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Internal Validation of the Non Codis Ministr NC01 and NC02 for Use in Forensic Casework

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Abstract

Miniplex has been designed to genotype degraded DNA samples when others commercial kits for human identification were unable to generate a complete genetic profile. Validation experiments following the Scientific Working Group on DNA Analysis Methods (SWGDAM) Guidelines were designed to evaluate the performance of Non-CODIS (NC) 01 and 02. This study describes an internal validation study of a set of six Miniplex loci D10S1248, D14S1434, D22S1045 (NC01) and D4S2364, D2S441, D1S1677 (NC02) to demonstrate their robustness in cases where current short tandem repeats genotyping usually fails. Allelic ladders were constructed with combined DNA templates from different human samples. Stutter averages, ranges, and standard deviations are shown. Peak height ratios in heterozygote loci were found to range from 0.57 to 1.00 when samples were amplified within the optimum DNA template. NC panels demonstrated the ability to produce full profiles from samples with lower DNA input (from 31.25pg to 1ng). No detectable DNA profiles to NC systems were observed in non-human mammalian or microorganism samples. Miniplexes NC01 and NC02 generated full profile in all degraded samples stored for up to 84 days under adverse environmental conditions, and they were able to produce profiles for the minor component in the mixture experiments. We also performed a casework sample study testing an old bone sample and noticed that the Miniplex systems were efficient.

Keywords: Forensic science; DNA typing; Short tandem repeat; non CODIS; NC01; NC02

Abbreviations: DNA: Deoxyribonucleic Acid; SWGDAM: Scientific Working Group on DNA Analysis Methods; NC: Non-CODIS; NC01: Miniplex loci D10S1248 D14S1434 D22S1045; NC02: Miniplex loci D4S2364 D2S441 D1S1677; MiniSTR: Mini Short Tanden Repeats; CODIS: Combined DNA Index System; STR: Short Tandem Repeats; FBI: Federal Bureau of Investigation; PCR: Polymerase Chain Reaction; EDTA: Ethylenediaminetetraacetic Acid; RFU : Relative Fluorescence Units; SD: Standard Deviations; PHR: Peak Height Ratio

Introduction

The identification of bodies from mass disasters is a challenge for forensic scientists due to its advanced state

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of decomposition, charring, or fragmentation. Consequently, the only possibility of identification is through DNA analyses [1,2]. However, DNA is usually degraded when samples have been exposed to light, humidity, high temperatures, and bacterial and/or fungal contaminations. Several studies have obtained more effective results from degraded samples when the DNA segment to be analyzed has a smaller size (called Mini Short Tanden Repeats; MiniSTR) [3-9]. Coble & Butler (2005) demonstrated successful results of degraded DNA specimens using six loci arranged into two MiniSTR triplexes [6]; these Miniplexes designated NC01 and NC02 (Non-CODIS 01 and 02), located far from Combined DNA Index System (CODIS) markers regions, include the four recommended autosomal markers in a set of six locus (NC01: D10S1248, D14S1434, D22S1045 and NC02: D4S2364, D2S441, D1S1677). Nevertheless, validated protocols and interpretation guidelines are required for all technologies used in forensic DNA casework analyses. This work describes an internal validation of Miniplexes NC01 and NC02 to determine their robustness in cases where current short tandem repeats (STR) genotyping typically fails.

Material and Methods

The experiments were performed according to the guidelines issued by the Director of the Federal Bureau of Investigation (FBI) [10] and based on the Scientific Working Group on DNA Analysis Methods (SWGDAM) [11].

We verified three important parameters for result interpretation:

a. Stutter Percentage Determination,
b. Heterozygous Peak Height Ratio and
c. Sizing Precision.

Additionally, we performed five validation studies:

a. Sensitivity;
b. Species Specificity;
c. Stability;
d. Mixture and
e. Casework Sample.

Polymerase Chain Reaction (PCR) PCR-based procedures were used as described in Coble et al. [6] and Hill et al. [12]. Population data from Southern Brazil was obtained in 2011 [13]. This investigation was approved by the Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul, Brazil (CEP-PUCRS # 08/04349).

