Evaluation of the iPLEX(®) Sample ID Plus Panel designed for the Sequenom MassARRAY(®) system. A SNP typing assay developed for human identification and sample tracking based on the SNPforID panel

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Evaluation of the iPLEX® Sample ID Plus Panel designed for the Sequenom MassARRAY® system. A SNP typing assay developed for human identification and sample tracking based on the SNPforID panel

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A B S T R A C T

Sequenom launched the first commercial SNP typing kit for human identification, named the iPLEX® Sample ID Plus Panel. The kit amplifies 47 of the 52 SNPs in the SNPforID panel, amelogenin and two Y-chromosome SNPs in one multiplex PCR. The SNPs were analyzed by single base extension (SBE) and Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS).

In this study, we evaluated the accuracy and sensitivity of the iPLEX® Sample ID Plus Panel by comparing the typing results of the iPLEX® Sample ID Plus Panel with those obtained with our ISO 17025 accredited SNPforID assay. The average call rate for duplicate typing of any one SNPs in the panel was 90.0% when the mass spectra were analyzed automatically with the MassARRAY® TYPER 4.0 genotyping software in real time. Two reproducible inconsistencies were observed (error rate: 0.05%) at two different SNP loci. In addition, four inconsistencies were observed once. The optimal amount of template DNA in the PCR was ≥10 ng. There was a relatively high risk of allele and locus drop-outs when ≤1 ng template DNA was used. We developed an R script with a stringent set of "forensic analysis parameters" based on the peak height and the signal to noise data exported from the TYPER 4.0 software. With the forensic analysis parameters, all inconsistencies were eliminated in reactions with ≥10 ng DNA. However, the average call rate decreased to 69.9%.

The iPLEX® Sample ID Plus Panel was tested on 10 degraded samples from forensic case-work. Two samples could not be typed, presumably because the samples contained PCR and SBE inhibitors. The average call rate was generally lower for degraded DNA samples and the number of inconsistencies higher than for pristine DNA. However, none of the inconsistencies were reproduced and the highest match probability for the degraded samples typed with the panel was $1.7E-9$ using the stringent forensic analysis parameters.

Although the relatively low sensitivity of the iPLEX® Sample ID Plus Panel makes it inappropriate for typing of trace samples from crime scenes, the panel may be interesting for relationship testing and for identification of e.g. samples in biobanks because of the low reagent costs, the limited hands-on time of the iPLEX® assay and the automatic analysis of the mass spectra.

1. Introduction

SNPs have attracted a lot of attention within the forensic community in the last decade. Several SNP panels have been developed for human identification and relationship testing [1–7] and recently, SNP panels have also been suggested for forensic phenotyping purposes [8–11]. Assays were developed for these panels that involved a large multiplex PCR and a large single base extension (SBE) multiplex. The SBE products were subsequently detected by capillary electrophoresis (CE). PCR and CE are widely used in modern forensic genetic laboratories. Thus, PCR–SBE–CE assays were easily implemented and also validated for case work in some laboratories [12–15]. However, analyses of the electropherograms were challenging because the SBE products from the same locus were detected at different spectral wavelengths and appeared in different dye windows of the electropherogram, and because the strengths of the fluorophore emissions from dyes used in the SBE reaction were unbalanced. Consequently, the peak height of one allele may be up to six times higher than the peak height of the other allele in the same locus [12,16]. Furthermore,

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small sized peaks from PCR products or PCR primers extended with ddNTPs in the SBE reaction were often detected and these products may be misinterpreted as true alleles [17,18].

The SNPforID consortium (www.snpforid.org) developed a PCR–SBE–CE assay for human identification that made it possible to amplify 52 autosomal SNPs from diminutive amounts of genomic DNA [1]. An optimized version of this assay with 49 of the 52 SNPs was accredited according to the ISO 17025 standard in 2007 [12,16]. The SNPforID panel was used by Applied Biosystems to develop the GenPlex™ HID system that amplified 48 of the 52 SNPs and amelogenin. This assay was tested by the forensic community as a possible alternative to the PCR–SBE–CE assay [19–21]. However, a commercial forensic kit was never made available.

