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TECHNICAL NOTE

Development and validation for identity testing of I-DNADuo, a combination of I-DNA1 and a new multiplex system, I-DNA2

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Abstract The I-DNADuo multiplex system combination is composed of previously validated I-DNA1 and a new short tandem repeat (STR) multiplex named I-DNA2 that analyses 11 STR loci plus amelogenin. I-DNADuo, with amplicon sizes ranging from 57 to 298 bp, is specifically designed to analyse amelogenin and 15 STR loci (ten of them plus amelogenin in duplicate), including all the STR loci of the CODIS, ISSL and ECL databases, and seven of the eight in GCL. The validation of I-DNADuo shows that it is a highly sensitive, robust multiplex system for obtaining individual genetic profiles and for detecting and preventing allelic dropouts.

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Introduction

Short tandem repeat genotyping in forensic genetics is mainly applied to human identification and has important medical and legal repercussions [1]. Most countries use genetic profiles in criminal investigations and are adapting their legislations in order to store those profiles in databases such as the Combined Index System (CODIS), Interpol Standard Set of Loci (ISSL), European Core Loci (ECL) and German Core Loci (GCL) [2, 3].

In this context, we have developed a multiplex system called I-DNADuo composed of previously validated I-DNA1 and a new short tandem repeat (STR) multiplex named I-DNA2. This combination of two multiplex systems analyses 15 loci STRs plus amelogenin. I-DNADuo includes all the STR loci in CODIS, ISSL, ECL and seven of the eight in GCL (the exception is SE33). I-DNADuo provides results in duplicate for ten loci plus amelogenin since they are shared by I-DNA1 and I-DNA2. In addition, eight of the loci analysed in duplicate are genotyped using different primer pairs in the I-DNA1 and I-DNA2 multiplex systems. This makes it possible to detect and prevent the allelic dropouts that may be caused by the presence of mutations in the 3′ end of the primers.

I-DNADuo validation is based on the validation of I-DNA1 [4] and I-DNA2 (performed following the SGWDAM guidelines) [5] to test primer set optimisation, concordance

with other multiplex systems, sensitivity, stutter percentage and the heterozygous peak height ratio (PHR). The results show that I-DNADuo is a useful tool for identity testing.

Materials and methods

Primers for amplifying 11 STR loci (D5S818, D7S820, D13S317, D18S51, D21S11, CSF1PO, FGA, TH01, TPOX, D2S1338 and vWA) plus amelogenin were designed with Perlprimer software (http://perlprimer.sourceforge.net/) based on the reference sequences whose Genbank accession numbers are listed in Table 1. These primers are protected by patents P201031269 and P201031270. The regions flanking the STRs analysed were studied as described in Odriozola et al. [4].

We used standard 9947A DNA (Applied Biosystems, Foster City, CA, USA), DNA 007 (Applied Biosystems) and K562 DNA (Promega® Corporation, Madison, WI, USA) to: (1) evaluate amplicon intensity and confirm the absence of nonspecific PCR products by denaturing high-performance liquid chromatography (DHPLC); (2) set up the PCR conditions; and (3) run sensitivity tests. These assays were performed and amplification products were analysed by capillary electrophoresis following the methodology described in Odriozola et al. [4]. Similarly, the allelic ladder for I-DNA2 was developed and its precision and accuracy were assessed via the methods used in the I-DNA1 validation by analysing 162 Caucasoid individuals (Basque Country, Spain) [4].

The reliability of the genetic profiles obtained with I-DNA2 was tested by comparing first the genetic profiles obtained after analysing the same 162 individuals with I-DNA2 and Identifiler® (Applied Biosystems™) and second the step profiles obtained with I-DNA2 and I-DNA1 of 600 individuals: 318 Caucasoid Europeans (Basque Country, Spain) and 282 individuals from Colombia (133 Negroid and 149 Hispanic).

Genetic profiles generated with I-DNA1 and I-DNA2 were compared using *I-DNA One-Two Utility* software (a0b1c0xyz@gmail.com).