DNA samples

We used 100 anonymous human DNA samples, a DNA pool from human-associated microorganisms (10^5 copies each: *Candida albicans*, *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis*, *Streptococcus mutans*, *Bacillus* subtilis and *Lactobacillus rhamnosus*), the AmpFlSTR® Control DNA 007 and Control DNA 9947A (Applied Biosystems, Foster City, CA). Also, DNA samples from rat, dog, cat, horse, chicken, and cow were tested. To test for changes caused by the environment, fresh human blood samples without Ethylenediaminetetraacetic acid (EDTA) were placed in cotton fabric to simulate blood stains found at the crime scene. These samples were kept in different environments for different periods of time (1 to 84 days): at room temperature, 4°C, -20ºC, 50°C, and under illumination of Grolux® 40 Watt lamp for horticulture (Sylvania, Vineyard, Brazil) during 16 hours to simulate sunlight in a summer day.

DNA quantification and extraction

DNA quantity of human samples was determined using Quantifiler® Human DNA Quantification Kit in the 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA) according to the manufacturer's protocol. DNA concentration of non-human samples was determined by agarose gel analyses (LCG, São Paulo, Brazil). Human blood DNA was extracted by a standard method [14] and human bone DNA and non-human DNA were extracted by PrepFiler™ Forensic DNA Extraction Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's protocol.

PCR and amplification analysis

Amplifications were performed using the primer sequences and the parameters described by Coble et al. [6] and Hill et al. [12]. All samples were amplified in re- or tri-plicate. Amplification using AmpFlSTR® Identifiler™ and AmpFlSTR® MiniFiler™ PCR Amplification Kits (Applied Biosystems, Warrington, UK) were performed according to the manufacturer's protocol. All PCR products were analyzed in the Applied Biosystems 3130*xl* or ABI PRISM® 3100-Avant (Applied Biosystems, Foster City, USA). Samples were prepared in formamide/LIZ® solution (0.3 µL of GeneScan™ 500 LIZ® size standard and 8.7 µL of deionized Hi-Di™ Formamide) (Applied Biosystems), injected at 3 kV for 10 seconds and electrophoresed at 15 kV for 1500 seconds in Performance Optimized Polymer-4 (POP-4™ polymer, Applied Biosystems) with a run temperature of 60°C. Electrophoresis results were analyzed using GeneMapper® *ID-X* Software v1.2.1 (Applied Biosystems, Foster City, USA). Unless stated otherwise, allele peaks were interpreted when the peak heights were greater than or equal to 50 relative fluorescence units (RFU). Bin width ranged from ±0.5 to ±1.0 bp for the six MiniSTRs analyzed in this study.

Results and Discussion

Allelic Ladders, GeneMapper Panels, and Bins to NC01 and NC02 were built. Using the 100 human DNA pool, the NC01 alleles were: D10S1248: 12-13-14-15-16-17; D14S1434: 10-11-12-13-14-15-16-17; D22S1045: 11-12-13-14-15-16-17-18-19, and the NC02 alleles were: D4S2364: 8-9-10; D2S441: 10-11-11.3-12-12.3-13-14-15; D1S1677: 10-12-13-14-15-16-17 (Figure 1). A representative Miniplexes NC01 and NC02 profile was generated using 1ng of Control DNA 007. Its genotype was D10S1248: 12-15; D14S1434: 11-14; D22S1045: 11-16; D4S2364: 9-10; D2S441: 14-15; D1S1677:13-13.

