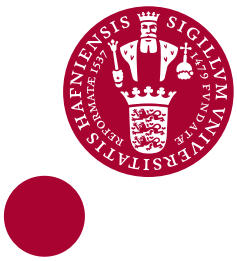

Forensic genetic SNP typing of low-template DNA and highly degraded DNA from crime case samples

Børsting, Claus; Mogensen, Helle Smidt; Morling, Niels

Published in:
Forensic science international. Genetics

DOI:
10.1016/j.fsigen.2013.02.004

Publication date:
2013

Forensic Science International: Genetics 7 (2013) 345–352

Contents lists available at SciVerse ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Forensic genetic SNP typing of low-template DNA and highly degraded DNA from crime case samples

Claus Børsting *, Helle Smidt Mogensen, Niels Morling

Section of Forensic Genetics, Department of Forensic Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Frederik V's Vej 11, DK-2100, Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 9 August 2012
Received in revised form 8 November 2012
Accepted 12 February 2013

Keywords:
Crime case work
Low-template DNA
Degraded DNA
Single nucleotide polymorphisms
SNP*for*ID

ABSTRACT

Heterozygote imbalances leading to allele drop-outs and disproportionally large stutters leading to allele drop-ins are known stochastic phenomena related to STR typing of low-template DNA (LtDNA). The large stutters and the many drop-ins in typical STR stutter positions are artifacts from the PCR amplification of tandem repeats. These artifacts may be avoided by typing bi-allelic markers instead of STRs. In this work, the SNP*for*ID multiplex assay was used to type LtDNA. A sensitized SNP typing protocol was introduced, that increased signal strengths without increasing noise and without affecting the heterozygote balance. Allele drop-ins were only observed in experiments with 25 pg of DNA and not in experiments with 50 and 100 pg of DNA. The allele drop-in rate in the 25 pg experiments was 0.06% or 100 times lower than what was previously reported for STR typing of LtDNA. A composite model and two different consensus models were used to interpret the SNP data. Correct profiles with 42–49 SNPs were generated from the 50 and 100 pg experiments, whereas a few incorrect genotypes were included in the generated profiles from the 25 pg experiments. With the strict consensus model, between 35 and 48 SNPs were correctly typed in the 25 pg experiments and only one allele drop-out (error rate: 0.07%) was observed in the consensus profiles.

A total of 28 crime case samples were selected for typing with the sensitized SNP*for*ID protocol. The samples were previously typed with old STR kits during the crime case investigation and only partial profiles (0–6 STRs) were obtained. Eleven of the samples could not be quantified with the Quantifiler™ Human DNA Quantification kit because of partial or complete inhibition of the PCR. For eight of these samples, SNP typing was only possible when the buffer and DNA polymerase used in the original protocol was replaced with the AmpFℓSTR® SEfiler Plus™ Master Mix, which was developed specifically for challenging forensic samples. All the crime case samples were successfully typed with the SNP*for*ID multiplex assay and the match probabilities ranged from 1.1×10^{-15} to 7.9×10^{-23}. In comparison, four of the samples could not be typed with the AmpFℓSTR® SEfiler Plus™ kit and the match probabilities were higher than 10^{-7} for another six samples.

1. Introduction

Increasing the typing success of challenging samples is an essential part of all forensic genetic research. The sensitivity of typing assays and the ability to type highly degraded DNA samples is constantly being improved, and today, low-template DNA (LtDNA) analyses [1,2] and assays specifically designed to type highly degraded DNA [3–9] are used for forensic genetic case work by many laboratories.

LtDNA typing is complicated by the occurrence of stochastic phenomena that results in skewed amplification of alleles and loci. The result is frequent heterozygote imbalances and, in the extreme situation, allelic or locus drop-out. The number of drop-outs may be reduced by increasing the sensitivity of the typing assays. Different methods have been used for LtDNA typing of STRs: (1) increased number of PCR cycles (usually from 28 to 34 PCR cycles), or (2) increased number of analyzed PCR products by adding more PCR products, increasing the injection time and/or the injection voltage of the capillary electrophoresis instrument, or (3) post-PCR purification of the PCR products [10–18]. However, the sensitizing methods had two major drawbacks: (1) the signal from stutters increased and (2) the number of drop-in alleles (any identified allele that is not present in the original sample DNA) increased dramatically from approximately zero with the standard protocol to 1–3% of the approved alleles with the sensitized protocols. The majority of drop-in alleles were

* Corresponding author. Tel.: +45 3532 6225; fax: +45 3532 6289.
E-mail address: claus.boersting@forensic.ku.dk (C. Børsting).