Table 1 Genbank accession, allele range, fragment sizes, primer concentration, fluorescent dye for each locus of I-DNA2 and difference in length between I-DNA1 and I-DNA2 amplicons

Locus	Genbank accession	Allele range	Product size (bp)	Primer sequence[a] (5′–3′)	Primer (μM)	I-DNA2–I-DNA1 (bp)
CSF1PO	X14720	6–15	92–128	6FAM-GAAGATATTAACAGTAACTG CCTT ACCCTGTTCTAAGTACTTCC	0.06	17
FGA	M64982	16–51.2	156–298	6FAM-CTCACAGATTAAACTGTA ACCA GTGATTTGTCTGTAATTGCCA	0.08	5
TPOX	M68651	6–16	61–101	NED-CTTAGGGAACCCTCACTG GCAGCGTTTATTTGCCCAA	0.1	0
D18S51	AP001534	7–27	109–189	NED-TGAGTGACAAATTGAGACCT GTACAATAACAGTTGCTACTATTTCT	0.3	−104
vWA	M25858	11–24	216–268	NED-ACTCCTCAGACTGATCCTATA AGATGATAAATACATAGGATGGATG	0.18	95
TH01	D00269	5–14	57–93	VIC-AACACAGACTCCATGGTG GTTCCTCCCTTATTTCCCT	0.05	0
D21S11	M84567	12–38	107–211	VIC-CCCAAGTGAATTGCCTTC ATAGGAGGTAGATAGACTGGA	0.11	−85
D7S820	AC004848	6–15	224–260	VIC-GAATTATAACGATTCCACATTT ATCC GTTGGTCAGGCTGACTATG	0.14	41
D2S1338	AC010136	11–28	88–156	PET-GGAAACAGAAATGGCTTGG GTAAGTTAAAGGATTGCAGGAG	0.15	–
D13S317	AL353628	5–17	164–212	PET-GTTCATTTCTTTAGTGGGCA GTCCTTCAACTTGGGTTGAG	0.15	92
Amelogenina	[17]	X, Y	220, 226	PET-AAAGCTACCACCTCATCCT GGCTTGAGGCCAACCAT	0.11	99
D5S818	AC008512	7–17	238–278	PET-TAGCAAGTATGTGACAAGGG GTCAGATGCTAATAGGCTGTT	0.08	111

[a] These primers are protected by patents P201031269 and P201031270

Results and discussion

I-DNADuo is a combined multiplex system capable of co-amplifying 15 STR loci plus amelogenin, ten of them plus amelogenin in duplicate, in the range of 57–298 bp. It is composed of the recently developed and validated I-DNA1 multiplex system [4] and I-DNA2, a new multiplex system for the simultaneous amplification of amelogenin and 11 STR loci.

Validation

Primer set optimisation

Seventy-eight primers were designed and those capable of producing amplicons no bigger than 300 bp were selected. For the calculation of amplicon sizes, alleles on the STRBase web site were considered [6]. Additionally, primer pairs were chosen whose melting temperatures (58.9±0.87°C and 58.8±0.91°C, respectively) and lengths (20.4±2.3 and 21.3±2.88 bp, respectively) were similar to I-DNA1 in order to allow subsequent PCR amplification in the same thermocycling conditions for both the multiplex systems that make up I-DNADuo [4].

Primers identical to those used in I-DNA1 were chosen to amplify TH01 and TPOX due to the extremely small size of their amplicons [4]. The rest of the I-DNA2 primer pairs were newly designed. Table 1 shows the primer sequences and the range of alleles considered for each locus. To assess the intensity of amplicons and to check that there were no nonspecific amplifications, the primer set was optimised by analysing the amplified products by DHPLC as described in Odriozola et al. [4] (Electronic supplementary material (ESM) Fig. 1).

Once the best set of primers for I-DNA2 was selected, the forward primer of each pair was marked with a fluorochrome as indicated in Table 1. This primer selection strategy for I-DNA2 allows it to be combined with I-DNA1 (ESM Fig. 2) in I-DNADuo.

