragment sizing. For the sensitivity study, three sets of human remains were diluted to eight different concentrations of each sample (500 pg, 250 pg, 125 pg, 62.5 pg, 31.25 pg, 15.63 pg, 7.8 pg, 3.9 pg) and amplified with InnoTyper 21. Additionally, a set of 16 human remains, including bone and teeth, that previously produced no or very partial profiles using STR and mtDNA were amplified with InnoTyper 21. The samples were injected on a 3130xl Genetic Analyzer with POP-6™ polymer (Applied Biosystems) and analyzed using a 50 or 100 RFU analytical threshold.

2.22. Population database study

A total of 592 samples obtained under an IRB exemption for discarded, anonymous samples from five sample populations (African-American, $n = 207$; Hispanic, $n = 40$; US Caucasian, $n = 205$; United Kingdom Caucasians, $n = 96$, and Asian, $n = 44$) were typed for the InnoTyper loci. Population and statistical analyses, including F_{ST}, for the INNUL markers amplified by the InnoTyper 21 kit were performed for major population groups with either Genetic Data Analysis (GDA) software [44] or in-house developed software. Departures from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium were tested using Fisher's exact test. Bonferroni's correction for multiple comparisons was performed according to Weir and Cockerham [45].

3. Results and discussion

3.1. Accuracy, precision, and reproducibility

Accuracy is the degree to which the result of a measurement conforms to its actual value. Precision characterizes the degree of mutual agreement among a series of individual measurements, values and/or results. Precision depends only on the distribution of random errors and does not relate to the true value or specified value. Sizing accuracy and precision of the InnoTyper 21 system were assessed first with measurements of the allelic ladder from six injections run on the Applied Biosystems 3130 Genetic Analyzer with POP-4 polymer. Supplemental Table 1 shows the mean size of each allele and its SD, which was shown to be less than 0.05 bp for every allele, demonstrating highly precise sizing for every allele in the InnoTyper 21 kit. Accuracy also was assessed with seventy-five amplified samples (twenty-five DNA samples amplified in triplicate) to measure the deviation of each sample allele from the corresponding allelic ladder allele. The InnoTyper 21 Allelic Ladder was loaded once for every fifteen samples (4 injections per an allelic ladder). Supplemental Table 2 shows the sizing precision results obtained from multiple runs of the sample alleles analyzed with the Local Southern sizing method. The total of 2301 sample alleles were separated into each InnoTyper 21 allele and analyzed

to evaluate various sizing parameters (maximum, minimum, range, mean, and SD). Across all sizing methods used (Local Southern, 2nd order, or 3rd order least squares), the largest range observed was ACA1766-N, which was 0.32 bp ($n = 51$). With the Local Southern sizing method, four alleles exceeded 0.2 bp: MLS26-N (0.29 bp, $n = 66$), ALU79712-I (0.23 bp, $n = 45$), ACA1766-N (0.32 bp, $n = 51$, and SB19.12-I (0.26 bp, $n = 39$). With the 2nd order sizing method, the same four alleles exceeded 0.2 bp: MLS26-N (0.3 bp, $n = 66$), ALU79712-I (0.25 bp, $n = 45$), ACA1766-N (0.32 bp, $n = 51$, and SB19.12-I (0.21 bp, $n = 39$). With the 3rd order sizing method, seven alleles exceeded 0.2 bp (data not shown). Therefore, the Local Southern and 2nd order sizing methods were more precise, and both exceeded the precision observed with the 3rd order sizing method. The rest of the sample alleles showed ranges less than 0.2 bp. Fig. 3 shows the size differences observed between the sample alleles and the mean of the allelic ladder alleles on the Applied Biosystems 3130 Genetic Analyzer with POP-4 polymer, analyzed with the Local Southern and 2nd order sizing methods. The deviations of the sample allele sizes from the allelic ladder sizes, regardless of which sizing method was used, were all less than ±0.3 bp, showing highly accurate sizing results for each allele. All sizing methods tested produced 100% concordance and generated acceptable data. The genotypes of each DNA sample amplified in triplicate produced the same profile, showing the reproducibility of this system.

While the accuracy and precision results are quite impressive and similar to those obtained for STR typing results, such high quality results are not necessarily required for INNUL alleles. Unlike STRs, INNULs have only two alleles and have amplicon allele size difference >2 bases. Therefore, the precision and accuracy of the system exceeds the requirements for correct allele calls.

3.2. Species specificity

Primers in the InnoTyper 21 kit have been designed to amplify human genomic DNA targeting young *Alu* elements. A variety of animal and microbial DNA samples were tested to assess the human specificity of the kit's performance. At lower DNA input (5 ng) no allelic artifacts were observed in the non-primate species DNA. At higher amounts of non-primate species DNA (10 ng) some

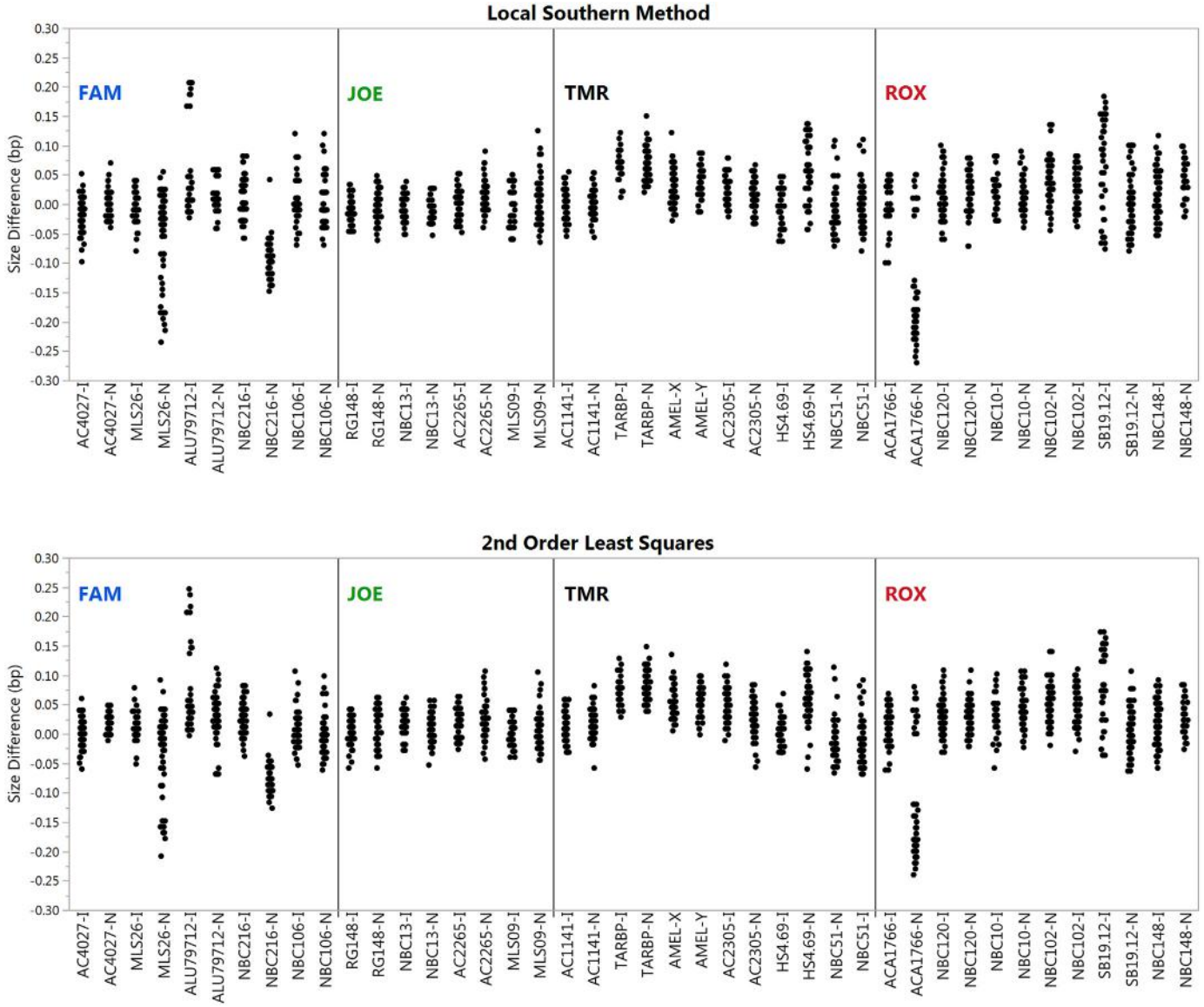

Fig. 3. Accuracy shown by size deviation and fluorophore of 2301 sample alleles from the allelic ladder alleles run on the Applied Biosystems® 3130 Genetic Analyzer and analyzed with the Local Southern and 2nd order sizing methods.

non-allelic artifacts were observed in the electropherograms listed in Table 2. Only one reproducible low level allelic peak (<100 RFU) was observed with chicken DNA when 10 ng input DNA was amplified (Table 2). On the other hand, of the primate DNA samples, chimpanzee and to a lesser extent orangutan and green monkey yielded partial profiles (Table 2). DNA from chimpanzee produced an interesting profile with homozygous peaks at every locus with InnoTyper 21. This result was expected due to genomic similarity among higher primates, especially those closest to humans. Interestingly, none of the peaks produced from the non-human primate species, chimpanzee or orangutan, were insertion alleles. Fig. 4 shows the electropherogram of the InnoTyper 21 profile obtained from the chimpanzee sample. As observed in Fig. 4, all peaks detected were no-insertion (N) peaks. Some of the peak heights were low, likely due to some sequence variation at the primer binding site. This observation supports the hypothesis that the young *Alu* elements are evolutionary insertions in the human genome, and therefore only the ancestral genomic state (no insertion peak) is present in higher primates [38].

