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INTERNAL VALIDATION OF A SIX NON-CODIS MINISTR LOCI (D1S1627, D3S4529, D5S2500, D6S1017, D8S1115 AND D9S2157) SYSTEM

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ABSTRACT

Non-CODIS (NC) miniSTR genotyping systems were developed to produce smaller amplicons by moving the primers as close as possible to the repeat region of interest. These systems are valuable when profiling degraded or compromised DNA samples. This study details the internal validation for a six NC miniSTR system comprised of the loci: D1S1627, D3S4529, D5S2500, D6S1017, D8S1115 and D9S2157. These loci demonstrated the ability to produce consistent, accurate and precise genotype profiles for low concentrations of template DNA. Template DNA concentrations as low as 50pg were successfully amplified and typed. Differentiation of major and minor components were easily identifiable in miniplex 1, but in miniplex 2 complete profiles for each contributor was only observed from ratios ≤1:4. This internal validation allowed for the determination of the reliability as well as the limitations of this NC miniSTR genotyping system.

Keywords: Non-CODIS, Variation, Genetic polymorphism, Microsatellite markers, Tandem repeats, Internal validation.

1. INTRODUCTION

The development of commercial multiplex STR kits has made it easy over the years to identify biological samples by DNA typing. However problems have been reported when kits were used to analyze degraded DNA samples or DNA samples which had PCR inhibitors present (Hummel *et al.*, 1999; Butler *et al.*, 2003; Romano *et al.*, 2006). A direct relationship between amplicon size and amplification efficiency was demonstrated previously (Chung *et al.*, 2004; Opel *et al.*, 2006). To overcome this shortfall, STR primers which would be able to successfully generate genetic profiles from degraded/low copy DNA were investigated. The redesigning of STR loci primers to produce shorter amplicons resulted in the successful typing of samples which had previously failed to produce conclusive results (Ricci *et al.*, 1999; Grubweiser *et al.*, 2003). This led to the development of 26 novel miniSTR loci known as the Non-CODIS (NC) loci. These NC loci produced shorter PCR products ranging between 50-150 base pairs (bp) and are located on chromosomes not common to the 13 COmbined DNA Index System (CODIS).

Multiplex typing systems must be optimized to the point where they meet certain performance standards. There are several governing bodies that ensure that high typing and analysis standards

are maintained. Among these is the International Society for Forensic Genetics (ISFG), the Scientific Working Group on DNA Analysis Methods (SWGDAM), and the European DNA Profiling Group (EDNAP). These organizations have proposed guidelines for the use and validation of multiplex PCR typing systems. Some common validation exercises include: (1) establishing that the typing system is sensitive and performs consistently using freshly prepared and stored DNA, (2) that identical results are obtained irrespective of the type of tissue from which DNA was extracted, (3) that the systems yield consistent results in several laboratories, and (4) that the system performs well when used to analyze samples similar to those encountered in forensic casework (Leat *et al.*, 2004). Validation plays a vital role in the forensic community since there is a constant influx of new DNA technology being developed. There are two types of validation processes; i.e. a developmental validation and an internal validation. Developmental validation is the more thorough of the two methods and it is used to determine the reliability and limitations of a novel method (Daniels *et al.*, 2004). This entails the determination of: accuracy, precision, reproducibility, species specificity, sensitivity, stability, PCR parameters and simulated casework studies (Daniels *et al.*, 2004). Developmental validations are generally performed by: manufacturers, academic institutions, technical organizations and government laboratories (Butler, 2005).

The purpose of an internal validation is solely to demonstrate the ability of a laboratory to perform a previously developmentally validated procedure (Daniels *et al.*, 2004). Both developmental and internal validations determine whether the analytical procedure will be adequate for its intended use. This paper outlines the internal validation for six NC miniSTR loci: D1S1627, D3S4529, D5S2500, D6S1017, D8S1115 and D9S2157.

2. MATERIALS AND METHODS

2.1. DNA Extraction and Quantification

DNA samples were collected either as whole blood or buccal swab samples. DNA was extracted as previously described (Lahiri and Nurnberger, 1991). DNA isolated from cell line 9947A was also used in this validation study. All DNA samples were quantified using a Nanodrop ND 1000 UV-Vis spectrophotometer.