In 2012, Sequenom launched the iPLEX® Sample ID Plus Panel that amplifies 47 of the 52 SNPs in the SNPforID multiplex, amelogenin and two Y-chromosome markers, XKRY-1 and HFSY-2. The iPLEX® protocol involves a large multiplex PCR, a large multiplex SBE reaction and detection of the SBE products by Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). The protocol requires very little hands-on time. All reactions (PCR, Shrimp Alkaline Phosphatase (SAP) treatment, SBE and ion-exchange) are conducted in the same plate up to the point in the protocol, where the SBE products are automatically transferred to the SpectroCHIP® MALDI-TOF MS target. The mass spectra are analyzed and the SNP calls evaluated in real-time by the TYPER software on the MassARRAY® analyzer system. Furthermore, quality assurance (QA) markers in the PCR mix, the SAP mix and the SBE mix are used as internal controls for the different pipetting steps. The QA markers are targets for an SBE primer and three equally sized peaks from the extension products are used as an indication of a successful experiment.

In this work, we evaluated the iPLEX® Sample ID Plus Panel by comparing the SNP typing results with those obtained with an ISO 17025 accredited PCR–SBE–CE protocol [12].

2. Materials and methods

2.1. Samples, DNA purification and DNA quantification

A total of 94 samples from individuals in relationship cases were selected. All samples were previously typed with the SNPforID multiplex assay [12] as part of the case work investigation. DNA was purified from 200 μL of blood using the QIAamp DNA blood mini kit (Qiagen) as recommended by the manufacturer. DNA was eluted in 50 μL of AE buffer.

Seven degraded crime case samples were selected based on STR typing results from the case work investigation. For three of these samples, DNA was extracted from two different areas of the sample material and both preparations were investigated. The crime case samples were purified either by a standard phenol/chloroform extraction protocol [22] or by chelex-100 resin [23]. All DNA concentrations were determined by real-time PCR using the Quantifiler™ Human DNA Quantification kit (Life technologies-Applied Biosystems, LT-AB) on an AB 7900 (LT-AB) according to the manufacturer's recommendations. The work was approved by the Danish ethical committee (H-1-2011-081).

2.2. SNP typing

Samples were genotyped using the iPLEX® Sample ID Plus Panel (Sequenom). The PCR contained: 0.8 μL H_2O, 0.5 μL PCR buffer (20 mM $MgCl_2$), 0.4 μL 25 mM $MgCl_2$, 0.1 μL 25 mM dNTP mix, 1 μL forward/reverse primer mix (500 nM each), 0.5 μL QC competitor, 0.5 μL QA spike, 0.2 μL PCR enzyme and 1 μL sample DNA. The PCR was performed in an GeneAmp® PCR system 9700 thermal cycler (LT-AB) with the following conditions: denaturation at 94 °C for 2 min followed by 45 cycles of 94 °C for 20 s, 62 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 3 min. The PCR products were treated with a cocktail of 1.53 μL H_2O, 0.17 μL 10× SAP Buffer and 0.3 μL Shrimp Alkaline Phosphatase (1.7 U/μL) (SAP) (Sequenom) in a GeneAmp® PCR system 9700 thermal cycler (LT-AB) at 37 °C for 40 min followed by 85 °C for 5 min. The SBE reaction contained 7 μL SAP treated PCR products and 2 μL iPLEX® pro mix (Sequenom). The iPLEX® pro mix contained 0.2 μL 10× iPLEX® pro buffer plus, 0.2 μL iPLEX® pro Termination mix, 0.94 μL primer mix (0.74–1.46 μM, Sequenom), 0.041 μL iPLEX®-enzyme, 0.5 μL EXT QA spike, and 0.119 μL H_2O. The SBE reaction was performed on a GeneAmp® PCR system 9700 thermal cycler (LT-AB) with the following conditions: denaturation at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 52 °C for 5 s, 80 °C for 5 s, 52 °C for 5 s, 80 °C for 5 s, 52 °C for 5 s, 80 °C for 5 s, 52 °C for 5 s, 80 °C for 5 s, 52 °C for 5 s, 80 °C for 5 s, followed by 72 °C for 3 min. A total of 41 μL of molecular grade water and ion exchange resin (Sequenom) was added to each sample.