3.3. Sensitivity study

To determine the range of DNA quantities able to produce reliable results and to establish the system's limit of detection, sensitivity studies were performed with pristine samples. Triplicate results of the two serially diluted genomic DNA samples are summarized in Table 3 for mean profile recovery (%), mean peak height (RFU), and mean PHR. Full profiles were recovered with as low as 100 pg template input for both DNA samples. The input amounts less than 100 pg exhibited drop-out alleles resulting in partial profile recoveries. Over 93% of the profiles were recovered at 50 pg input DNA for both DNA samples. No drop-in alleles were observed across the tested range of input DNA. The PHR at 400 pg DNA input exhibited relatively well balanced heterozygous peaks. PHR decreased as the amount of input DNA decreased, as expected; however, for the heterozygous markers where both alleles were detected above analytical threshold, PHRs were above 0.6 down to 12.5 pg of input DNA. In those markers exhibiting allele dropout, the highest peak height of the surviving sister allele was 226 RFU. Fig. 5 shows representative electropherograms of the amplified NIST SRM 2372 Component A samples at the indicated amounts. Reliable DNA profiles were obtained across the range of input DNA tested. Sample quantities above 400 pg of input DNA (data not shown) begin to exhibit the effects of excess template DNA, such as PCR artifacts being formed as well as electrophoresis related artifacts, such as pull up.

Table 2
Cross reactivity of InnoTyper 21 on DNA extracts from various organisms.

Species	DNA input (ng)	Reproduced allelic peaks above 100 RFU		Reproduced non-allelic or allelic peaks above 50 RFU			
		Number of peaks	Allele calls	Fluorophore	Average size (bp)	Allele calls	Average peak height (RFU)
Human	0.5	33	AC4027-I, MLS26-IN, ALU79712-N, NBC216-I, NBC106-IN, RG148-IN, NBC13-N, AC2265-IN, MLS09-IN, AC1141-I, TARBP-IN, AMEL-XY, AC2305-IN, HS4.69-IN, NBC51-N, ACA1766-IN, NBC120-IN, NBC10-N, NBC102-N, SB19.12-IN, NBC148-I	–	–	–	–
Chimpanzee	1	20	MLS26-N, ALU79712-N, NBC216-N, NBC106-N, RG148-N, NBC13-N, AC2265-N, MLS09-N, AC1141-N, TARBP-N, AMEL-X, AC2305-N, HS4.69-N, NBC51-N, ACA1766-N, NBC120-N, NBC10-N, NBC102-N, SB19.12-N, NBC148-N	FAM	68.6	AC4027-N	83
Orangutan	1	15	AC4027-N, MLS26-N, RG148-N, NBC13-N, AC2265-N, MLS09-N, AMEL-X, AC2305-N, HS4.69-N, NBC51-N, ACA1766-N, NBC120-N, NBC10-N, NBC102-N, NBC148-N	FAM	117.92	NBC106-OL	2749
Green monkey	1	7	NBC216-N, AC1141-N, AMEL-X, HS4.69-N, NBC51-N, NBC10-N, NBC102-N	–	–	–	–
Deer	5	0	–	–	–	–	–
	10	0	–	–	–	–	–
Dog	5	0	–	–	–	–	–
	10	0	–	TMR	115.12	OMR	109
				ROX	115.2	OMR	90
Cat	5	0	–	–	–	–	–
	10	0	–	FAM	112.68	OMR	92.5
Rat	5	0	–	–	–	–	–
	10	0	–	–	–	–	–
Mouse	5	0	–	–	–	–	–
	10	0	–	FAM	78.17	OMR	96.5
				FAM	80.76	MLS26–OL	65.5
				FAM	103.98	NBC216–OL	527
Chicken	5	0	–	–	–	–	–
	10	0	–	FAM	88.4	OMR	85
				ROX	113.39	SB19.12-N	66.5
Fish	5	0	–	–	–	–	–
	10	0	–	TMR	83.4	OMR	84
Mosquito	5	0	–	–	–	–	–
	10	0	–	–	–	–	–
S. cerevisiae	5	0	–	–	–	–	–
	10	0	–	–	–	–	–
S. aureus	5	0	–	–	–	–	–
E. coli	5	0	–	–	–	–	–
R. eutropha	10	0	–	–	–	–	–
R. rubber	10	0	–	–	–	–	–

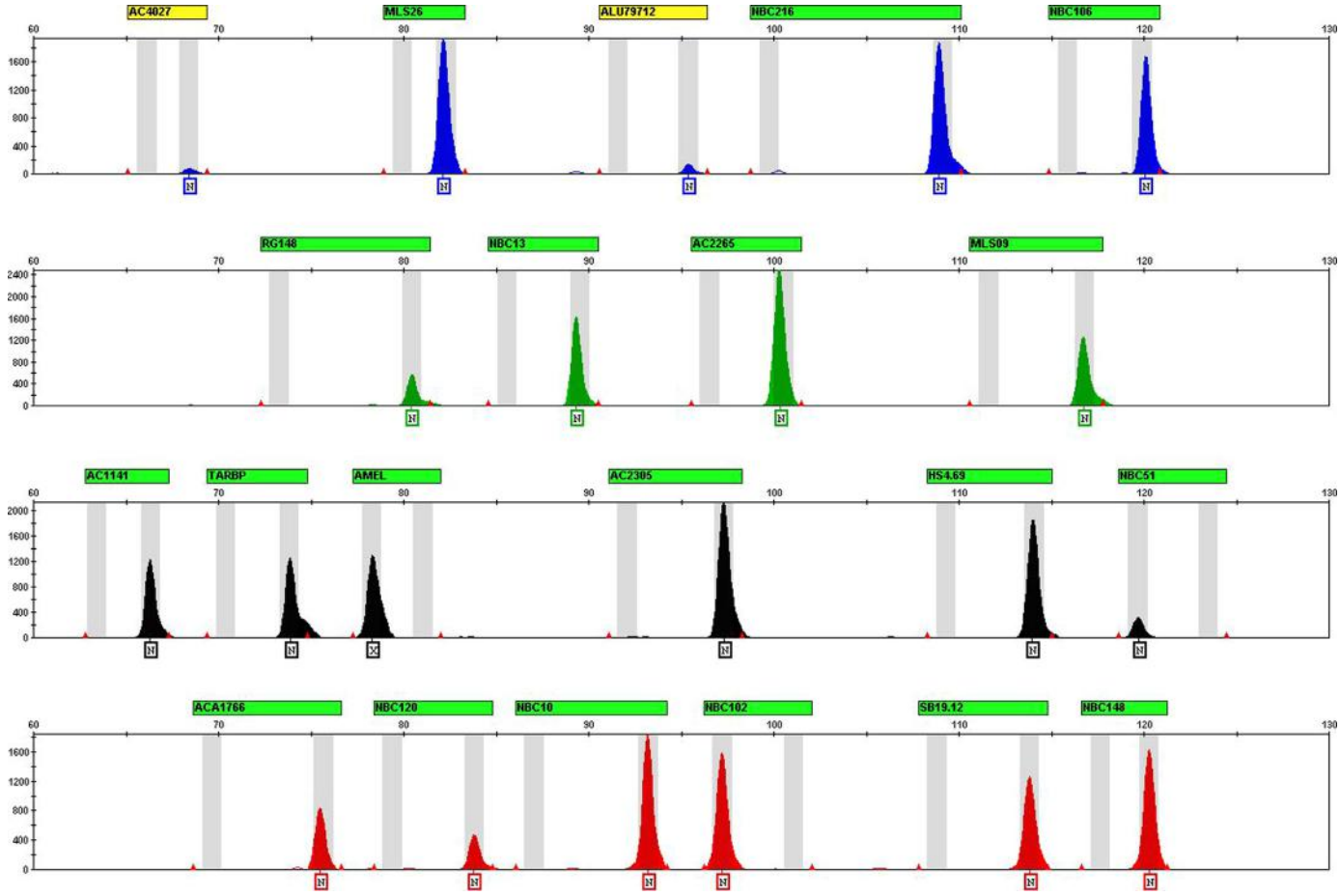

Fig. 4. Representative electropherogram of chimpanzee DNA amplified at 1 ng. All peaks produced were no-insertion alleles.

Table 3
Effects of varying input DNA on profile recovery, peak height, and peak height ratio.

DNA input (pg)	Mean profile recovery (%)		Mean peak height (RFU)		Mean peak height ratio
	NIST A	1212	NIST A	1212	
400	100.0 ± 0.0	100.0 ± 0.0	1381 ± 95	1112 ± 216	0.84 ± 0.11
200	100.0 ± 0.0	100.0 ± 0.0	738 ± 40	601 ± 47	0.77 ± 0.14
100	100.0 ± 0.0	100.0 ± 0.0	381 ± 34	301 ± 59	0.66 ± 0.16
50	95.2 ± 2.3	93.7 ± 1.4	183 ± 20	155 ± 13	0.67 ± 0.19
25	79.4 ± 9.9	71.4 ± 10.4	107 ± 12	106 ± 4	0.68 ± 0.20
12.5	40.5 ± 10.4	48.4 ± 9.9	81 ± 4	85 ± 1	0.62 ± 0.11
6.25	11.9 ± 4.1	20.6 ± 5.5	63 ± 11	66 ± 8	N/A

3.4. Degradation study

Amplicon sizes of widely accepted STR markers can be greater than 200 bp and alleles have been reported to be as long as 500 bp [8], making these large size markers less suitable for analysis of highly degraded DNA samples. However, the largest amplicon in InnoTyper 21 is 123 bp (NBC51 insertion allele). In this study, performance levels of InnoTyper 21, Identifiler Plus [7], and MiniFiler [9] were compared for analyzing degraded DNA samples. Nine different levels of degraded genomic DNA ranging from a DI 0.89 to 241.69 were amplified in triplicate at 200 pg template input DNA (based on the "short" quantitation value from InnoQuant®), and the profile recoveries and peak heights were assessed (Table 4). InnoTyper 21 was able to recover over 95% of the profile up to a DI of 75.16, which was ~ 1.4 and ~ 6.6 times higher than what could be achieved with MiniFiler and Identifiler Plus, respectively (Tables 4–7).