2.2. PCR Amplification

DNA samples were analysed using NC miniSTR primer sets previously (Hill *et al.*, 2008). Primers were synthesized by Applied Biosystems and all forward primers were labelled with either 6FAM™, VIC™ and NED™ dyes which enabled colour separation on an ABI 377 genetic analyzer. All reverse primers were unlabelled and had an additional 5' guanine base added to produce fully adenyalted PCR products (Hill *et al.*, 2008). Complete primer sequences and final concentrations of each of the loci used in this study are presented in Table 1.

Amplification of samples was performed in reaction volumes of 10µl using a master mix containing: 1X Supertherm PCR buffer (containing 1.5mM $MgCl_2$); 250µM of each deoxynucleotide triphosphate (Roche, dNTP's: dATP, dCTP, dGTP, dTTP); 1µl of template DNA at the various concentrations, 160µg/ml BSA and 1U Supertherm Gold Taq DNA polymerase. Primers were added to produce final concentrations as indicated in Table 1. Amplification reactions were performed using a 96-well Gene-Amp® PCR system 9700 thermal cycler (Applied Biosystems). Thermal cycling conditions for miniplex 1 were: 95°C for 10 minutes; 35 cycles of: 94°C for 1 minute, 59°C for 1 minute and 72°C for 1 minute followed by a final incubation at 60°C for 45 minutes and 4°C forever. Thermal cycling conditions for miniplex 2 were: 95°C for 10 minutes; 35 cycles of: 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute followed by a final incubation at 60°C for 45 minutes and 4°C forever.

2.3. Fragment Analysis on ABI 377 Genetic Analyzer

Amplified samples were electrophoretically separated using an ABI Prism® 377 Genetic Analyzer (Applied Biosystems) and GeneScan™ 500 LIZ™ size standard (Applied Biosystems, Warrington, UK). Samples were prepared for analysis by mixing 1µl of dye mix (i.e. 5µl de-ionized formamide, 1µl dextran blue and 1µl of GS500 LIZ size standard [Applied Biosystems]) with 1µl of PCR product. Mixtures were heat denatured on a thermocycler at 95°C for 5 minutes and then immediately snap cooled on ice. 1µl of sample was loaded on a 36cm gel, and filter set G at 2400 scans per hour.

All data was collected with the ABI 377 collection software (Applied Biosystems) and analyzed using the GeneScan 3.1 (Applied Biosystems) software. Size fragments were converted to allele numbers by making use of the Genotyper 2.5 (Applied Biosystems) software. Allele designations were assigned according to recommendations of the DNA Commission of the ISFG (Bar *et al.*, 1997) with the aid of allelic ladders. Allele numbers were assigned to samples according to the known allele numbers in allelic ladders.

3. RESULTS AND DISCUSSION

3.1. Reproducibility

To test genotyping consistency random human DNA samples were selected for each miniplex set and typed on three separate occasions with a minimum of one week lapse between each experiment. Consistent genotype profiles were obtained for all samples tested within each miniplex set (Table 2 and 3).

3.2. Precision and Accuracy

Central to STR genotyping is the ability of the method to measure the size of the amplified product (Krenke *et al.*, 2005; Mulero *et al.*, 2006). "Sizing precision allows for determining accurate and reliable genotypes" (Mulero *et al.*, 2006). In this study precision refers to the

reproducibility of the size measured for the amplified product and accuracy refers to the ability of the method to obtain a correct size and genotype (Daniels *et al.*, 2004; Krenke *et al.*, 2005). The standard DNA sample 9947A was selected for this study.

DNA sample was amplified and typed on three different occasions with a minimum of one week lapse between each typing session. Precision was calculated for each locus and expressed as a standard deviation. Accuracy was expressed as the variation in size (bp) between the allele(s) of the sample compared to those same allele(s) within the allelic ladder of the same gel run. Allele sizes were determined using an internal lane standard, GeneScan LIZ 500.