1872-4973/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsigen.2013.02.004

identified in the typical stutter positions. This indicated that the drop-ins were generated by the PCR and were not a result of contamination. By typing the samples two or more times and interpreting the combined results by various consensus models, the number of drop-outs and drop-ins in the generated sample profile were reduced, but not eliminated [10,12,15,16,18]. As a consequence, a "statistical" approach was developed to compensate for the stochastic phenomena of LtDNA analyses [2]. Drop-out and drop-in probabilities were introduced in the calculation of the statistical weight of the evidence, which linked conventional DNA and LtDNA typing in an elegant way and eliminated the need for an "LtDNA threshold", which was very difficult to define. The challenges of the statistical approach were to estimate the drop-out and drop-in probabilities of the sample, locus, or allele under investigation, and although different methods have been tested [19–22], it remains uncertain how these probabilities should be estimated for real case work samples.

The increased sizes of stutters and the increased number of drop-ins observed with the sensitized protocols are phenomena related specifically to the PCR amplification of tandem repeats. Typing of other loci such as single nucleotide polymorphisms (SNPs) and insertion/deletions (indels) are not likely to generate high numbers of PCR artifacts, which should make SNPs and indels more suitable for sensitized LtDNA protocols.

The SNP*for*ID multiplex assay [4] was validated for relationship testing in our ISO17025 accredited laboratory in 2007 [23]. The assay proved to be a valuable supplement to STR typing in cases, where the conclusions based on STRs were ambiguous [24–32]. All 49 SNP loci were amplified in one PCR reaction and the SNPs were detected by two single base extension (SBE) reactions and capillary electrophoresis [4,23,32]. The amplicon lengths ranged from 59 to 115 bps and 38 of the amplicons were shorter than 100 bps. Locus specific guidelines for data analysis based on the peak height(s) of the detected allele(s) were developed during the validation of the SNP*for*ID assay [23]. The guidelines ensured a clear and quantitative distinction between heterozygous and homozygous allele calls and made it easy to identify unusual genotype calls which allowed the analyst to focus on these individual results. Furthermore, they ensured consistency in the data analysis and simplified training of new analysts. Finally, the guidelines made it possible to identify mixtures or contaminated samples [29].

Here, a sensitive SNP typing protocol that increases SBE signal strengths without increasing noise and without affecting the heterozygote balance is introduced. The protocol allows SNP typing of LtDNA with high accuracy and improves the DNA typing success of challenging crime case samples.

2. Materials and methods

2.1. Samples and DNA preparations

For the LtDNA experiments, whole blood samples from 10 Danish individuals and six commonly used reference DNA samples were selected. DNA was extracted from 200 μl whole blood using the the QIAamp DNA blood mini kit (Qiagen). The reference DNA samples were 9947a, 9948a and AmpFℓSTR male control DNA 007 from the AmpFℓSTR PCR amplification kits (LT-AB), the K562 leukaemia cell line (Promega), the male control DNA XY1 (Biotype) and the female control DNA XX74 (Biotype) [33].

A total of 28 crime case samples were selected based on previous STR typing results (Supplementary Table S1). For twelve of these samples, DNA was extracted from two different areas of the sample material and both preparations were investigated. DNA was extracted from the crime case samples either by a standard phenol/chloroform extraction protocol [34] or by chelex-100 resin [35].

2.2. Quantification

All DNA concentrations were determined by real-time PCR using the Quantifiler™ Human DNA Quantification kit (LT-AB) on an AB 7900 (LT-AB) according to the manufacturer's recommendations.

For the LtDNA experiments, quantification was performed in duplicate on the original DNA preparation. The DNA was subsequently diluted to a concentration of 100 pg/μl and quantified again in duplicate. The average concentration of the diluted DNA was 106 pg/μl (range: 63–144 pg/μl).

For the crime case samples, the final dilution of the DNA preparation that could be typed with the SNP*for*ID multiplex was quantified in duplicate (Supplementary Table S1).