Studies were performed on the highest DI sample (241.69) to determine the effect of low concentrations on a highly degraded sample. The DI 241.69 sample was serially diluted and amplified with InnoTyper 21 and MiniFiler at 400 pg to 25 pg of total input DNA. Fig. 6 and Supplemental Table 3 show the mean profile recovery and mean peak heights obtained from typing the serially diluted highly degraded sample. Supplemental Tables 4 and 5 show drop-out heat maps for the replicate amplifications with InnoTyper 21 and MiniFiler, respectively. These results clearly indicate that although allele dropout is more extensive with both low quantity and quality DNA, the InnoTyper 21 kit recovers a higher percentage of alleles than MiniFiler with these highly compromised samples.

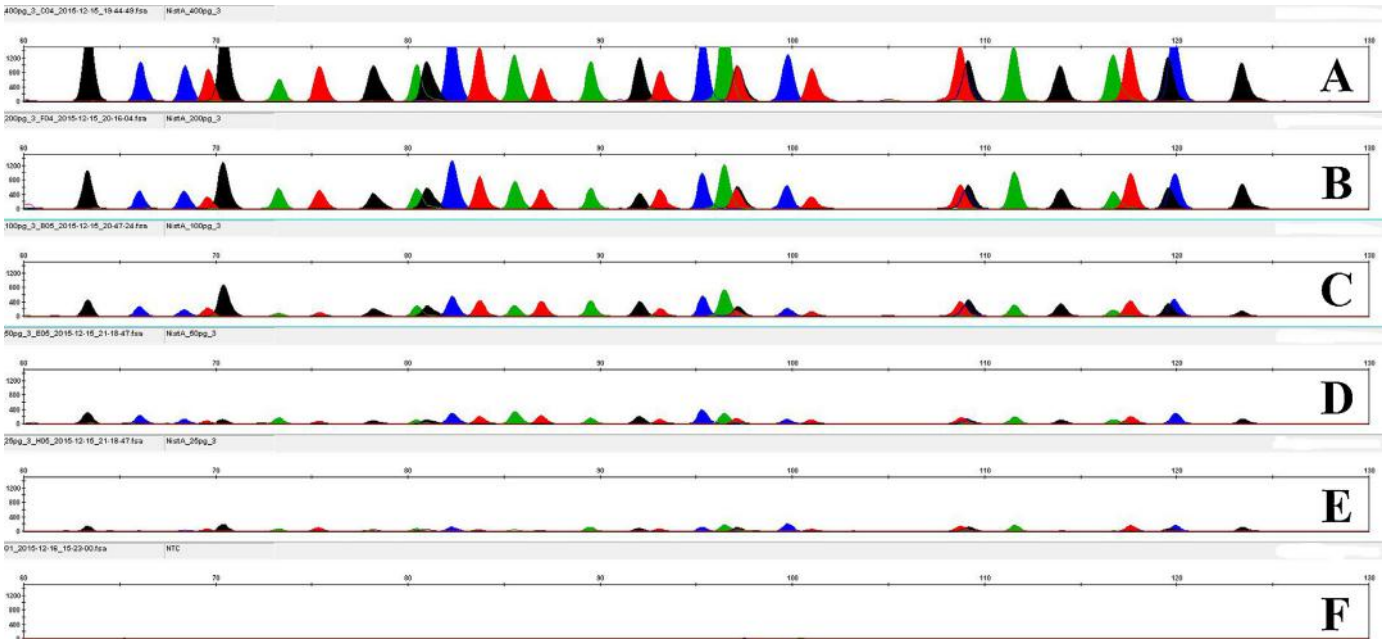

Fig. 5. Representative electropherograms from the sensitivity study using NIST SRM 2372 Component A. Panel A, B, C, D, and E show the peak heights of 400 pg, 200 pg, 100 pg, 50 pg, and 25 pg input DNA, respectively. Panel F shows the electropherogram of the non-template control. The Y-axis scale is set to 1500 RFU.

Table 4
Effects of degradation on profile recoveries and peak heights of InnoTyper 21, MiniFiler, and Identifiler Plus.

	Mean profile recovery (%)				Mean peak height (RFU)		
DI	InnoTyper 21	MiniFiler	Identifiler Plus	DI	InnoTyper 21	MiniFiler	Identifiler Plus
0.89	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	0.89	596 ± 20	963 ± 91	530 ± 17
2.16	100.0 ± 0.0	100.0 ± 0.0	64.6 ± 4.8	2.16	346 ± 78	397 ± 58	185 ± 6
2.48	100.0 ± 0.0	100.0 ± 0.0	62.5 ± 0.0	2.48	360 ± 46	336 ± 62	181 ± 19
13.14	99.2 ± 1.4	83.1 ± 5.9	39.6 ± 1.8	13.14	312 ± 34	236 ± 18	137 ± 48
15.16	99.2 ± 1.4	87.0 ± 6.4	33.3 ± 3.6	15.16	335 ± 53	249 ± 18	122 ± 21
62.42	96.8 ± 1.4	72.2 ± 0.0	13.5 ± 1.8	62.42	298 ± 16	218 ± 16	111 ± 14
66.16	98.4 ± 2.7	75.9 ± 3.2	16.7 ± 3.6	66.16	269 ± 33	234 ± 34	102 ± 15
75.16	96.0 ± 3.6	70.4 ± 3.2	14.6 ± 4.8	75.16	317 ± 27	222 ± 13	120 ± 24
241.69	81 ± 7.2	63.0 ± 3.2	4.2 ± 3.6	241.69	249 ± 5	148 ± 11	80 ± 75

3.5. Inhibition study

Inhibition studies were performed with hematin, humic acid, and melanin to evaluate performance of the InnoTyper 21 kit in the presence of PCR inhibitors. DNA samples from crime scenes may contain inhibitors that can affect amplification of DNA samples. 400 pg of the kit control DNA was amplified in triplicate in the presence of various concentrations of the inhibitors (Table 8). Complete inhibition was observed as the concentration of hematin reached 75 μM. With humic acid, a 50% peak height reduction was observed at a concentration of 30 ng/μL of humic acid. Melanin up to 10 ng/μL concentration did not produce allele drop-out, but there was slight reduction in peak heights observed with higher melanin concentrations.

3.6. Mixture study

Two pristine female and male genomic DNA samples were mixed at various ratios and analyzed for peak height ratio (PHR). The genotypes of the major and minor contributor DNA, the observed PHRs, and theoretical PHRs are summarized in Supplemental Table 6. The mixtures of a homozygous allele of the major component and heterozygous alleles of the minor component seen in TARBP and AMEL showed a peak height imbalance at all the tested ratios, especially with the low amount of the minor component. This mixing pattern results in a sister allele originating from the heterozygous genotype of the minor DNA. The heterozygous alleles of the minor DNA keeps the PHR less than 0.33 (theoretical PHR) all the way up to the 1:1 mixing ratio. The observed PHRs ranged from 0.04 to 0.44, and the theoretical PHRs range from 0.03 to 0.33 at the tested ratios. Another pattern that produces heterozygous alleles is a mixture of an opposite homozygous allele. The PHR less than 0.33 (theoretical) can be obtained at the ratios, 15:1, 7:1, and 3:1. The 1:1 ratio gives a PHR equal to 1.0. The mixtures of heterozygous alleles of the major donor with a homozygous allele of the minor donor as seen in the markers AC4027, MLS26, NBC106, RG148, NBC13, AC2265, and HS4.69 showed the decrease in PHR as the contribution of the minor donor increases. At the mixture ratio 1:1, the observed PHRs ranged between 0.25 and 0.33. The ratios other than 1:1 have relatively high PHR in both observed and theoretical. PHRs of twenty-five single source samples amplified at 500 pg in triplicate were summarized in Supplemental Table 7. The PHR of each marker is relatively high (mean ≥ 0.78, median ≥ 0.80). The lowest PHR observed was 0.51 in NBC13 and AC1141.

Detection of mixed samples in a bi-allelic system depends solely upon notable differences in peak height, such as would be seen with low PHRs. If both major and minor components exhibit

Table 5
Heat map of InnoTyper 21 degradation study results. Alleles are listed by size from the smallest to the largest base pair size. The table indicates the number of replicates (out of three replicate amplifications of each sample) exhibiting allele dropout for a particular allele. Green (or darkest shaded) cells indicate no dropout was observed; red (or lighter shaded) cells indicate the number of replicates where dropout was observed.