The precision study indicated that across all loci the standard deviation did not exceed 0.141 bases (Table 4). In this study the highest standard deviation of 0.141 bases was shown by the locus D8S1115 (Table 4). This is well below the accepted norm of 0.2 bases standard deviation (Online STR Database, 2012). Capillary based genetic analysers and DNA sequencers generally report standard deviations below 0.15 bases (Online STR Database, 2012). In this study the gel based ABI PRISM 377 DNA sequencer (Applied Biosystems) was used. This method is deemed to be less accurate than the capillary based systems (Online STR Database, 2012). Thus this study proved that the miniplex systems investigated could generate reproducible results with high precision since standard deviation across all markers fell well below the 0.2 base limit.

In the accuracy test alleles across all loci fell within ±0.5bp of the same allele in the allelic ladder (Table 5). ABI Prism DNA Genotyper Analysis Software (version 3.7) allows for ±0.5bp variation between the unknown fragment and the fragment in the allelic ladder. In each set of analysis conducted, the standard sample, 9947A, fell well within the ±0.5bp variation allowed. Furthermore, the standard sample, 9947A, did not show the presence of any variant alleles.

3.3. Sensitivity and Stochastic Studies

DNA obtained from crime scenes are generally compromised in one form or another. In some instances the DNA recovered can be both degraded and the amount of template can also be low. It is envisioned that by shortening the size of the amplicon, amplification efficiency of degraded and low copy DNA would be improved. The Non-CODIS miniplex system investigated in this study was designed so as to aid in the amplification of degraded and low concentrations of DNA.

The sensitivity of miniplex 1 (n=9) and miniplex 2 (n=8) was investigated. Decreasing amounts of DNA (500pg, 200pg, 100pg, 50pg, 25pg, and 12.5pg) was added to a standard 10µl PCR reaction. GeneScan analysis threshold was set at a minimum of 150RFU.

The lower limit of template DNA necessary for a complete profile was 25pg and 50pg in miniplex 1 and miniplex 2 respectively (Figure 1 and 2). At lower concentrations miniplex sets displayed preferential amplification of the smaller allele and/or allele drop out. At concentrations of 500pg allele drop in and pull up was observed in some samples. Good signal intensities with average RFU values ranging between 800-1800 for miniplex 1 loci at a template concentration of

25pg was observed (Figure 3). Miniplex 2 loci displayed average RFU values ranging between 800-2500 at a template concentration of 50pg (Figure 4).

3.4. Mixture Studies

Casework samples may in some instances contain DNA from more than one contributor. Thus it is important to test the ability of a miniplex system to distinguish between the major and minor contributors. Deciphering the genotypes of individuals in mixed samples can be very difficult especially in instances where the contributors share common alleles. In cases like these, analysis of peak ratios becomes an important tool in differentiating between the DNA originating from the minor and major contributors (Chung *et al.*, 2004). For this mixture study, two DNA samples were selected for each miniplex. The samples selected shared no common alleles for the loci tested. DNA samples were prepared at a concentration of 100pg (for miniplex 1) and 200pg (for miniplex 2) and then mixed at ratios of: 1:19, 1:9, 1:4 and 1:1. 1µl of mixed DNA, (for each of the above mentioned ratios), was cycled under the standard PCR conditions in a final reaction volume of 10µl.

The results from this study showed that across all loci (i.e. D1S1627, D5S2500 and D8S1115) and all dilutions (i.e. 1:19, 1:9, 1:4 and 1:1) in miniplex 1 both the minor and major components were detectable within the mixed samples (Table 6).

At a ratio of 1:19 a complete profile was only observed for the major component in miniplex 2 (Table 7). The minor component displayed allele drop out for all loci (i.e. D3S4529, D6S1017 and D9S2157) at this ratio (Table 7). In miniplex 2 the minor component was detectable for the all loci from a ratio of 1:4 (Table 7).

These mixture studies displayed that it is possible to discriminate between individuals within a mixed sample for miniplex 1 and miniplex 2. In miniplex 2 this is however dependant on the ratio of genomic DNA within the mix since two complete profiles could only be detected with certainty for all loci from a ratio of 1:4 and less.