Samples were rotated for approximately 5 min on a tube rotator (VWR) and centrifuged at 3600 rpm for 5 min. All SBE products were spotted twice on the SpectroCHIP array (Sequenom) using the RS1000 nanospotter (Sequenom). Results were visualized on the MassARRAY® analyzer 4 system (Sequenom) using the autorun settings. All samples were typed in duplicate. There were no significant difference between the call rates from the two spots (supplementary table S1, $p = 0.11$). All analyses were performed with the results from the first spot from each sample.

2.3. Data analysis

The MassArray TYPER 4.0 genotyping software analyzed the results in real time using a Gaussian mixture model for cluster analyses. The credibility of the SNP calls were evaluated as a posterior probability using a non-disclosed formula in the MassArray TYPER 4.0 software and the SNP calls were divided into 3 groups; Conservative, Moderate and Aggressive genotype calls. A forth group named Low Probability SNP calls contained genotypes with skewed allele balances or low signal to noise ratios. The Low Probability SNP calls were not accepted as genuine SNP genotypes by the TYPER 4.0 genotyping software. If no extended SBE primers were detected at a locus, the genotype call was categorized as No alleles.

For further analyses of the data, the Plate Data File with signal to noise ratios (SNR) and peak heights were exported from TYPER 4.0 and imported into the statistical computing software R v.2.11.0 (http://CRAN.R-project.org/doc/FAQ/R-FAQ.html; ISBN 3-900051-08-9). The allele balance (AB) was calculated in R as AB = (height of allele 1 − height of allele 2)/(height of allele 1 + height of allele 2). A set of "forensic analysis parameters" for analysis were defined; peak height > 1.5, SNR > 5 and |AB| > 0.8 for homozygotes and |AB| < 0.2 for heterozygotes. |AB| > 0.8 translates to a genotype call where the peak height of one allele was at least 9 times higher than the peak height of the other allele. |AB| < 0.2 translates to a heterozygous genotype call where the peak height of one allele was maximally 1.5 times the peak height of the other allele. The signal from the G allele of the SNP rs2111980 was weak (supplementary Fig. S1). For rs2111980, |AB| > 0.9 for homozygotes and 0.3 < AB < 0.7 for heterozygotes. The heterozygous genotype calls of rs2111980 were manually changed and marked as a user defined call.

3. Results

The iPLEX® Sample ID Plus Panel was evaluated by duplicate typing of purified DNA from 94 individuals. Two experiments were performed independently of each other on two different days. Two

of the 94 samples were only typed once. In one of the experiments, the SAP quality assurance marker in one sample was not detected and the spotting of another sample failed completely. The two samples were subsequently excluded from the analyses described below.

The results were analyzed using the MassArray TYPER 4.0 genotyping software in real-time mode. The average call rate for duplicate typing of any one SNP in the iPLEX® Sample ID Plus Panel was 90.0% (for details on each SNP, see supplementary Table S2), 6.5% were typed successfully once and 3.5% were not typed in either experiment. Only one discrepancy between the first and second experiment was observed (in rs873196). The genotyping successes of three SNPs were poor. The SNP rs735155 was only typed in duplicate in one individual (see below) and the call rates for duplicate typing of rs1029047 and rs1031825 were only 57% and 54%, respectively. Without these three SNPs, the average call rate for duplicate typing of any SNP in the panel would be 93.5%.

The signal from the rs2111980 G allele was weaker than the signal from the A allele (supplementary Fig. S1) which resulted in a peak height ratio of approximately 3:1 (A:G) for heterozygous individuals. The TYPER 4.0 software did not automatically accept heterozygous allele calls for rs2111980 as genuine and thus, the heterozygous genotype calls were manually changed and marked as user defined allele calls (Fig. 1).