Marker	Allele	Allele Size (bp)	Degradation Index 0.89	2.16	2.48	13.14	15.16	62.42	66.16	75.16	241.69
AC1141	I	63.25									
AC1141	N	66.11									
AC4027	I	66.18									
AC4027	N	68.57									
ACA1766	I	69.32									
TARBP	I	70.24									
RG148	I	73.38									
ACA1766	N	75.11									
AMEL	X	77.95									
NBC120	I	78.98									
MLS26	I	79.92									
RG148	N	80.64									
MLS26	N	82.38									
NBC13	I	85.45									
NBC13	N	89.48									
NBC10	N	92.87									
ALU79712	N	95.48									
AC2265	I	96.46								1	
NBC102	N	96.86									
AC2305	N	97.03									
NBC216	I	99.81									
AC2265	N	100.56									1
SB19.12	I	108.33									2
HS4.69	I	108.83									1
SB19.12	N	113.25									2
HS4.69	N	113.67									1
NBC106	I	115.91							1		2
MLS09	N	116.73									1
NBC148	I	117.23								1	3
NBC51	N	119.46									2
NBC106	N	119.95						3	1	1	3
NBC148	N	119.95				1	1	1		2	3

Table 6
Heat map of MiniFiler degradation study results. Alleles are listed by size from the smallest to the largest base pair size. The table indicates the number of replicates (out of three replicate amplifications of each sample) exhibiting allele dropout for a particular allele. Green (or darkest shaded) cells indicate no dropout was observed; red (or lighter shaded) cells indicate the number of replicates where dropout was observed.

Marker	Allele	Allele Size (bp)	Degradation Index 0.89	2.16	2.48	13.14	15.16	62.42	66.16	75.16	241.69
D16S539	11	98.66									
AMEL	X	100.46									
CSF1PO	11	105.59									
D16S539	13	106.6									
CSF1PO	12	109.5									
D13S317	11	113.97									
D2S1338	17	126.32									
D2S1338	19	134.41									
D18S51	12	143.06									
D7S820	9	161.27						1	1		2
D7S820	10	165.31						1	1	3	3
D18S51	20	175.42				1		3	2	2	3
FGA	21	165.73				1	1	2	2	3	3
FGA	24	177.02				2	2	3	3	3	3
D21S11	28	201.63				3	2	3	1	2	3
D21S11	32.2	219.13				2	2	2	3	3	3

the same homozygous allele or heterozygous alleles, there will be no indicators of the proportions of the contributors. However, mixing of the opposite homozygous alleles or a homozygous allele with heterozygous alleles can result in notable peak imbalance with certain mixture ratios. Based upon the theoretical PHR calculation of the two-source mixture and comparison of PHR of single source DNA with that of the mixture, a PHR $\leq$ 0.33 appears to be an appropriate cutoff for interpretation of two source samples.

Table 7
Heat map of Identifiler Plus degradation study results. Alleles are listed by size from the smallest to the largest base pair size. The table indicates the number of replicates (out of three replicate amplifications of each sample) exhibiting allele dropout for a particular allele. Green(or darkest shaded) cells indicate no dropout was observed; red (or lighter shaded) cells indicate the number of replicates where dropout was observed.

Marker	Allele	Allele Size (bp)	Degradation Index 0.89	2.16	2.48	13.14	15.16	62.42	66.16	75.16	241.69
AMEL	X	105.67									1
D19S433	13	116.79						1	3	3	3
D3S1358	14	118.87						1			3
D3S1358	15	122.84						1	2	1	3
D19S433	15	124.89					1	3	2	3	3
D8S1179	10	130.22						3	1	1	3
D8S1179	14	147.45				2	1	2	2	2	3
D5S818	11	150.68						3	2	3	3
vWA	16	173.99			1	1	3	3	3	3	3
vWA	18	182.07			2	3	3	3	3	3	3
TH01	9.3	185.5					1	3	3	3	3
D21S11	28	199.76			1	3	3	3	3	3	3
D21S11	32.2	217.71		2	2	3	3	3	3	3	3
D13S317	11	227.94				3	3	3	3	3	3
FGA	21	229.72		3	2	3	3	3	3	3	3
TPOX	11	241.51				2	3	3	3	3	3
FGA	24	241.85		3	2	3	3	3	3	3	3
D7S820	9	266.64		3	3	3	3	3	3	3	3
D7S820	10	270.67		3	3	3	3	3	3	3	3
D16S539	11	275.78		1	2	3	3	3	3	3	3
D18S51	12	281.7		3	3	3	3	3	3	3	3
D16S539	13	283.83		1	1	3	3	3	3	3	3
D2S1338	17	314.23		3	3	3	3	3	3	3	3
D18S51	20	314.59		3	3	3	3	3	3	3	3
D2S1338	19	322.37		3	2	3	3	3	3	3	3
CSF1PO	11	324.11		3	3	3	3	3	3	3	3
CSF1PO	12	328.15		3	3	3	3	3	3	3	3

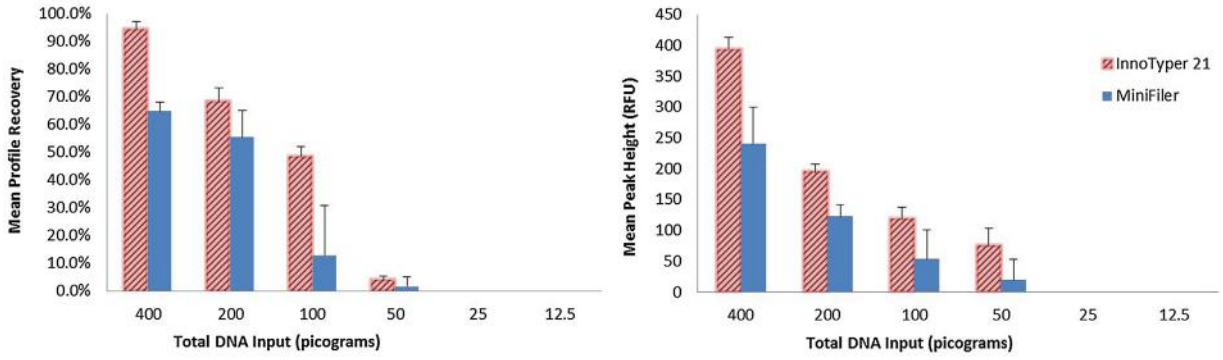

Fig. 6. Mean profile recoveries and peak heights (RFU) of InnoTyper 21 and MiniFiler with various quantities of a sample with severely degraded template DNA (Degradation Index of 241.69).

Table 8
Amplification efficiency of control DNA 1212 in the presence of increasing amounts of hematin, melanin, and humic acid.

Hematin (μM)	Mean profile recovery (%)	Mean peak height (RFU)	Melanin (ng/μL)	Mean profile recovery (%)	Mean peak height (RFU)	Humic acid (ng/μL)	Mean profile recovery (%)	Mean peak height (RFU)
0	100.0	1156 ± 112	0	100.0	1222 ± 219	0	100.0	1410 ± 133
15	100.0	1181 ± 118	1.25	100.0	1343 ± 129	5	100.0	1053 ± 198
30	100.0	1079 ± 160	2.5	100.0	1554 ± 236	10	100.0	1066 ± 147
45	100.0	621 ± 51	3.75	100.0	1448 ± 78	15	100.0	1033 ± 167
60	25.4 ± 14.6	119 ± 38	5	100.0	1382 ± 232	20	100.0	599 ± 70
75	0.0	0	6.25	100.0	1173 ± 26	25	94.5 ± 1.4	398 ± 49
90	0.0	0	7.5	100.0	1004 ± 68	30	52.4 ± 14.9	201 ± 26
105	0.0	0	10	100.0	966 ± 47			

However, detection of mixtures of more than two will be more complicated and is challenging with a bi-allelic system with only 21 markers. In addition, stochastic effects can impact the PHR with low level samples and caution should be exercised when attempting to deconvolve mixtures based on PHR when the quantity of template DNA (total or of a contributor) is low. Overall, the performance of InnoTyper 21 on mixed samples behaved as expected.

3.7. Case-type samples study

The results obtained using InnoTyper 21 were generally consistent with those obtained from other typing systems. The significant exception was for the highly degraded samples, with which InnoTyper 21 had a higher portion of profile recovery. An example of this is non-probative sample NO1-010, a degraded semen sample with a DI value of 7.41. Identifiler Plus produced a partial profile with allelic dropout observed at six STR loci, and InnoTyper 21 produced a full profile (Fig. 7). This was the general trend observed across the degraded samples tested as part of the non-probative study. All reagent blanks produced no results.

A summary of the non-probative sample results can be found in Supplemental Table 8, which shows the sample description, DI values, total input DNA used for InnoTyper 21 typing, the InnoTyper 21 result, and average peak heights for all the non-probative samples tested. The overall average PHR for the single source case-type samples was 0.92 with a standard deviation of 0.03. Peak heights for the non-probative samples were well-balanced and correlated with the input DNA in the sample. Overall, peak heights were within acceptable ranges. Also, the DI values in conjunction with the total input DNA correlated well with the observed peak heights of the non-probative samples. The non-probative sample study supports the effectiveness of using InnoTyper 21 for typing highly degraded samples.