2.3. STR typing

STR typing of the crime case samples was performed using the AmpFℓSTR® SGM Plus, AmpFℓSTR® Identifiler or the AmpFℓSTR® SEfiler Plus kits (LT-AB). PCR was performed according to the manufacturer's recommendations using 28, 28 and 30 cycles in the PCR, respectively. PCR reactions were performed in a GeneAmp® PCR system 9700 thermal cycler (LT-AB). A total of 1.5 μl PCR product was mixed with 15 μl Hi-Di™ formamide (LT-AB) and 0.1 μl GeneScan™ 400 HD ROX™ size standard (LT-AB) or 0.3 μl GeneScan™ 500 HD Liz® size standard (LT-AB). Analyses of the amplified PCR products were performed with an ABI 3130*xl* Genetic Analyzer (LT-AB) with 36 cm capillary arrays, POP-4 polymer, and 6 or 20 s injections at 3000 V (LT-AB). Data were analyzed independently by two analysts using GeneScan® analysis software v. 3.7 and Genotyper® analysis software v. 3.7 (LT-AB), and the results were compared. The minimum peak height was set to 50 relative fluorescence units (RFUs) for all dyes. The AmpFℓSTR® SEfiler Plus kit was used for all crime case investigations in our laboratory up to November 2011 [36].

2.4. SNP typing

SNP typing was performed as previously described [23,32] except for two changes in the protocol. For LtDNA typing, the SBE reactions were performed with 100 cycles instead of 30 cycles. For typing of some of the crime case samples (Supplementary Table S1), PCR was performed in 25 μl reactions containing 1–4 μl DNA extract, 10 μl AmpFℓSTR® SEfiler Plus™ Master Mix (LT-AB), 8 mM $MgCl_2$, 700 μM of each dNTP and 0.01–0.17 μM of each primer (DNA Technology). If the signal strength from the crime case samples was low, SBE reactions were performed with 100 cycles. PCR and SBE reactions were performed in a GeneAmp® PCR system 9700 thermal cycler (LT-AB). Two μl SBE products were mixed with 20 μl Hi-Di formamide (LT-AB) and 0.1 μL GeneScan™ 120 Liz® size standard (LT-AB). The SBE products were analyzed by capillary electrophoresis using 3130*xl* Genetic Analyzers (LT-AB) with 36 cm capillary arrays and POP-4 polymer (LT-AB) as previously described [23,32]. Every genotype call was evaluated based on the peak height(s) of the allele(s) according to pre-defined guidelines for allele calling [23]. Genotype calls that did not fulfill the guidelines for allele calling were scrutinized and evaluated individually. If the peak height was less than 300 RFU, the result was only accepted if the noise in that part of the electropherogram was less than 50 RFU. Heterozygous allele calls were never accepted if the peak height ratio was more than two times higher than the maximum or less than half of the minimum value of the pre-defined interval for heterozygous allele calls [23].

2.5. Interpretation models

Sample profiles were generated using a composite model and two consensus models. Only accepted genotype calls were used to build the profiles. In the composite model, the generated profiles included all detected alleles ($x = 1$, $n = 4$, where x = minimum number of observations of an allele and n = number of experiments). In the relaxed consensus models, the generated profiles included all alleles that were detected at least twice ($x = 2$, $n = 4$). If one allele was detected 2, 3 or 4 times and the second allele only once, the results for that locus were not included in the consensus profile. In the strict consensus model, the profiles were generated as above for the relaxed consensus model with one addition. Homozygous genotypes were only included in the consensus profile if homozygous genotype calls were observed at least three times ($x = 3$, $n = 4$).

3. Results

3.1. Increasing the sensitivity of the SNPforID multiplex

The standard conditions of the SNP*for*ID assay were 35 PCR cycles, 30 SBE cycles and 20 s injection at 3 kV [4,23,32], which resemble the sensitized protocols used for STR typing. To increase the sensitivity of the SNP*for*ID assay further, we decided to increase the number of SBE cycles from the standard 30 cycles to 100 cycles. The SBE reaction is a linear amplification of the SBE products and it should not be subjected to stochastic phenomena because the hybridization targets of the SBE primers are amplified PCR products. Therefore, an increase in the number of SBE cycles should increase signals without adverse effects.