The consensus SNP profiles generated from duplicate typing with the iPLEX® Sample ID Plus Panel were compared to those obtained with an ISO 17025 accredited investigation of the same SNPs [12]. Two reproduced inconsistencies were detected; one in the rs717302 locus and one in the rs735155 locus (observed in two different individuals).

Furthermore, one inconsistency was observed in the rs873196 locus in one of the two experiments, and three inconsistencies were observed (two in rs717302 and one in rs1031825) in loci where the second experiment did not generate any result. Six of the eight inconsistencies were allele drop-outs and two were allele drop-ins (both in rs735155). Three of the eight inconsistencies were detected in the three poorly performing SNPs, rs735155, rs1029047 and rs1031825, and four of the remaining five inconsistencies were found in rs717302.

The typing results of the sex markers AMEL, XKRY-1 and HFSY-2 matched the known gender of all 94 individuals.

3.1. Forensic analysis parameters

The distribution of the five different genotype categories (Conservative, Moderate, Aggressive, Low-probability and No-alleles) reported by the TYPER 4.0 software is shown in Fig. 1A. The peak height, signal to noise and allele balance are clearly important parameters for the evaluation of the typing result.

The genotypes of the eight inconsistencies were defined as Conservative (2), Moderate (5) and Aggressive (1). We decided to develop a stringent set of "forensic analysis parameters" based on the peak height and the signal to noise data exported from the TYPER 4.0 software. This way of analyzing the data eliminated all inconsistencies. The R script is available in supplementary Table S3. The minimum requirements for peak height, signal to noise and allele balance may be altered by the user. Also, individual requirements for a given locus may be defined, e.g. for the rs2111980 locus, where the allele balance was approximately 3:1,
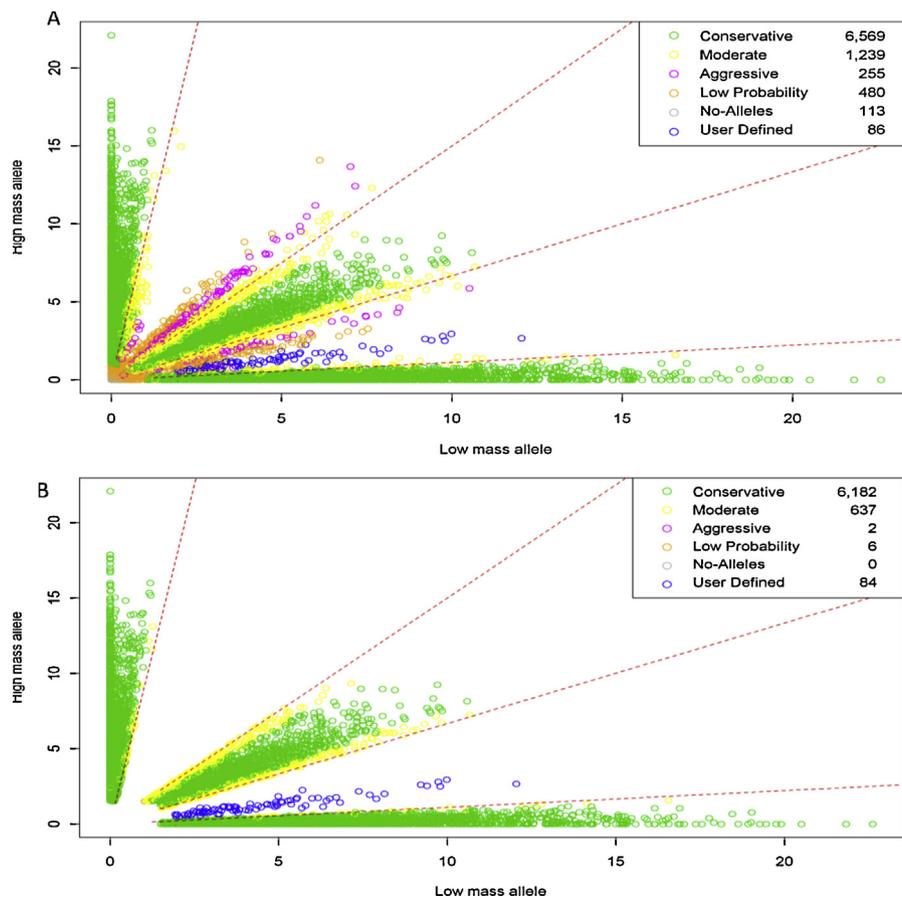
Fig. 1. Distribution of genotype calls. (A) TYPER 4.0 software analysis parameters. (B) Forensic analysis parameters. Red lines represent the ratio restrictions used for the forensic analysis parameters (homozygote allele balance (AB) 0.8 and heterozygote AB 0.2).