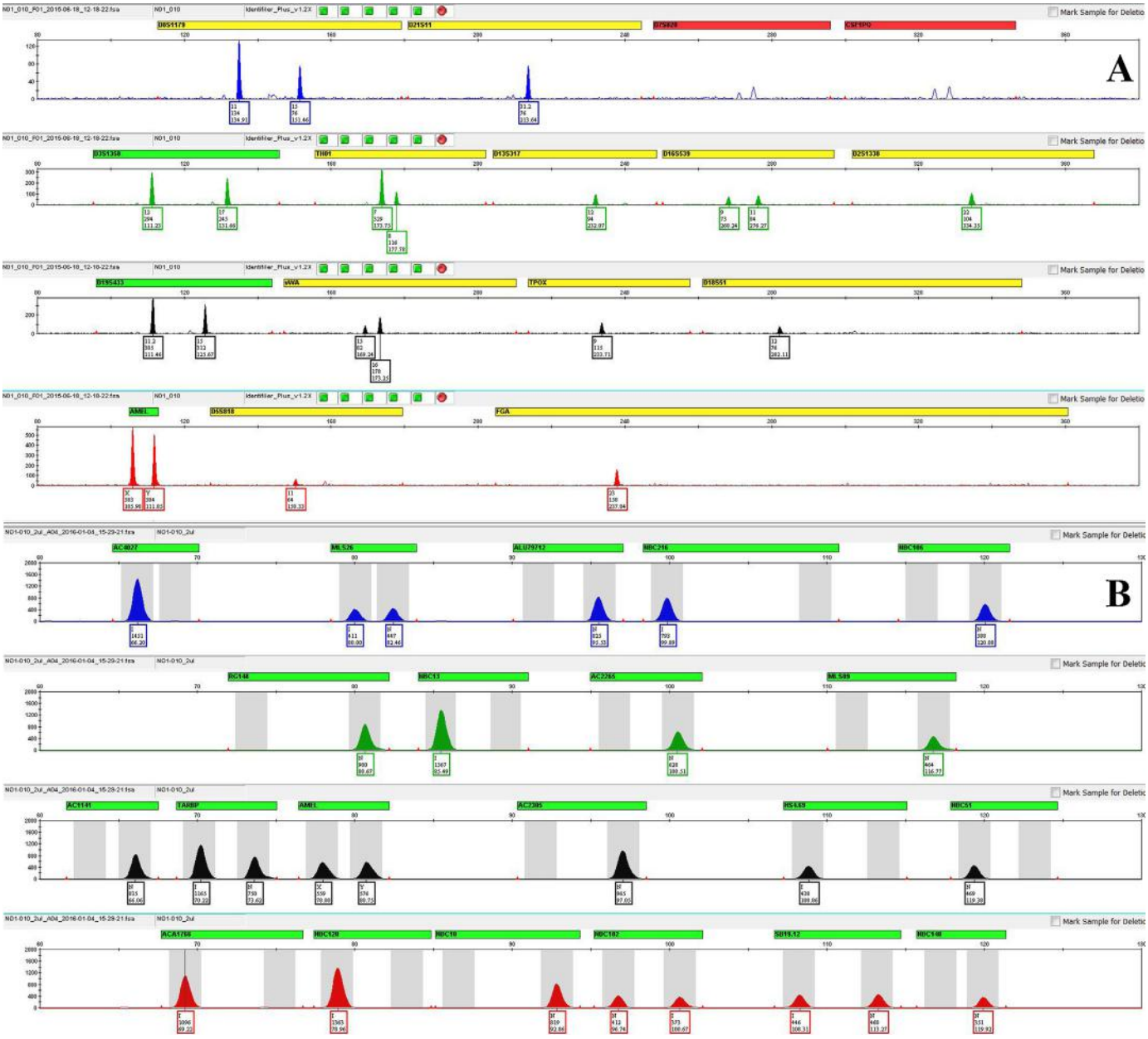

Fig. 7. Non-probative degraded semen sample tested with Identifiler Plus (A) and InnoTyper 21 (B). The Y axis RFU scale is 600 or less for Identifiler Plus in Panel A and 2000 for InnoTyper 21 in Panel B.

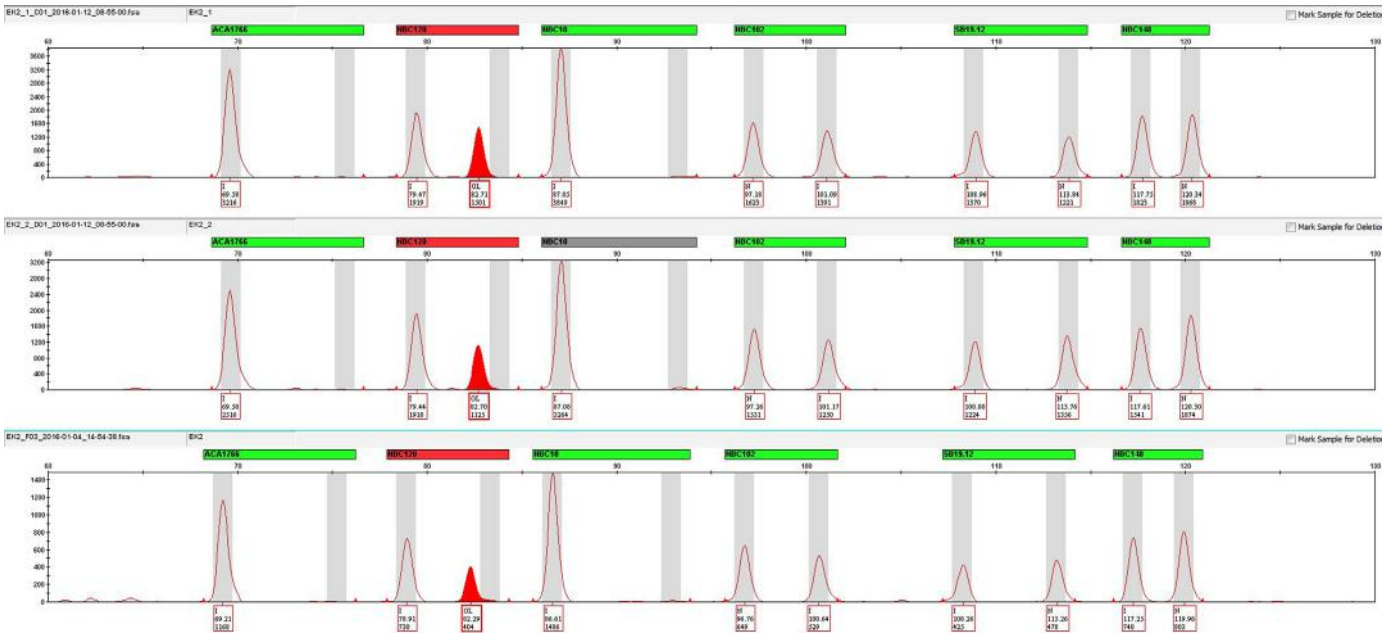

Fig. 8. Microvariant allele observed at locus NBC120 in non-probative blood sample amplified three times.

Of interest, a microvariant allele in marker NBC120 was observed in a blood sample in the non-probative study (Fig. 8). This "off-ladder" allele is approximately one base pair smaller in size than the corresponding no insertion ladder allele and was confirmed by multiple amplifications as well as sequencing. The sequencing results verify a single base pair deletion downstream from the forward primer. This type of rare occurrence has been observed in the past with other typing systems and is likely due to variants in or around the primer-binding site [27]. Refer to the microvariant discussion in the Population Database Study for further details.

3.8. Analysis of highly degraded non-probative human remains

The results of the sensitivity study performed with human remains shows full, concordant profiles were obtained with as low as 31.25 pg of total input DNA, according to the quantitation values from Quantifiler Duo (Table 9). Below 31.25 pg of total DNA input,

Table 9
Results of the sensitivity study performed with three human remains. Darkened cells indicate complete locus dropout; shaded cells indicate allele dropout with the surviving sister allele RFU indicated.

Sample	Total Input (pg)	AC 4027	MLS26	ALU 79712	NBC216	NBC106	RG148	NBC13	AC 2265	MLS09	AC 1141	TARBP	AMEL	AC 2305	HS4.69	NBC51	ACA 1766	NBC120	NBC10	NBC102	SB19.12	NBC148
UNT1	3.9		N	300	I		I,N	N	I,N	N	N	I	66		I	N						
	7.81	I,N	N	I,N	I			N	I,N	N	I	I	112		I	N		N			84	
	15.63	I,N	N	I,N	I	I,N	I,N	N	184	N	I	I	X,Y	I	I	N	I	N	N	N,I	69	I
	31.25	I,N	N	I,N	I	I,N	I,N	N	I,N	N	I	I	X,Y	I	I	N	I	N	N	N,I	I,N	I
	62.5	I,N	N	I,N	I	I,N	I,N	N	I,N	N	I	I	X,Y	I	I	N	I	N	N	N,I	I,N	I
	125	I,N	N	I,N	I	I,N	I,N	N	I,N	N	I	I	X,Y	I	I	N	I	N	N	N,I	I,N	I
	250	I,N	N	I,N	I	I,N	I,N	N	I,N	N	I	I	X,Y	I	I	N	I	N	N	N,I	I,N	I
	500	I,N	N	I,N	I	I,N	I,N	N	I,N	N	I	I	X,Y	I	I	N	I	N	N	N,I	I,N	I
UNT6	3.9	N	N		325	I,N	N	N	I	I,N	I	I	X	88	N	I	67	N	N			I
	7.81	N	N	148	I,N	I,N	N	N	I	I,N	I		X	I,N	N	I		N		I		
	15.63	N	N	I,N	I,N	I,N	N	N	I	I,N	I	I	X	I,N	N	I	96	N	N	I	124	I
	31.25	N	N	I,N	I,N	I,N	N	N	I	I,N	I	I	X	I,N	N	I	I,N	N	N	I	I,N	I
	62.5	N	N	I,N	I,N	I,N	N	N	I	I,N	I	I	X	I,N	N	I	I,N	N	N	I	I,N	I
	125	N	N	I,N	I,N	I,N	N	N	I	I,N	I	I	X	I,N	N	I	I,N	N	N	I	I,N	I
	250	N	N	I,N	I,N	I,N	N	N	I	I,N	I	I	X	I,N	N	I	I,N	N	N	I	I,N	I
	500	N	N	I,N	I,N	I,N	N	N	I	I,N	I	I	X	I,N	N	I	I,N	N	N	I	I,N	I
UNT7	3.9	N	I	187		N	N	153	I,N	N		I	X,Y		112	96	70	75				I
	7.81	N	I	I,N	I,N	N	N	252	352	N	I	I	237	I,N	154	N,I	73	52		I		I
	15.63	N	I	I,N	664	N	N	I,N	I,N	N	I	I	X,Y	I,N	I,N	N,I	193	I,N	I,N	I		I
	31.25	N	I	I,N	I,N	N	N	I,N	I,N	N	I	I	X,Y	I,N	I,N	N,I	I,N	I,N	I,N	I	I	I
	62.5	N	I	I,N	I,N	N	N	I,N	I,N	N	I	I	X,Y	I,N	I,N	N,I	I,N	I,N	I,N	I	I	I
	125	N	I	I,N	I,N	N	N	I,N	I,N	N	I	I	X,Y	I,N	I,N	N,I	I,N	I,N	I,N	I	I	I
	250	N	I	I,N	I,N	N	N	I,N	I,N	N	I	I	X,Y	I,N	I,N	N,I	I,N	I,N	I,N	I	I	I
	500	N	I	I,N	I,N	N	N	I,N	I,N	N	I	I	X,Y	I,N	I,N	N,I	I,N	I,N	I,N	I	I	I