PCR amplification parameter optimisation

I-DNADuo has the advantage that the two multiplex reactions can take place under the same thermocycling

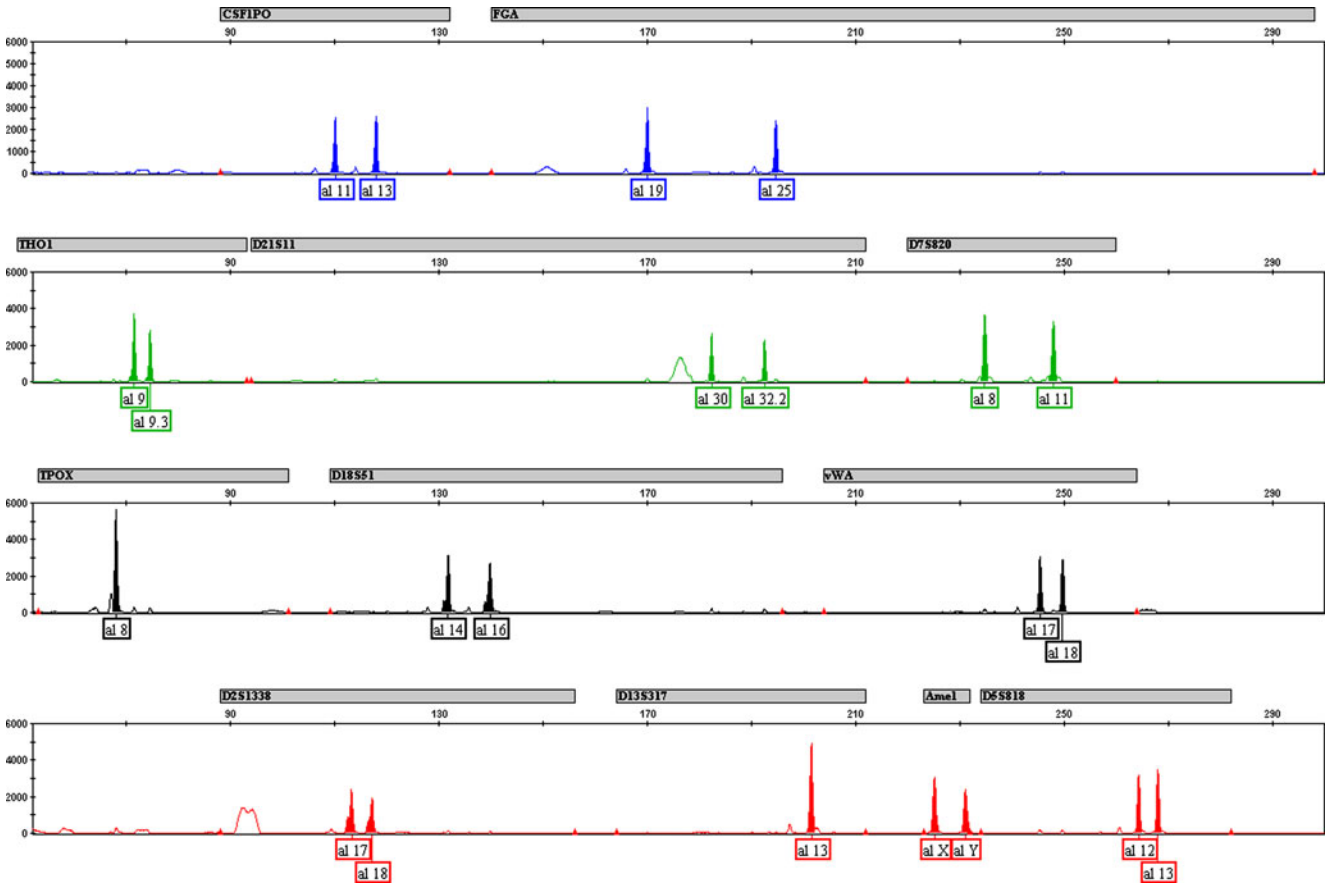

Fig. 1 Typical electropherogram of 1 ng amplified DNA with I-DNA2. Signals represent the alleles indicated *below the respective peaks*. *Boxes above peaks* indicate each locus name