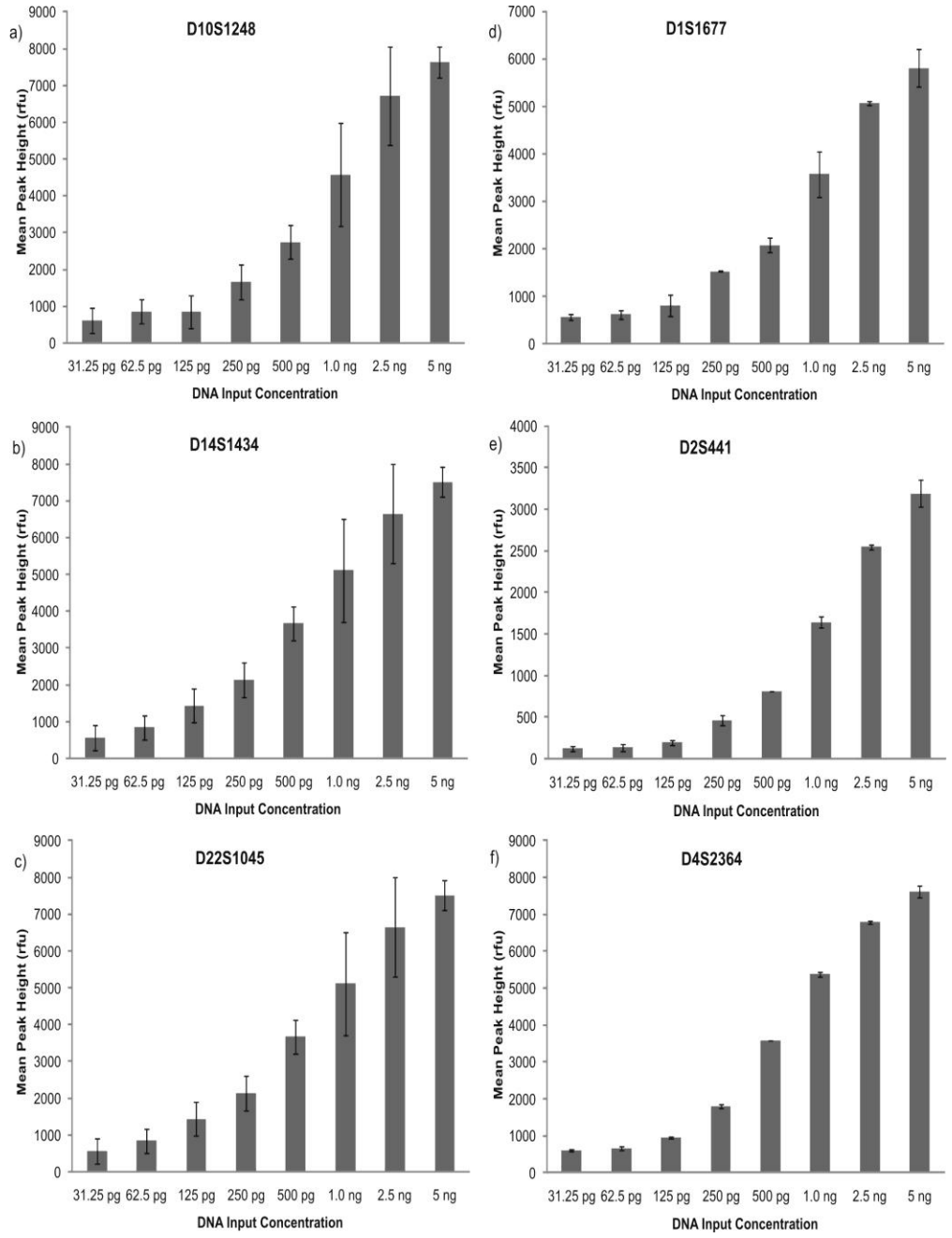

Figure 1: Improved sensitivity of Miniplexes NC01 (a, b, c) and NC02 (d, e, f). Average peak heights obtained for eight dilutions of a Control DNA 007 sample. (a) D10S1248; (b) D14S1434, (c) D22S1045, (d) D1S1677, (e) D2S441 and (f)D4S2364.

Stutter percentage determination

The 100 anonymous human DNA samples were used (1ng per reaction). Stutters were determined for those samples with peak heights between 100 and 6000 RFUs. The threshold minimum stutter peak height was 10 RFUs. Stutter averages, ranges, and standard deviations (SD) are shown in Table 1. All loci yielded one repeat unit loss stutter; -4bp for tetra nucleotide loci D10S1248, D14S1434, D4S2364, D2S441, D1S1677, and -3bp for the tri nucleotide loci D22S1045. The stutter % medians in this work were similar for other autosomal loci, ranging from 4% to 10%. The upper limit of the stutter value range was 22%. D4S2364, D2S441 and D22S1045 stutter % medians were lower than that of other three loci for 100 human samples.

Locus	Count	Stutter average (%)	Stutter range (%)	SD
NC01				
D10S1248	67	9.4	5.8-20.3	2.5
D14S1434	62	10.0	4.1-17.1	3.0
D22S1045	69	7.3	1.5-16.4	4.2
NC02				
D4S2364	35	3.3	0.6-13.2	3.0
D2S441	106	4.0	1.0-13.1	1.9
D1S1677	78	9.9	3.3-21.4	3.0

Table 1: Stutter averages, ranges, and standard deviations (SD) for Miniplexes NC01 and NC02. The proportion of the stutter product relative to the main allele (% stutter) was measured by dividing the height of the stutter peak by the height of the main allele peak.

Heterozygous peak height ratio (PHR)

The PHR data was obtained from amplifying 100 DNA reference samples. In the heterozygous pair the peak height of the smaller peak was divided by the peak height of the larger peak. When samples were amplified with optimum DNA template range of 0.5ng-1ng, for the 435 heterozygous peak pairs, the PHR data showed a final average of 88% and the medians were between 87-97% with a range from 57% to 100% (Table 2). The difference in peak height ratios by ABI 3130*xl* was low, what is in accordance with PHR values in other autosomal loci [15].