Table 10
Results of the analysis of highly degraded human remains previously tested with other typing systems. P/C IC indicates samples were previously tested with Profiler/Cofiler using increased cycle number, MF indicates samples were previously tested with MiniFiler.

Sample name	Sample type	Estimated age	Quantity Amplified based on Quantifiler Duo (pg)	Previous results		InnoTyper 21 Loci		
				MtDNA	Number of STR Loci	No. of loci above 100 RFU	No. of loci exhibiting allele dropout	Average RFU
UNT 11	Tooth	50–80 years	Undet.	NR	PC/IC = 0	5	16	756
UNT 12	Tooth	50–80 years	Undet.	NR	PC/IC = 0	1	20	517
UNT 13	Tooth	50–80 years	Undet.	Inconclusive	PC/IC = 0	18	8	285
UNT 14	Tooth	50–80 years	Undet.	NR	PC/IC = 0	0	21	n/a
UNT 15	Tooth	50–80 years	Undet.	NR	PC/IC = 0	6	15	265
UNT 16	Tooth	50–80 years	Undet.	Partial	PC/IC = 0	2	19	181
UNT 17	Tooth	50–80 years	Undet.	Partial	PC/IC = 0	2	20	361
UNT 18	Femur	23 years	13.9	HV1 & HV2	PC/IC = 0	19	3	367
UNT 19	Metacarpal	42 years	5.0	HV1 & HV2	PC/IC = 0	18	3	576
UNT 20	Femur	Unknown	Undet.	HV1 & HV2	PC/IC = 0	16	3	267
UNT 21	Femur	Unknown	Undet.	HV1 & HV2	PC/IC = 0	5	2	134
UNT 22	Humerus	Unknown	Undet.	HV1 & HV2	PC/IC = 5, MF = 0, Total = 5	17	4	366
UNT 23	Femur	Unknown	16.2	HV1 & HV2	PC/IC = 6, MF = 7, Total = 11	20	1	721
UNT 24	Tibia	41 years	Undet.	HV1 & HV2	PC/IC = 2, MF = 5, Total = 7	20	1	385
UNT 25	Rib	5 years	Undet.	HV1 & HV2	PC/IC = 5, MF = 2, Total = 6	17	4	330
UNT 26	Tibia	23 years	39.8	HV1 & HV2	PC/IC = 5, MF = 5, Total = 8	21	0	487

stochastic effects such as drop out and artifacts were observed. For example, there was one allele drop-in detected in one marker of one sample at 3.9 pg of total input DNA. Results between 250 pg and 62.5 pg exhibited well balanced profiles with minimal background noise. 500 pg of input DNA resulted in artifacts of excessive template DNA. Results of this study show that sensitivity levels of the InnoTyper 21 kit remain high even when used with highly degraded human remains.

Samples previously tested with other typing systems yielded promising results with InnoTyper 21. Of 16 samples tested, 12 samples produced InnoTyper 21 results of at least 100 RFU at 5 or more loci (Table 10). The results indicate that InnoTyper 21 can be utilized as a complementary system to STRs to increase the statistical significance of a match. For example, sample UNT22 previously produced a 5-locus STR profile with a genotype frequency of approximately 1 in 3.8 million individuals of the African American population and approximately 1 in 42.9 million

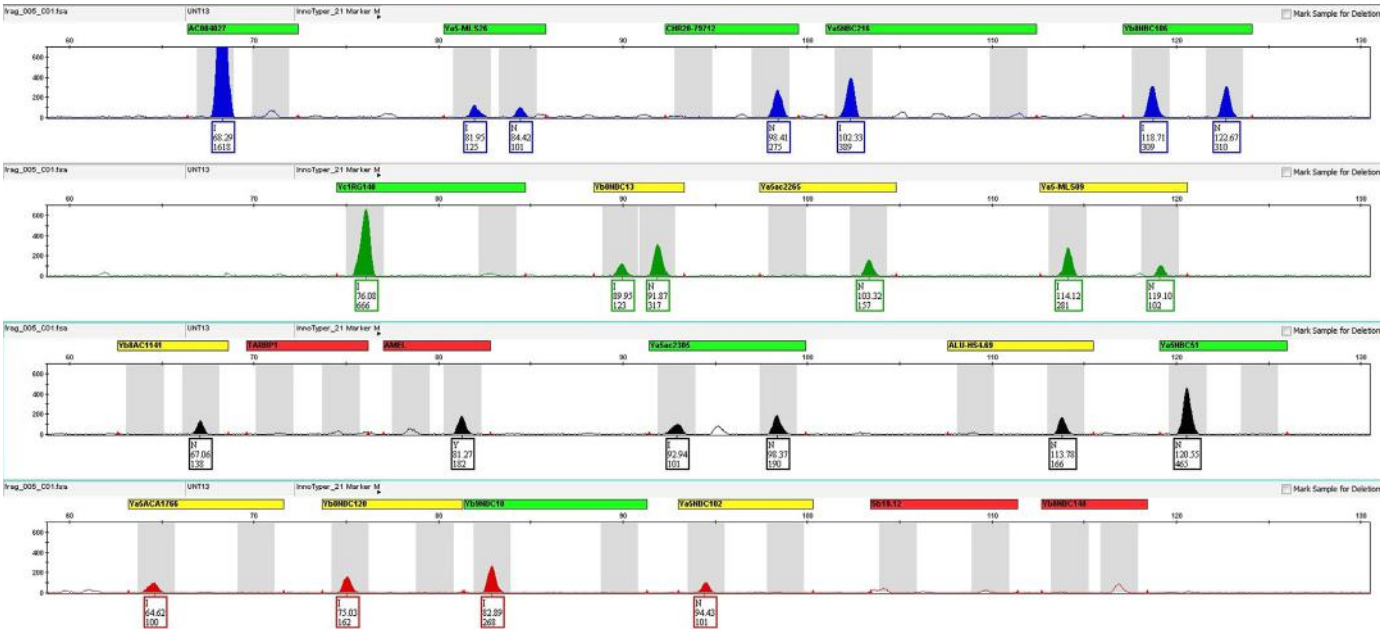

Fig. 9. InnoTyper 21 result of 50–80 year old human remains sample UNT13 analyzed at 100 RFU analytical threshold.

individuals of the Caucasian population, while the 17-locus InnoTyper 21 profile produced by this sample has a genotype frequency of approximately 1 in 31.4 million individuals of the African American population and approximately 1 in 169.8 million individuals of the Caucasian population (using 2pq and p^2). Fig. 9 shows an InnoTyper 21 electropherogram of sample UNT13, a bone sample that previously produced no result with STR testing, and inconclusive result with mtDNA testing. Approximately 10 μL of sample UNT13 was amplified with InnoTyper 21 (it should be noted that the final version of the kit allows for the addition of up to 16 μL of total input DNA), and results show a 17-locus InnoTyper 21 profile with an average RFU of 285, and a statistical profile frequency of 1 in 14 billion Caucasians and 1 in 254 million African Americans (using 2pq and p^2). Results for all remains tested are shown in Table 10.

These results indicate that InnoTyper 21 can yield useful nuclear DNA results from challenging samples up to 80 years old that previously produced partial or no results with other typing systems.

3.9. Population database study

Five sample populations (African-American, $n = 207$; Southwest Hispanic $n = 40$; Caucasian, $n = 301$ (i.e., US, n= 205; UK, $n = 96$); and East Asian, $n = 44$) were typed for the InnoTyper 21 loci. The allele frequencies and observed and expected heterozygosity are listed in Table 11. All loci were polymorphic in all five sample populations. Heterozygosity (observed) across the markers and populations ranged from a low of 0.073 for the ALU79712 locus in Asians to a high of 0.65 for the NBC216 locus in Hispanics. The average heterozygosity was similar for all five sample populations. Population parameters for the five groups are provided in Table 12. The power of discrimination (PD) across the populations ranged from a low of 0.136 at the ALU79712 locus in Asians to a high of

Table 11
Population specific InnoTyper 21 allele frequencies and heterozygosity for five major population groups.