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Table-1. Primer sequences, dye labels and final concentration of primers used in miniplex reactions 1 and 2

STR locus	Miniplex set	Primer sequence (5'-3')	Primer concentration (µM)
D1S1627	1	F-[VIC]-CATGAGGTTTGCAAATACTATCTTAAC R-GTTTTAATTTTCTCCAAATCTCCA	0.3
D3S4529	2	F-[VIC]-CCCAAAATTACTTGAGCCAAT R-GAGACAAAATGAAGAAACAGACAG	0.1
D5S2500	1	F-[NED]-CTGTTGGTACATAATAGGTAGGTAGGT R-GTCGTGGGCCCCATAAAATC	0.1
D6S1017	2	F-[NED]-CCACCCGTCCATTTAGGC R-GTGAAAAAGTAGATATAATGGTTGGTG	0.1
D8S1115	1	F-[6FAM]-TCCACATCCTCACCAACAC R-GCCTAGGAAGGCTACTGTCAA	0.1
D9S2157	2	F-[6FAM]-CAAAGCGAGACTCTGTCTCAA R-GAAAATGCTATCCTCTTTGGTATAAAT	0.5

Table-2. Genotype profiles obtained for human DNA samples analysed with miniplex 1 loci

Sample ID	Miniplex 1 loci								
	D1S1627			D5S2500			D8S1115		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
9947A	13:14	13:14	13:14	14:23	14:23	14:23	9:18	9:18	9:18
C106	11:14	11:14	11:14	17:18	17:18	17:18	9:14	9:14	9:14
C26	12:14	12:14	12:14	17:18	17:18	17:18	14:16	14:16	14:16
As17	13:14	13:14	13:14	14:18	14:18	14:18	9:16	9:16	9:16
A146	10:13	10:13	10:13	18:23	18:23	18:23	15:16	15:16	15:16
As12	10:13	10:13	10:13	14:18	14:18	14:18	9:16	9:16	9:16

Table-3. Genotype profiles obtained for human DNA samples analysed with miniplex 2 loci

Sample ID	Miniplex 2 loci								
	D3S4529			D6S1017			D9S2157		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
9947A	15:15	15:15	15:15	9:10	9:10	9:10	7:13	7:13	7:13
A130	15:17	15:17	15:17	10:13	10:13	10:13	11:12	11:12	11:12
A149	13:15	13:15	13:15	8:10	8:10	8:10	12:15	12:15	12:15
As2	14:16	14:16	14:16	8:10	8:10	8:10	7:13	7:13	7:13
As3	13:14	13:14	13:14	10:12	10:12	10:12	13:14	13:14	13:14
C9	13:14	13:14	13:14	8:9	8:9	8:9	7:10	7:10	7:10

Table-4. Precision study showing average allele size and standard deviation of the control sample 9947A for six Non-CODIS miniSTR loci

Locus	Mean size allele 1 (bp)	Standard deviation allele 1 (bp)	Mean size allele 2 (bp)	Standard deviation allele 2 (bp)
D1S1627	93.027	0.086	96.113	0.045
D3S4529	120.957	0.098	120.957	0.098
D5S2500	86.687	0.133	123.083	0.062
D6S1017	91.857	0.082	95.733	0.058
D8S1115	65.560	0.141	93.310	0.037
D9S2157	74.120	0.100	92.270	0.135

Table-5. Accuracy study presenting the variation between allele sizes of the control sample 9947A in comparison to the same allele sizes in the allelic ladders for each of the Non-CODIS miniSTR loci

Locus	Run number	Allele size(s) (bp) of sample 9947A	Allele size(s) (bp) within the allelic ladder	Variation of size (bp)*
D1S1627	1	93.13; 96.17	93.21; 96.27	0.08; 0.1
	2	93.03; 96.06	93.08; 96.23	0.05; 0.17
	3	92.92; 96.11	92.65; 96.06	-0.27; -0.05
D3S4529	1	120.83	120.91	0.08
	2	120.97	120.94	-0.03
	3	121.07	120.87	-0.2
D5S2500	1	86.76; 123.03	86.81; 123.14	0.05; 0.11
	2	86.80; 123.17	86.96; 123.02	0.16; -0.15
	3	86.50; 123.05	86.50; 122.83	0.0; -0.22
D6S1017	1	91.75; 95.72	91.87; 95.78	0.12; 0.06
	2	91.95; 95.67	91.98; 95.95	0.03; 0.28
	3	91.87; 95.81	91.87; 95.95	0.0; 0.14
D8S1115	1	65.66; 93.36	65.74; 93.37	0.08; 0.01
	2	65.66; 93.30	65.30; 93.35	-0.36; 0.05
	3	65.36; 93.27	65.35; 93.37	-0.01; 0.1
D9S2157	1	74.07; 92.31	74.17; 92.37	0.1; 0.06
	2	74.26; 92.43	74.14; 92.63	-0.12; 0.2
	3	74.03; 92.28	74.02; 92.29	-0.01; 0.01