100 pg DNA from 16 samples were amplified in duplicate with the SNP*for*ID multiplex PCR. The PCR products were subsequently used in SBE reactions with 30 or 100 SBE cycles. In Fig. 1A, the peak heights of the 942 approved homozygous genotype calls were compared. If the peak height >6000 relative fluorescence units (RFUs) with 30 SBE cycles, the average increase in peak height with 100 SBE cycles was only 1.08 ± 0.13. This was not surprising since these genotype calls already approached saturation with 30 SBE cycles. In contrast, the average increase in peak height with 100 cycles was 2.28 ± 0.61 and 2.46 ± 0.59 if the peak height <6000 RFUs and <2500 RFUs, respectively, in the experiment with 30 SBE cycles. The baseline noise did not increase with increasing number of SBE cycles (data not shown).

The average increases in signal ranged from 1.15 to 3.25 for the different loci. Five loci showed average increases in signals close to the highest theoretical increase of 100/30 = 3.33, that may be obtained under the assumption that one PCR product was targeted by one SBE primer per cycle. Three loci showed average increases in signals that were less than 1.5. The differences between the loci could be explained in part by the different concentrations of SBE primers, which may indicate that the amount of SBE primers become limiting for the SBE reaction in the last cycles. However, differences in hybridization efficiency of the SBE primers were also likely to play a role for the locus specific increases in signal strengths.

In Fig. 1B, the peak height ratios of the 632 approved heterozygous genotype calls were compared. The peak height ratios with 30 cycles were very similar to the peak height ratios with 100 cycles (linear regression model: $y = 1.04x + 0.04$, $r^2 = 0.96$). This indicated that the number of SBE cycles may be increased without affecting the heterozygote balance and that the guidelines for data analysis based on the peak height(s) of the detected allele(s) may also be applied in LtDNA analyses.

Table 1
Analysis of LtDNA results.[a]

	Failed allele calls	Allele drop-outs[b]	Allele drop-ins	Locus drop-outs
25 pg	466 (7.4)	199 (8.0)	4 (0.06)	40 (1.28)
50 pg	341 (5.4)	48 (1.93)	0 (0)	1 (0.03)
100 pg	158 (2.5)	3 (0.12)	0 (0)	0 (0)

[a] The results in percent are shown in parentheses.
[b] The percentages were calculated for the heterozygous phenotypes only.

3.2. LtDNA analyses

Four independent experiments with 25 pg, 50 pg and 100 pg DNA from sixteen samples were performed with the sensitized SNP*for*ID multiplex assay. The electropherograms were analyzed according to our validated protocol [23] and the results were compared to the known SNP profiles (Table 1). The typing efficiency and the quality of the phenotyping results clearly

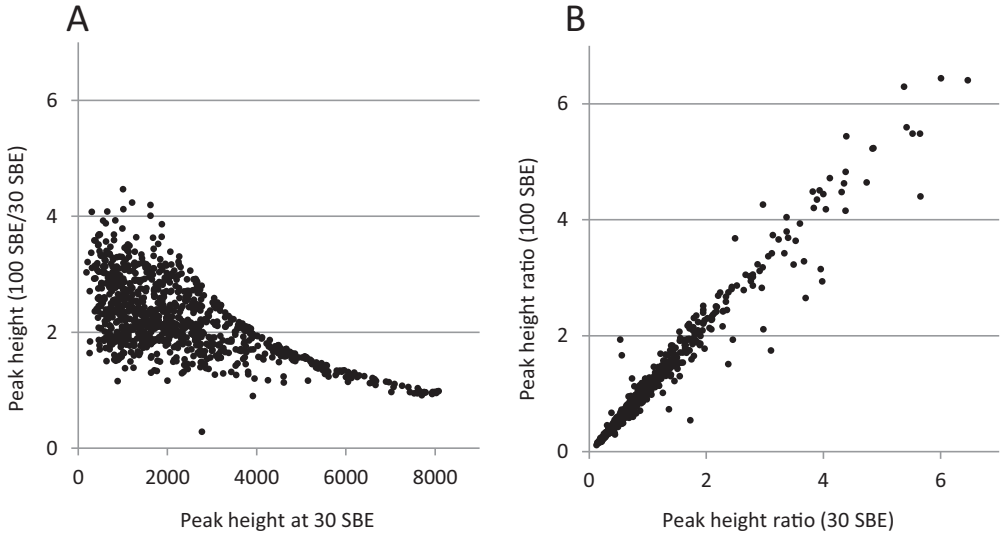

Fig. 1. Results from SNP typing of 16 samples typed in duplicate. A total of 100 pg of DNA was used in the PCR. The peak heights of the approved homozygous allele calls (A) and peak height ratios of heterozygous allele calls (B) from experiments with 30 and 100 SBE cycles were compared.

decreased with decreasing amount of DNA in the PCR. The number of genotype calls that were not approved was tripled and there was a dramatic increase in the number of allele and locus drop-outs. However, it was noteworthy, that the drop-in rate was very low and that drop-ins were only observed in the 25 pg experiments.