	African American ($N = 207$)				Southwest Hispanic ($N = 40$)				UK Caucasian ($N = 96$)	
	Insertion Frequency	Null Frequency	He	Ho	Insertion Frequency	Null Frequency	He	Ho	Insertion Frequency	Null Frequency
AC4027	0.53860	0.4614	0.49702	0.53600	0.65000	0.3500	0.45500	0.35	0.40620	0.5938
MLS26	0.14980	0.8502	0.25472	0.23200	0.51250	0.4875	0.49969	0.525	0.36460	0.6354
ALU79712	0.30920	0.6908	0.42719	0.43500	0.50000	0.5000	0.50000	0.45	0.45830	0.5417
NBC216	0.59660	0.4034	0.48134	0.50700	0.53750	0.4625	0.49719	0.425	0.73960	0.2604
NBC106	0.57490	0.4251	0.48878	0.43500	0.37500	0.6250	0.46875	0.65	0.42710	0.5729
RG148	0.53620	0.4638	0.49738	0.50200	0.36250	0.6375	0.46219	0.475	0.33330	0.6667
NBC13	0.21980	0.7802	0.34298	0.33300	0.36250	0.6375	0.46219	0.475	0.33850	0.6615
AC2265	0.39130	0.6087	0.47637	0.46400	0.76250	0.2375	0.36219	0.275	0.72400	0.2760
MLS09	0.23670	0.7633	0.36135	0.36700	0.38750	0.6125	0.47469	0.475	0.37500	0.6250
AC1141	0.22950	0.7705	0.35366	0.34300	0.71250	0.2875	0.40969	0.375	0.59370	0.4063
TARBP	0.28500	0.7150	0.40755	0.37700	0.36250	0.6375	0.46219	0.425	0.55730	0.4427
AC2305	0.30680	0.6932	0.42535	0.43000	0.66250	0.3375	0.44719	0.375	0.61460	0.3854
HS4.69	0.31880	0.6812	0.43433	0.41500	0.20000	0.8000	0.32000	0.3	0.39060	0.6094
NBC51	0.59420	0.4058	0.48225	0.45400	0.53750	0.4625	0.49719	0.575	0.55210	0.4479
ACA1766	0.72220	0.2778	0.40125	0.39100	0.80000	0.2000	0.32000	0.35	0.60940	0.3906
NBC120	0.59660	0.4034	0.48134	0.46900	0.53750	0.4625	0.49719	0.375	0.41150	0.5885
NBC10	0.65940	0.3406	0.44918	0.44900	0.48750	0.5125	0.49969	0.525	0.40620	0.5938
NBC102	0.39610	0.6039	0.47841	0.45400	0.58750	0.4125	0.48469	0.425	0.44270	0.5573
SB19.12	0.39610	0.6039	0.47841	0.48300	0.17500	0.8250	0.28875	0.3	0.29170	0.7083
NBC148	0.54350	0.4565	0.49622	0.51700	0.91250	0.0875	0.15969	0.125	0.90100	0.0990
Mean ± 1 SD	0.43 ± 0.1693	0.57 ± 0.1693	0.436 ± 0.0655	0.43 ± 0.0732	0.521 ± 0.1929	0.479 ± 0.1929	0.428 ± 0.092	0.413 ± 0.1171	0.497 ± 0.1601	0.503 ± 0.1601

	[0,10-13]UK Caucasian ($N = 96$)		US Caucasian ($N = 205$)				East Asian (US) ($N = 44$)			
	He	Ho	Insertion Frequency	Null Frequency	He	Ho	Insertion Frequency	Null Frequency	He	Ho
AC4027	0.48240	0.479	0.43900	0.5610	0.49256	0.507	0.47727	0.52273	0.49897	0.50000
MLS26	0.46333	0.479	0.33900	0.6610	0.44816	0.493	0.40698	0.59302	0.48269	0.58140
ALU79712	0.49652	0.438	0.49020	0.5098	0.49981	0.502	0.03660	0.96340	0.07052	0.07317
NBC216	0.38518	0.292	0.72680	0.2732	0.39712	0.38	0.27027	0.72973	0.39445	0.43243
NBC106	0.48937	0.542	0.44630	0.5537	0.49423	0.512	0.51136	0.48864	0.49974	0.56818
RG148	0.44442	0.417	0.28780	0.7122	0.40994	0.449	0.70455	0.29545	0.41632	0.31818
NBC13	0.44784	0.448	0.36590	0.6341	0.46403	0.488	0.14290	0.85710	0.24496	0.28571
AC2265	0.39965	0.365	0.74390	0.2561	0.38103	0.356	0.83720	0.16280	0.27259	0.32558
MLS09	0.46875	0.479	0.41950	0.5805	0.48704	0.468	0.82550	0.17450	0.28810	0.25581
AC1141	0.48244	0.625	0.61460	0.3854	0.47373	0.478	0.60465	0.39535	0.47810	0.46512
TARBP	0.49343	0.51	0.58290	0.4171	0.48626	0.502	0.35227	0.64773	0.45635	0.47727
AC2305	0.47373	0.438	0.56100	0.4390	0.49256	0.488	0.85710	0.14290	0.24496	0.28571
HS4.69	0.47606	0.385	0.38540	0.6146	0.47373	0.459	0.68605	0.31395	0.43077	0.48837
NBC51	0.49457	0.417	0.51460	0.4854	0.49957	0.522	0.63953	0.36047	0.46106	0.48837
ACA1766	0.47606	0.469	0.63660	0.3634	0.46268	0.444	0.78210	0.21790	0.34084	0.33333
NBC120	0.48434	0.406	0.40980	0.5902	0.48373	0.459	0.39286	0.60714	0.47704	0.45238
NBC10	0.48240	0.458	0.44390	0.5561	0.49371	0.537	0.25581	0.74419	0.38075	0.37209
NBC102	0.49343	0.594	0.39020	0.6098	0.47589	0.459	0.20450	0.79550	0.32536	0.31818
SB19.12	0.41322	0.354	0.30980	0.6902	0.42765	0.424	0.41463	0.58537	0.48543	0.43902
NBC148	0.17840	0.177	0.86340	0.1366	0.23588	0.234	0.22090	0.77910	0.34421	0.34884
Mean ± 1 SD	0.451 ± 0.0719	0.439 ± 0.0996	0.499 ± 0.1556	0.501 ± 0.1556	0.454 ± 0.0622	0.458 ± 0.0693	0.481 ± 0.2509	0.519 ± 0.2509	0.38 ± 0.1133	0.39 ± 0.1216

Ho = Observed heterozygosity; He = Expected heterozygosity.

Table 12
Population specific InnoTyper 21 population parameters across five populations.