* Variation of size (bp) = Allelic ladder size – Allele size of sample 9947A

Table-6. Genotype profiles obtained from the amplification of mixed DNA samples with miniplex 1 loci

Ratio of minor to major DNA component	Genotype profiles obtained for minor DNA component			Genotype profiles obtained for major DNA component		
	D1S1627	D5S2500	D8S1115	D1S1627	D5S2500	D8S1115
Neat	10:12	17:17	9:9	13:13	14:14	16:16
1:1	10:12	17:17	9:9	13:13	14:14	16:16
1:4	10:12	17:17	9:9	13:13	14:14	16:16
1:9	10:12	17:17	9:9	13:13	14:14	16:16
1:19	10:12	17:17	9:9	13:13	14:14	16:16

0 indicative of an allele displaying an RFU value less than the threshold limit of 150.

Table-7. Genotype profiles obtained from the amplification of mixed DNA samples with miniplex 2 loci.

Ratio of minor to major DNA component	Genotype profiles obtained for minor DNA component			Genotype profiles obtained for major DNA component		
	D3S4529	D6S1017	D9S2157	D3S4529	D6S1017	D9S2157
Neat	15:17	10:13	11:12	13:14	8:9	7:10
1:1	15:17	10:13	11:12	13:14	8:9	7:10
1:4	15:17	10:13	11:12	13:14	8:9	7:10
1:9	0:17	0:0	0:0	13:14	8:9	7:10
1:19	15:0	0:0	0:0	13:14	8:9	7:10

0 indicative of an allele displaying an RFU value less than the threshold limit of 150.

Figure-1. Electropherograms illustrating DNA template concentration titration for miniplex 1. (a) Amplification of 500pg of DNA, allele drop in and pull up can be observed. Loci are performing optimally when amplified with DNA concentration of 200pg and 50pg (panels b and c respectively). (d) Locus D5S2500 (black) is not performing optimally when amplified with 12.5pg of DNA. Sensitivity limit for miniplex 1 was set at 25pg.