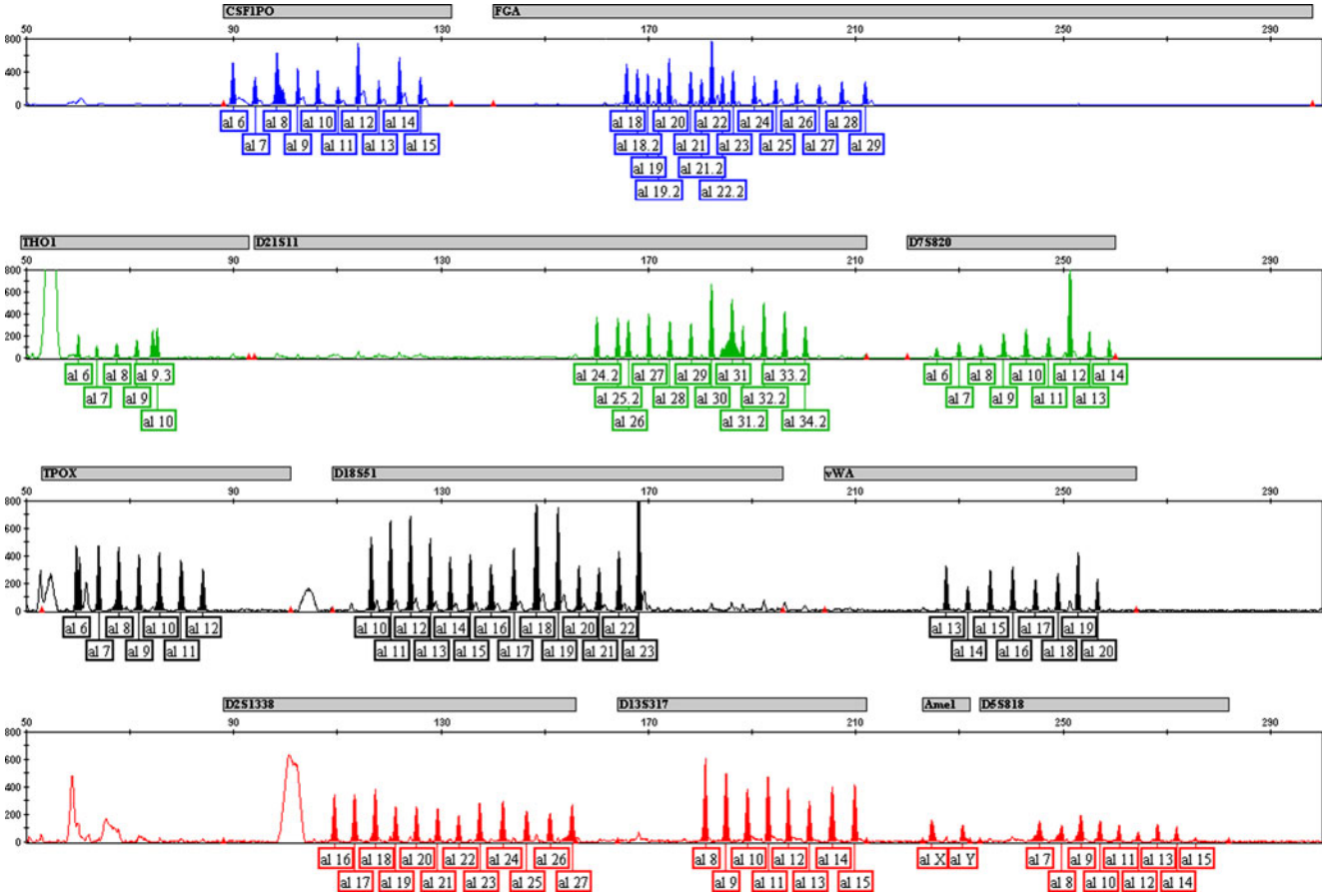

Fig. 2 Allelic ladder for the I-DNA2 multiplex system. The allele designation of each locus is assigned under the respective signals

conditions since multiplex system I-DNA2 was carefully designed to have similar optimal PCR conditions to I-DNA1. The best annealing results for I-DNA2 were obtained at temperatures of 58.9°C and 60.5°C. The second annealing temperature was selected on the grounds that it was more restrictive and was identical to that used in I-DNA1 PCR amplification [4]. Similarly, both multiplex systems have the same optimal number of cycles (30). Incomplete adenylation was removed more efficiently by adding a guanine to the 5′ end of the reverse primer (marked as G in boldface, Table 1) than by increasing final extension time or adding pig-tailing.