Table 1
The effect of different analysis parameters on the call rate and the distribution of genotype calls.

Call	TYPER 4.0 calls	SNR > 5	Peak height > 1.5	SNR > 5 and peak height > 1.5	Allele balance	Forensic analysis parameters
Conservative	13,155	−75	−647	−663	−120	−754 (5.7%)
Moderate	2519	−87	−271	−280	−984	−1215 (48.2%)
Aggressive	519	−30	−77	−82	−493	−517 (99.6%)
Low probability	932	−490	−534	−549	−678	−924 (99.1%)
No-alleles	235	−235	−235	−235	−235	−235 (100%)
User defined	171	0	−7	−7	0	−7 (4.1%)

the acceptable allele balance for heterozygotes was set to 0.3–0.7 (see Section 2).

The distribution of genotype categories after analysis with the forensic analysis parameters is shown in Fig. 1B. The effect of the individual parameters is shown in Table 1.

The numbers of acceptable Moderate and Aggressive calls were reduced considerably mainly by the more stringent requirements on the allele balance. Some Conservative calls were also eliminated, mostly by the increased peak height requirement. The average call rate for duplicate typing of any one SNP in the iPLEX® Sample ID Plus Panel with the forensic analysis parameters was 69.9% (for details on each SNP, see supplementary Table S2). The call rates of five SNPs (rs1029047, rs1490413, rs717302, rs733164 and rs737681) were reduced by more than half when the forensic analysis parameters were used.

3.2. Sensitivity of the iPLEX® Sample ID Plus Panel

The sensitivity of the iPLEX® Sample ID Plus Panel was tested by typing five samples in seven different dilutions (40–0.625 ng). The average call rates of the analyzed results using either the TYPER 4.0 software or the forensic analysis parameters are shown in Fig. 2. As expected, the average call rates were lower when the forensic analysis parameters were used. The optimal amount of DNA in the PCR seemed to be ≥10 ng. Fourteen inconsistencies were observed between SNP profiles analyzed with the TYPER 4.0 software and the SNP profiles generated with the ISO 17025 accredited investigation [12]. All the inconsistencies were detected in the experiments where 1.25 ng or 0.625 ng DNA