Drop-ins were observed four times at four different loci (Supplementary Table S2). Locus drop-outs were observed 40 times at 27 different loci, whereas failed allele calls and allele drop-outs were detected at all loci. Three loci (rs1357617 (A03), rs917118 (A07), rs737681 (A41)) accounted for more than 20% of the failed allele calls (Supplementary Table S2). The SBE primers used for these loci were the three shortest SBE primers used in the SNP*for*ID assay. Their sizes were similar to the sizes of the PCR primers. PCR primers may become extended during the SBE reaction, if the PCR primers are not degraded by Exonuclease I in the PCR clean-up step. This results in higher noise in the part of the electropherogram where the shortest SBE primers are detected, and therefore, the allele calls of the three SNPs failed more often. A total of 19 allele drop-outs were detected at the rs763869 (A08) locus, which is more than at any other locus in the multiplex (the average number was 5.1 per locus). rs763869 is detected as a C/T SNP and the average heterozygote balance is 1 [23]. The high number of allele drop-outs is puzzling, because rs763869 is usually easy to type and no locus drop-out was observed.

Failed allele calls and allele drop-outs were detected in all samples. However, most of the allele drop-outs and 90% of the locus drop-outs were found in the six reference DNA samples (Supplementary Table S3). This is most likely explained by differences in DNA purification methods or DNA solution buffers that may lead to slightly different PCR conditions.

3.3. Interpretation of the LtDNA results

A composite model and two different consensus models were used to interpret the results and the sample profiles were compared to the known SNP profiles (Tables 2–4). Reference profiles, analyzed results for the four experiments, and generated sample profiles are shown in the Supplementary Tables S4–S6.

In the 100 pg experiments, all SNP alleles were detected at least once and since there were no drop-ins, the composite SNP profiles were all complete and correct. Three alleles were only observed once and therefore, two of the SNP profiles generated with the relaxed consensus model were incomplete. When the strict consensus model was applied, seven of the sixteen generated profiles were incomplete, but at least 47 of the 49 SNPs were included in the consensus profiles and they were all typed correctly.

In the 50 pg experiments, four genotype calls in four different loci were failed in all four experiments and thus, four of the composite SNP profiles were incomplete. However, all the composite SNP genotypes were correct. Only five of the consensus SNP profiles were complete and they were all generated with the relaxed consensus model. Nevertheless, even with the strict consensus model, at least 42 of the 49 SNPs were included in the consensus SNP profiles and they were all typed correctly.

In the 25 pg experiments, incorrect genotypes were observed. Seven drop-outs and four drop-ins were included in the composite SNP profiles. The drop-ins were not reproducible and consequently, results for these loci were not included in either of the consensus SNP profiles. Also, the numbers of drop-outs were reduced to four and one when the results were interpreted with the relaxed and strict consensus models, respectively. The rs1024116 allele A from the reference sample 9947a dropped out in all four experiments, which was the only incorrect genotype observed with the strict consensus model. In the 50 pg and 100 pg experiments, the rs1024116 allele A was detected in all experiments and nothing unusual was observed. Allele drop-outs were not especially frequent in the rs1024116 locus, but four locus drop-outs were observed in the 25 pg experiments (Supplementary Table S2), which was the highest number for any SNP in the multiplex. It was also noteworthy that the highest number of locus drop-outs per sample was observed for 9947a (Supplementary Table S3). Between 35 and 48 of the 49 SNPs were included in the consensus SNP profile with the strict consensus model. The match probabilities for these consensus profiles ranged from 2.4×10^{-16} (sample 8) to 5.7×10^{-22} (sample XX74).

3.4. SNP typing of degraded DNA

A total of 28 crime case samples were selected for SNP typing with the SNP*for*ID multiplex assay. The samples were collected from a wide range of materials (Table 5) that are typical for crime case investigations. Samples were only selected if sufficient amounts of human DNA were detected and a series of experiments could be conducted. The samples were typed with either the AmpFℓSTR® SGM Plus® or the AmpFℓSTR® Identifiler® kit during the crime case investigations and only

Table 2
Interpretation of 25 pg experiments.