Locus	Number of observations	PHR Mean ± SD	PHR Median	PHR Minimum	PHR Maximum
NC01					
D10S1248	73	0.85 ± 0.10	0.88	0.60	1.00
D14S1434	72	0.94 ± 0.07	0.97	0.65	1.00
D22S1045	77	0.86 ± 0.10	0.87	0.57	1.00
NC02					
D4S2364	53	0.89 ± 0.09	0.91	0.64	1.00
D2S441	81	0.86 ± 0.11	0.89	0.46	0.99
D1S1677	79	0.85 ± 0.12	0.87	0.43	0.99

Table 2**:** Heterozygous Peak Height Ratios (PHR) for Miniplexes NC01 and NC02. For each heterozygous, pair the PHR was calculated by the height of lowest intensity allele / height of the highest intensity allele.

Sizing precision

Four injections of the amplified Miniplexes using the positive DNA 007control were performed in ABI 3130*xl*. The consistency of sizing was assessed by looking at the average base pair size and standard deviation of all alleles for each sample injected. The highest standard deviation observed for any allele was 0.06 in the D14S1434 locus (data not shown).

Sensitivity study

PCR amplification was performed in triplicates using internal Control DNA 007 sample that was serially diluted to provide amounts of 1ng, 500pg, 250pg, 125pg, 62.5pg and 31.25pg per amplification. The average peak heights are shown in Figure 1. DNA profiles were obtained with both NC01 and NC02 panels. Partial profiles missing anywhere from 1 allele were observed at 31.25pg in D22S1045 loci (data not shown).

Species specificity

A variety of animal and microbial species were tested to assess the human specificity of Miniplexes NC01 and NC02. No detectable DNA profiles or artifacts were observed when 10ng of DNA from non-human mammalian or microorganisms samples were amplified.

Stability study

The effect of DNA degradation was carried out to characterize amplification performance of either inhibited or degraded DNA. In this study, fresh human blood without EDTA was placed in cotton fabric in triplicates. These samples were kept under different conditions for different periods, as previously described. After 1, 3, 7, 14, 28, 56, and 84 days, DNA was extracted and quantified. The results showed that from day 28 samples lost 50% of DNA concentration if they were under high (50°C) or room temperatures, or if were exposed under the Grolux® light effect. This loss of DNA concentration interfered directly on the amplification by AmpFlSTR® Identifiler™ PCR Kit. However, the Miniplex NC01 and NC02 panels generated full profile for all samples (data not shown).

Mixture study

One set of mixture was prepared containing DNA from two genomic DNA samples; these mixture samples were normalized to a final amount of 1ng of DNA. Using Control DNA 9947A and Control DNA 007, respectively, the following sample ratios were prepared for each mixture: 1:0, 1:1, 1:2, 1:4, 1:6, 1:9, 1:19, and 0:1. Even with low amounts of the minor component (the 1:19 proportion; about 50pg of the minor DNA component in a total of 1ng) it was possible to detect some alleles from the contributor in the mixture sample.

Casework sample study

We tested a human bone sample recovered from a woodland area after 35 years of death. We examined this DNA bone sample by AmpFlSTR® Identifiler™ and AmpFlSTR® MiniFiler™ PCR Amplification Kits as well as by Miniplex NC01 and NC02 panels. The MiniFiler™ kit was efficient in getting partial profiles (amelogenin and five loci); the Miniplex NC01 and NC02 panels were able to generate full profiles for this casework sample (Supplemental Figure 7). The results from this sample were consistent with the proposal of Miniplex NC01 and NC02.

Conclusion

Miniplex panels were developed to provide a sensitive system to obtain genetic profile from degraded samples by analyzing smaller DNA segments. In this work, an internal validation based on the Scientific Working Group on DNA Analysis Methods (SWGDAM) Validation Guidelines was conducted in order to assess the reliability and performance of Miniplexes NC01 and NC02 in forensic casework. This internal validation made it possible to verify the Miniplexes robustness and to create standard operating procedures for analysis and interpretation of various types of forensic samples. Internal validation is essential to guarantee consistent and correct results to be used in court. Finally, the combined use of Miniplexes NC01 and NC02 with other commercial kits may provide a greater number of markers with sizes below 200bp which greatly increases the chance of a successful resolution of challenging forensic samples.

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