was used in the PCR. Twelve of the inconsistencies were allele drop-outs and two were allele drop-ins. When the forensic analysis parameters were used, four inconsistencies remained. All of them were allele drop-outs.
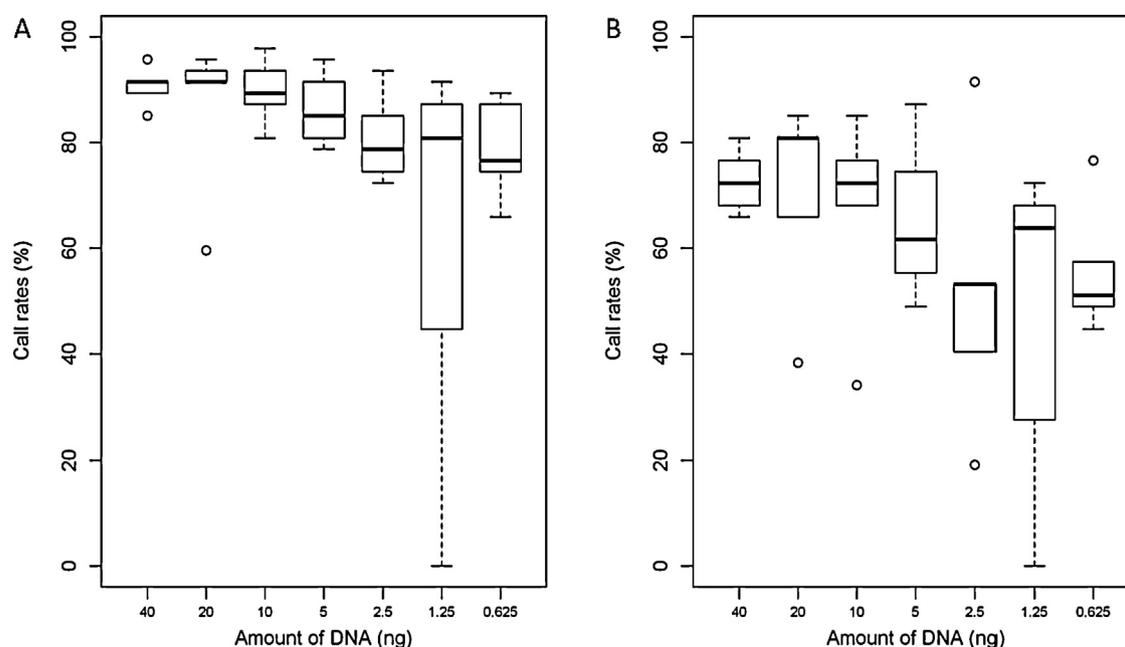
3.3. Typing of degraded DNA

Seven crime case samples were selected for duplicate typing with the iPLEX® Sample ID Plus Panel. For three of these samples, DNA was extracted from two different areas of the sample material and both preparations were investigated.

The samples were previously typed with the AmpFℓSTR® SEfiler Plus™ PCR amplification kit using the validated protocol for crime case samples [24] and partial STR profiles were obtained in all samples except for sample 8 (Table 2) [25]. Complete SNP profiles were obtained with the SNPforID PCR–SBE–CE assay [25].

The call rates for the duplicate typing using either the TYPER 4.0 software or the forensic analysis parameters are shown in Table 2 and the SNP profiles from the individual experiments are shown in supplementary Tables S4 and S5. As expected, the call rates were generally lower than those obtained with pristine DNA samples. Nevertheless, the match probabilities for the consensus profiles based on the forensic analysis parameters ranged from 1.7E−09 (sample 1) to 1.2E−16 (sample 6).

No reproducible results were obtained for sample 2 and 8 even though the samples were investigated four times. Partial SNP profiles were obtained once but the result could not be repeated. Interestingly, the signals from the QA markers were very weak in the experiments where no signals were detected,

Fig. 2. Sensitivity study. Call rates with the TYPER 4.0 software (A) and the forensic analysis parameters (B) using seven different dilutions of DNA (40–0.625 ng).

Table 2
Call rates of SNP and STR typing of degraded DNA.

Sample	Sample material	Call rate		
		AmpFℓSTR® SEfiler Plus™	TYPER 4.0	Forensic analysis parameters
1	FFPE[†] tissue[‡]	33%	81%	53%
2	FFPE[†] tissue[^]	66%	0	0
3	Nail[‡]	66%	60%	45%
4	Nail[§]	25%	77%	60%
5	Cigaret bud	92%	64%	53%
6	Cigaret bud	92%	89%	81%
7	Blood	50%	74%	60%
8	Muscle[§,^]	100%	0	0
9	Blood[‡]	75%	53%	45%
10	Blood	66%	79%	62%

[^] Partial SNP profiles were obtained in one out of four experiments.
[§] The internal PCR control in the Quantifiler® kit was partially inhibited.
[†] FFPE = formalin fixed paraffin embedded.
[‡] Used in a GenPlex™ HID system interlaboratory exercise [20].

which indicated that the SBE reactions were inhibited. The internal PCR control was inhibited in the real-time quantification reaction of sample 8 (data not shown) which confirmed the presence of PCR inhibitors in the DNA preparation, but no inhibition was observed for sample 2.