Finally, the optimum primer concentration was determined (Table 1) for a single balanced multiplex reaction capable of amplifying 11 STR loci plus amelogenin from 1 ng of template DNA (Fig. 1). The amplification conditions were as follows: one initial denaturing cycle lasting 15 min at 95°C, 30×30 s/cycle at 95°C, 90 s at 60.5°C and 1 min at 72°C, followed by a final extension cycle of 30 min at 60°C. In these conditions, the following fluorescence intensities were determined for each fluorochrome: 6-FAM™ (1933±424 RFU), VIC™ (2659±428 RFU), NED™ (2276±100 RFU) and PET® (1869±416 RFU). No overlapping between signals from different fluorochromes was detected. However, at higher than optimal primer concentrations, imbalance in heterozygotes and artefacts FAM91, FAM155, VIC51, VIC179, NED100, NED165, NED179, PET95 and PET97 were observed.

Allelic ladder

The allelic ladder for the I-DNA2 multiplex system was developed following exactly the same strategy described above for I-DNA 1 [4] (Fig. 2).

Precision and accuracy

The accuracy of the allellic ladder for I-DNA2 was evaluated by analysing 162 individuals to measure the deviation of each sample allele size from the corresponding ladder, as in the I-DNA1 validation [4]. The precision calculation reveals a standard deviation of <0.07 bp, and the allelic ladder accuracy calculation detects no alleles outside the range of the allelic ladder in any of the loci included in I-DNA2, as in I-DNA1 [4]. Therefore, the precision and accuracy of both multiplex systems (I-DNADuo) are similar.

Preliminary concordance studies

To test the reliability of I-DNA2, concordance studies were run between genetic profiles determined by this system and by other previously validated systems. One thousand nine hundred ninety-four allele calls were obtained by comparing I-DNA2 and Identifiler® (Applied Biosystems) on the grounds that this is a widely used system [7]. No discordances were detected between the two multiplex systems, i.e. there is a concordance of 100% between I-DNA2 and Identifiler® (Applied Biosystems) in this study.

The genetic profiles obtained in I-DNA1 are combined with those obtained in I-DNA2 as part of I-DNADuo, so an exhaustive concordance study between the two reactions was carried out, with 6,600 allele calls analysed by I-DNA2 and I-DNA1 being compared. A concordance of 99.9% between the genetic profiles of the two components of the kit I-DNADuo was observed, a result similar to those of other concordance studies [8, 9]. Previous studies have reported that these discordances were due to mutations which probably coincide with the 3′ end binding region of one of the primer pairs, so the combination of two kits with different primers is therefore of great interest in detecting and preventing allelic dropouts [10–12].

Sensitivity

The limiting quantity for applying I-DNA1 was determined by Odriozola et al. [4]. Analyses of 100, 50 and 25 pg of DNA using I-DNA2 were performed (ESM Fig. 3). The smallest quantity of DNA that enables complete profiles with I-DNA2 to be obtained is 100 pg, similar to that required by I-DNA1 [4]. In comparison, other multiplex systems such Minifiler™ Kit PCR (Applied Biosystems) [13] and NGM™ (Applied Biosystems) [14] require 125 pg of DNA to obtain full profiles. Otherwise, higher sensitivities were observed in PowerPlex® ESX and Powerplex® ESI (Promega Corporation) [15, 16] with a sensitivity of 62.5 pg.

Determination of stutter percentage and heterozygous PHR

The results of the determination of stutter percentage for each locus analysed by I-DNA2 in 162 Caucasoid individuals are shown in ESM Table 1. The lowest average value is in TH01 (4.3±1.6 and 4.0±2.1, respectively), whilst the highest is in D21S11 (12.9±2.9 and 11.4±2.2, respectively). I-DNA2 shows slightly higher values than I-DNA1 in CSF1PO (10.6%±2.2 and 6.3%±2.6, respectively) and FGA (12.1%± 3.0and 8.4%±2.0, respectively) [4].

PHR was calculated by analysing 600 individuals (ESM Table 2). Low standard deviations and average values very close to the mean observed in I-DNA1 were also found for all the loci analysed [4] when I-DNA2 was applied. Likewise, I-DNA2 shows an average PHR of 87%, similar to that reported for I-DNA1 [4].

In conclusion, I-DNADuo has shown itself to be a highly sensitive, robust multiplex system for obtaining individual genetic profiles and for detecting and preventing allelic dropouts. Moreover, I-DNADuo saves on performance cost and time because I-DNA1 and I-DNA2 can be used simultaneously in the same thermocycler. In short, I-DNADuo is a highly useful tool for human identification and for analysing the STR loci included in most databases, especially those in CODIS, ISSL and ECL.

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