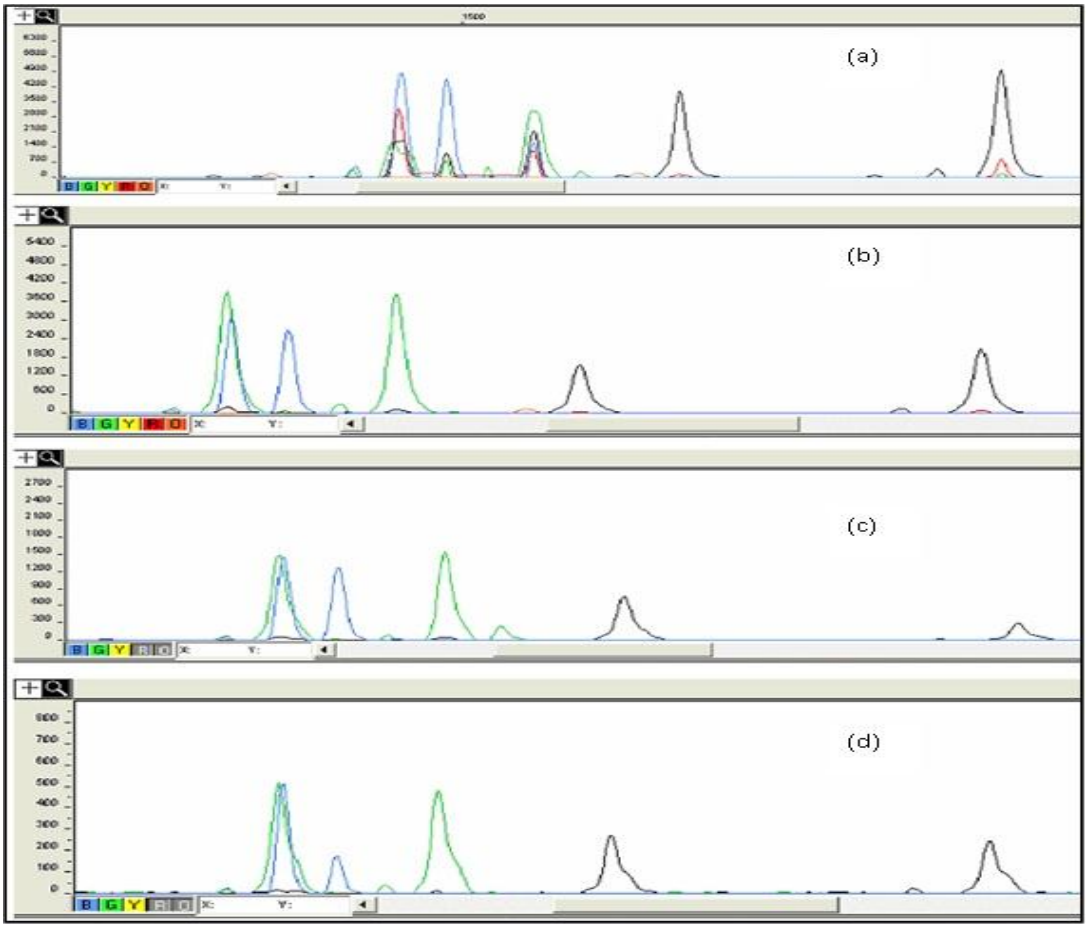

Figure-2. Electropherograms illustrating DNA template concentration titration for miniplex 2. (a) . Loci are performing optimally when amplified with DNA concentrations of 500pg 200pg and 50pg (panels a, b and c respectively). (d) At a concentration of 12.5pg, allele drop out was observed for the larger allele of the locus D9S2157 (blue). Sensitivity limit for miniplex 2 was set at 50pg.