Locus	African American (*N* = 207)				Southwest Hispanic (*N* = 40)				UK Caucasian (*N* = 96)				US Caucasian (*N* = 205)				East Asian (US) (*N* = 44)				
	PD	HWE (*p*-value)[a,b]	RMP	PE	PD	HWE (*p*-value)[a,b]	RMP	PE	PD	HWE (*p*-value)[a,b]	RMP	PE	PD	HWE (*p*-value)[a,b]	RMP	PE	PD	HWE (*p*-value)[a,b]	RMP	PE	F_{ST}[c]
AC4027	0.62350	0.26906	0.37650	0.18675	0.59950	0.16031	0.40050	0.17574	0.61560	1.00000	0.38440	0.18305	0.62120	0.77230	0.37880	0.18563	0.62397	1.00000	0.37603	0.18724	0.01439
MLS26	0.41210	0.86844	0.58790	0.11114	0.62480	0.53656	0.37520	0.18742	0.54780	0.82480	0.45220	0.15550	0.55770	0.21070	0.44230	0.15913	0.55706	0.34150	0.44294	0.18310	0.11799
ALU79712	0.58060	0.47125	0.41940	0.16797	0.62500	0.36031	0.37500	0.18750	0.60470	0.30310	0.39530	0.17800	0.59500	1.00000	0.40500	0.17387	0.13563	1.00000	0.86437	0.03402	0.02807
NBC216	0.61510	0.17844	0.38490	0.18275	0.62360	1.00000	0.37640	0.18679	0.62320	0.02950	0.37680	0.18663	0.62490	0.58940	0.37510	0.18745	0.54638	0.68410	0.45362	0.15833	0.09887
NBC106	0.61920	0.11906	0.38080	0.18466	0.60790	0.02531	0.39210	0.17944	0.61950	0.39470	0.38050	0.18481	0.62210	0.67610	0.37790	0.18605	0.58368	0.54270	0.41632	0.18744	0.08016
RG148	0.62370	1.00000	0.37630	0.18684	0.60390	1.00000	0.39610	0.17769	0.59260	0.64420	0.40740	0.17283	0.56780	0.22560	0.43220	0.16296	0.58264	0.14520	0.41736	0.16483	0.03994
NBC13	0.50950	0.67656	0.49050	0.14208	0.60390	1.00000	0.39610	0.17769	0.59480	1.00000	0.40520	0.17378	0.60510	0.54490	0.39490	0.17819	0.40816	0.58280	0.59184	0.10748	-0.00198
AC2265	0.61230	0.77188	0.38770	0.18145	0.52760	0.17750	0.47240	0.14830	0.55970	0.44320	0.44030	0.15989	0.54430e	0.36060	0.45570	0.15422	0.43916	0.57530	0.56084	0.11772	0.11967
MLS09	0.52680	1.00000	0.47320	0.14803	0.61140	1.00000	0.38860	0.18101	0.60790	1.00000	0.39210	0.17944	0.61830	0.57220	0.38170	0.18422	0.44565	0.58310	0.55435	0.12330	0.03192
AC1141	0.51970	0.68969	0.48030	0.14556	0.56760	0.68813	0.43240	0.16288	0.61560	0.00650	0.38440	0.18305	0.61080	1.00000	0.38920	0.18076	0.61871	1.00000	0.38129	0.18190	0.16113
TARBP	0.56600	0.30781	0.43400	0.16225	0.60390	0.73250	0.39610	0.17769	0.62170	0.83800	0.37830	0.18585	0.61780	0.66570	0.38220	0.18402	0.59194	1.00000	0.40806	0.17611	0.01745
AC2305	0.57930	1.00000	0.42070	0.16744	0.59440	0.32063	0.40560	0.17360	0.61080	0.52090	0.38920	0.18076	0.62120	0.88580	0.37880	0.18563	0.40816	0.57250	0.59184	0.10748	0.09161
HS4.69	0.58570	0.52938	0.41430	0.17001	0.48640	0.64031	0.51360	0.13440	0.61220	0.08650	0.38780	0.18137	0.61080	0.66400	0.38920	0.18076	0.56138	0.49290	0.43862	0.16900	0.05282
NBC51	0.61570	0.38625	0.38430	0.18298	0.62360	0.52438	0.37640	0.18679	0.62220	0.14460	0.37780	0.18614	0.62480	0.58070	0.37520	0.18739	0.59167	1.00000	0.40833	0.17739	0.08278
ACA1766	0.56100	0.47844	0.43900	0.16038	0.48640	0.51344	0.51360	0.13440	0.62170	1.00000	0.37830	0.18585	0.61210	0.55500	0.38790	0.18133	0.50756	1.00000	0.49244	0.14138	0.03241
NBC120	0.61510	0.76969	0.38490	0.18275	0.62360	0.12281	0.37640	0.18679	0.61680	0.14070	0.38320	0.18352	0.61650	0.46970	0.38350	0.18337	0.62245	0.75490	0.37755	0.18163	0.00037
NBC10	0.59570	1.00000	0.40430	0.17415	0.62480	1.00000	0.37520	0.18742	0.61560	0.67190	0.38440	0.18305	0.62180	0.25110	0.37820	0.18592	0.54516	1.00000	0.45484	0.15413	0.03307
NBC102	0.61350	0.73250	0.38650	0.18199	0.61700	1.00000	0.38300	0.18361	0.61220	0.06070	0.38780	0.18137	0.60430	0.65610	0.39570	0.17782	0.49174	1.00000	0.50826	0.13622	0.01929
SB19.12	0.61350	1.00000	0.38650	0.18199	0.45240	1.00000	0.54760	0.12353	0.57030	0.21370	0.42970	0.16392	0.58100	0.87400	0.41900	0.16810	0.63534	0.53320	0.36466	0.18380	0.09632
NBC148	0.62310	0.57969	0.37690	0.18655	0.28110	0.25438	0.71890	0.07347	0.30910	1.00000	0.69090	0.08124	0.38830	1.00000	0.61170	0.10403	0.51055	1.00000	0.48945	0.14248	0.20125
Overall	**1.73E−05**		**2.47E−08**	**0.9757**	**9.59E−06**		**3.52E−08**	**0.9740**	**2.14E−05**		**1.49E−08**	**0.9780**	**2.65E−05**		**1.34E−08**	**0.9785**	**9.55E−07**		**2.64E−07**	**0.9626**	**0.066893**

PD = Power of Discrimination; HWE = Hardy Weinberg equilibrium; RMP = Random Match Probability; PE = Probability of Exclusion.
[a] α-level of 0.05 is adjusted from 0.05 to 0.0025 when corrected for multiple tests (Bonferroni's correction).
[b] Calculated using GDA software [44].
[c] Calculated according to Weir BS with GDA [45].

0.635 at the SB19.12 locus in Asians. The combined PD for all loci per population was >0.999. There were only two departures from HWE expectations detected (at the loci NBC216 (p = 0.030) and AC1141 (p = 0.007) in the Caucasian population). When the critical value was adjusted by the Bonferroni correction [46], these departures were no longer significant.

Departures from linkage equilibrium [i.e., linkage disequilibrium (LD) between pairs of loci] were tested for each of the five populations. Five pairs of loci in the African-American population, 14 pairs in the Hispanic population, 26 pairs in the UK Caucasians, four pairs in US Caucasians, and two pairs in the US East Asian population demonstrated significant LD (at $p < 0.05$) (Supplemental Table 9). The higher than expected number of apparent LDs in the UK Caucasians could be associated with the deviation from HWE at the two loci demonstrating departures from HWE expectations, as described by Falush et al. [47] and Chakraborty [48] or due to substructure. The former possibility is supported as 13 of the 26 pairs involved the two loci NBC216 or AC1141. Regardless, when the critical value was adjusted for multiple comparisons (via the Bonferroni correction, [49]), only one pair of loci (MLS09/TARBP, $p = 0.002$) displayed significant LD.

Wright's F_{ST} was estimated to assess the population substructure [45]. For the five populations, the F_{ST} values are provided for each locus as there are some loci in which the values are relatively high and others in which the values are relatively low (Table 12). For example, the markers AC1141 and NBC148 have F_{ST} values >0.16, while for marker NBC120 the F_{ST} value is 0.0004. These data suggest (consistent with the allele frequency differences in Table 11) that some markers have very different allele frequencies between populations, while others are more similar. Regardless, the overall data support that these population data can be used to assess the rarity of an InnoTyper 21 profile.

The allele frequencies of the African American and Caucasian data reported herein were similar to those obtained by NIST [50] for the same populations. The allele frequencies for the Hispanics generally were similar but a few loci were notably different, likely due to the different origins of the populations (e.g., U.S. Southwestern Hispanics vs. U.S. Southeastern Hispanics).

Regarding mutation rates, because there is no known mechanism of RE insertions to revert back to native state (i.e., no insertion) and vice versa [13,14], *Alu*s have no reported insertion polymorphism "mutation" [23]. However, a mutation in a primer binding site may cause allele imbalance or complete drop-out of an allele. For this reason, a single InnoTyper 21 discrepancy in a kinship case should not be used as the sole basis to draw a conclusion of exclusion. An in-house study of 94 parentage cases comprising 158 known meiosis revealed no such mutations (data not shown). However, for practical purposes, if an InnoTyper 21 mutation is encountered in a kinship case, it seems reasonable to use the mutation rates of other similar systems, such as SNPs, which are considerably lower than STR mutation rates [26].

In addition, the system may exhibit a variant causing the insertion or no insertion allele to size differently than the known sizes, as has been previously observed in InnoTyper 21 as well as other systems [27]. Variant alleles have been observed in the InnoTyper 21 system in markers NBC106, NBC120, and RG148 both in-house as well as by other users [50], non-probative section above, and personal communication]. Virtual bins for those variant alleles confirmed by sequencing have been added to the InnoTyper 21 GMID/GMID-X panel and bin files, described as the number of base pairs larger or smaller than the corresponding ladder alleles (i.e. I-1 or N + 1). When encountered in both questioned and known samples of a forensic case, these microvariant alleles will increase the power of discrimination.

4. Conclusions

The InnoTyper 21 PCR amplification kit simultaneously amplifies 20 INNUL markers and the sex determining marker Amelogenin, making it a highly discriminating nuclear DNA detection system for human identification. Because the primers for the InnoTyper 21 loci were designed using a strategy that allows small product fragments to be formed (between 63 and 123 bp), degraded samples may yield more typing results with the InnoTyper® 21 kit than previously possible with current STR kits. Additionally, the InnoTyper 21 kit is more discriminating than mtDNA sequencing, which until now was one of the few options for characterizing degraded single source samples. Thus, a forensic analyst has alternatives in deciding which typing system to use, especially when confronted with a compromised DNA sample that yields sufficient DNA only for a single analysis.

The validation of the InnoTyper 21 kit encompassed optimization of PCR conditions and reagent concentrations for the amplification of pristine as well as compromised DNA. The performance criteria included overall peak heights, peak height ratios (PHRs), intralocus peak balance, and lack of cross-reactivity in the presence of nonhuman DNA. The PCR conditions and thermal cycling parameters of the InnoTyper 21 kit have been optimized to enhance sensitivity for detection of small amounts of DNA and particularly degraded DNA. The sensitivity studies demonstrated that an input amount of 400 pg of DNA does not produce off-scale peaks and that sufficient signal for complete and almost complete profiles were obtained with 50 pg or less of template DNA.

In summary, the results from the degradation, inhibition and non-probative samples demonstrate that the InnoTyper 21 kit can be extremely useful for amplifying DNA under conditions where other commercial autosomal STR kits often yield partial or no profiles. As such, this kit is a useful and robust complement to conventional STR kits and will be especially applicable to challenging cases such as those involving the identification of human remains, hair shafts, paraffin embedded tissues, and other sample types [51,52]. *Alu* elements are stable polymorphisms with a known ancestral state that are not deleted after being inserted. *Alu* elements are neutral genetic loci that are poorly transcribed and are identical by descent only, not by state, with no known mechanism for parallel independent insertions to occur, unlike STRs and SNPs. As such, these *Alu* markers are ideally suited for kinship analysis of degraded human remains.

Finally, while the developmental validation studies have been described herein, it is recommended that each laboratory conduct its own internal validation studies according to FBI Quality Assurance Standards for Forensic DNA Testing Laboratories and/or SWGDAM guidelines [53] and/or applicable standards for each jurisdiction.

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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.2017.03.017.

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