Twelve inconsistencies were observed between the SNP profiles analyzed with the TYPER 4.0 software and the SNP profiles generated with the SNP*for*ID PCR–SBE–CE assay [25]. They were detected in 11 different loci and none of them were reproduced (supplementary Table S4). Seven of the inconsistencies were observed in loci where the second experiment did not generate a result. All the inconsistencies were allele drop-outs. Four inconsistencies remained after the iPLEX® Sample ID Plus Panel results were analyzed with the forensic analysis parameters.

4. Discussion

Although the forensic community has invested a lot of research in the possible use of SNPs for forensic genetic testing, few laboratories have implemented or validated SNP typing assays [12–15]. The widely used PCR–SBE–CE assay has proved to be an excellent research tool. However, as a routine investigation in a forensic genetic context, the assay is deselected in favor of the constantly improving commercial STR kits. There are presently no commercial PCR–SBE–CE kits for forensic genetic testing. Thus, all maintenance of the assay including tests of new primer stocks, test and validation of each new primer mix etc. must be performed in-house. Also, there is no commercial software that can analyze the unbalanced signals with different fluorophores efficiently, or employ the locus specific guidelines that are important for standardization of the analyses [12] and for detection of mixtures [15].

These inconveniences were overcome with the iPLEX® Sample ID Plus Panel. All reactions in the iPLEX® protocol were performed in the same plate with little hands-on time, the peaks from the two alleles in each locus were of almost equal size and the analyses were performed automatically by the TYPER 4.0 software. The evaluation of the iPLEX® Sample ID Plus Panel described in this work showed that both pristine and degraded crime case samples may be typed accurately with the assay. Clearly, the call rates of some of the SNPs were not impressive. In particular, the rs1029047, rs1031825, rs1490413, rs717302, rs733164, rs735155 and rs737681 loci should either be removed from the panel or the individual SNP assays should be redesigned and optimized. The QA markers were a valuable addition to the assay and may work as an internal control for SBE (and PCR) inhibition. The QA markers required manual inspection, which seemed

awkward when they might as well be analyzed automatically with the TYPER 4.0 software.

The inconsistencies observed between the iPLEX® Sample ID Plus Panel profiles and the SNP profiles generated with the SNP*for*ID PCR–SBE–CE assay were a matter of grave concern. We developed a set of forensic analysis parameters and re-analyzed the data in the statistical software R using an in-house developed script (supplementary Table S3). The forensic analysis parameters were more stringent than the ones used by the TYPER 4.0 software and the majority of the inconsistencies were eliminated. However, the forensic analysis parameters reduced the average call rate considerably (from 90% to 69.9%). For some loci, the call rate was more than halved and the individual SNP assays for these loci should be optimized (see above).

High sensitivity is pivotal for crime case investigations because the trace samples collected from crime scenes often contain very little DNA (<1 ng). The iPLEX® Sample ID Plus Panel required ≥10 ng for optimal performance. This is 50–100 times more than the amount of DNA typically used in the PCR of forensic genetic STR kits. Furthermore, allele drop-outs seemed to occur frequently when the PCR was set up with 1 ng or less. Therefore, the iPLEX® Sample ID Plus Panel is not attractive for crime case investigations in its current form. However, it may be useful for relationship testing, especially when the costs of the assay are taken into consideration. The list-price of the iPLEX® Sample ID Plus Panel is 7 Euro/sample (including all reagents and the SpectroCHIP® MALDI-TOF MS target). This is one third of the list-price of the widely used AmpFℓSTR® NGM SElect™ (AB-LT) STR typing kit (not including the cost of the CE). Furthermore, MALDI-TOF MS platforms require less maintenance than the CE-platforms used in most forensic genetic laboratories. For the same reasons, the iPLEX® Sample ID Plus Panel may be a fast and cost-effective method for sample tracking of e.g. frequently used human cell lines, hospital or biobank samples, etc., where the amount of sample material is less of a concern.

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.2013.04.009.

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