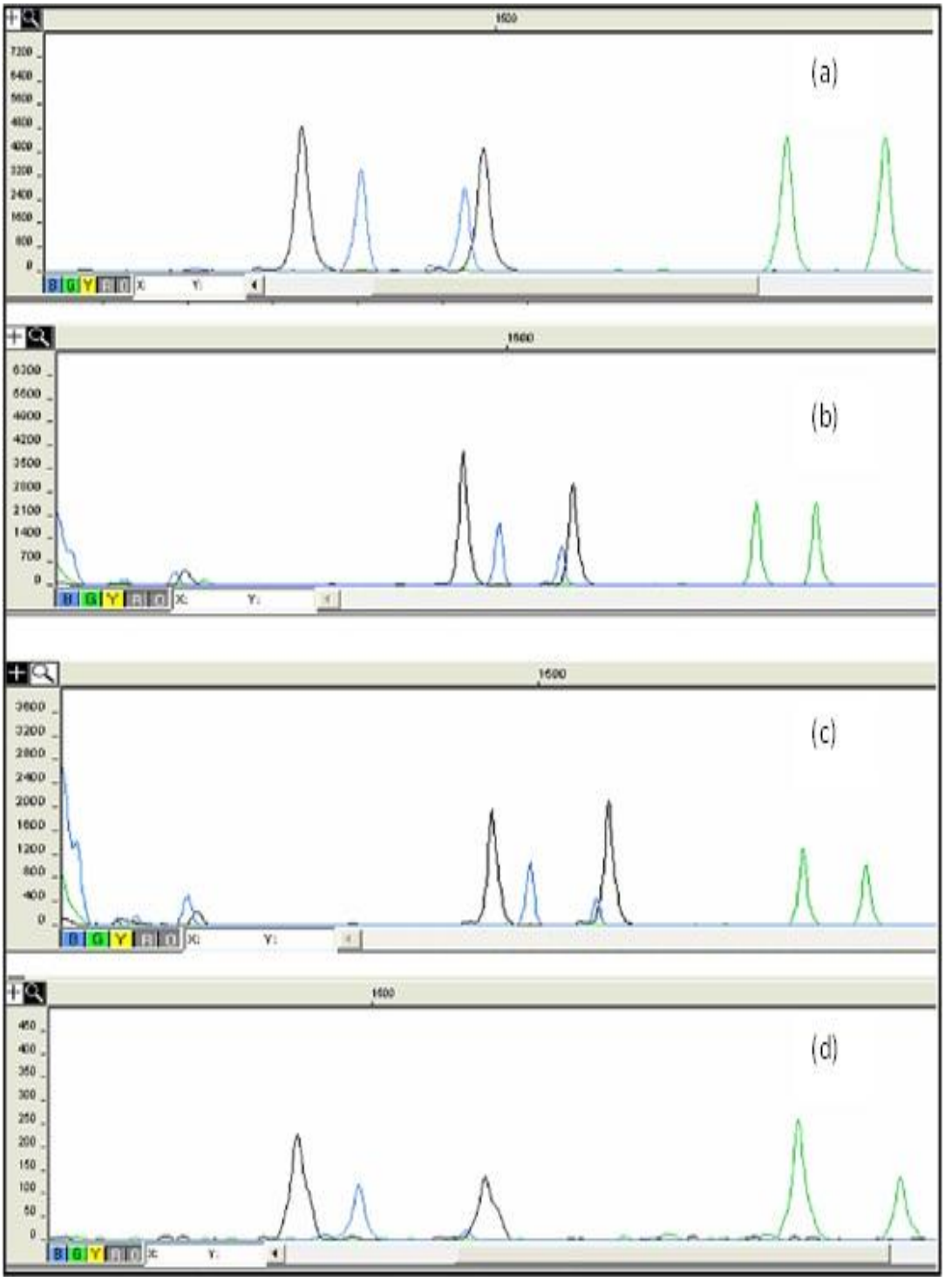

Figure-3. Sensitivity and stochastic study for miniplex 1. The average fluorescence signal intensity for D1S1627, D5S2500 and D8S1115 was plotted as function of template concentration. Good signal intensities ranging between 800-1800RFU were obtained at the minimal template concentration of 25pg. (n=9)

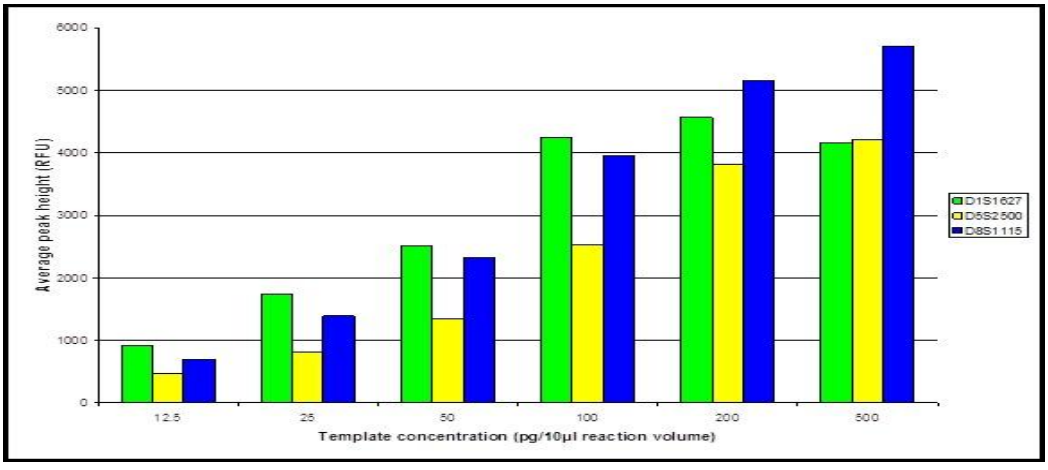

Figure-4. Sensitivity and stochastic study for miniplex 2. The average fluorescence signal intensity for D3S4529, D6S1017 and D9S2157 was plotted as function of template concentration. Good signal intensities ranging between 800-2500RFU were obtained at the minimal template concentration of 25pg. (n=8)