Sample	Composite model			Relaxed consensus model			Strict consensus model		
	Typed loci	Allele drop-out	Allele drop-in	Typed loci	Allele drop-out	Allele drop-in	Typed loci	Allele drop-out	Allele drop-in
OO7	48/49	1	0	47/49	1	0	43/49	0	0
9947a	48/49	2	1	42/49	1	0	39/49	1	0
9948	49/49	3	2	39/49	2	0	35/49	0	0
K562	48/49	0	0	41/49	0	0	38/49	0	0
XX74	49/49	0	0	47/49	0	0	47/49	0	0
XY1	49/49	0	0	42/49	0	0	37/49	0	0
S1	49/49	0	0	45/49	0	0	42/49	0	0
S2	49/49	0	0	49/49	0	0	48/49	0	0
S3	49/49	0	0	46/49	0	0	45/49	0	0
S4	48/49	0	0	44/49	0	0	43/49	0	0
S5	49/49	0	0	47/49	0	0	46/49	0	0
S6	49/49	0	0	46/49	0	0	42/49	0	0
S7	48/49	1	0	43/49	0	0	42/49	0	0
S8	49/49	0	1	42/49	0	0	42/49	0	0
S9	49/49	0	0	48/49	0	0	48/49	0	0
S10	48/49	0	0	47/49	0	0	46/49	0	0
Total	778/784	7	4	711/784	4	0	682/784	1	0

Table 3
Interpretation of 50 pg experiments.

Sample	Composite model			Relaxed consensus model			Strict consensus model		
	Typed loci	Allele drop-out	Allele drop-in	Typed loci	Allele drop-out	Allele drop-in	Typed loci	Allele drop-out	Allele drop-in
OO7	49/49	0	0	49/49	0	0	48/49	0	0
9947a	48/49	0	0	47/49	0	0	45/49	0	0
9948	48/49	0	0	46/49	0	0	43/49	0	0
K562	49/49	0	0	47/49	0	0	42/49	0	0
XX74	49/49	0	0	48/49	0	0	47/49	0	0
XY1	49/49	0	0	49/49	0	0	48/49	0	0
S1	49/49	0	0	49/49	0	0	47/49	0	0
S2	49/49	0	0	48/49	0	0	45/49	0	0
S3	48/49	0	0	47/49	0	0	47/49	0	0
S4	49/49	0	0	49/49	0	0	47/49	0	0
S5	49/49	0	0	49/49	0	0	48/49	0	0
S6	49/49	0	0	48/49	0	0	47/49	0	0
S7	49/49	0	0	48/49	0	0	47/49	0	0
S8	49/49	0	0	47/49	0	0	47/49	0	0
S9	48/49	0	0	48/49	0	0	48/49	0	0
S10	49/49	0	0	48/49	0	0	48/49	0	0
Total	780/784	0	0	767/784	0	0	744/784	0	0

partial profiles (<6 STRs) were obtained (Supplementary Table S1). For twelve of these samples, DNA was extracted from two different areas of the sample material and both preparations were investigated.

A series of experiments were performed to optimize the reaction conditions for each sample. The sensitized SNP*for*ID protocol with 100 SBE cycles increased the typing success of all the samples by increasing the signal strengths 2–3 times. Seventeen of the samples had to be diluted 10–50 times to obtain results, presumably because the DNA preparation contained PCR inhibitors in the form of highly fragmented DNA, salts, fabric dyes or chemicals. Some of the samples had to be diluted to a level, where only weak signals (<1000 RFU) could be detected even with the sensitized protocol. Therefore, another protocol was implemented, where the PCR was performed with the AmpFℓSTR® SEfiler Plus™ Master Mix. This master mix was developed specifically for challenging forensic samples with PCR inhibitors (AmpFℓSTR® SEfiler Plus™ user guide: http://www3.appliedbiosystems.com/cms/groups/applied_markets_support/documents/generaldocuments/cms_047059.pdf). The typing successes of eight samples (Table 5) were greatly improved when the PCR was performed with the SEfiler Plus master mix (see Supplementary Figure S1 for example) and it was even possible to add more sample DNA to the PCR without inhibiting the reaction. All the samples were successfully typed. The lowest number of SNPs that were detected was 41 and the highest match probability was 1.1×10^{-15} for sample 8 (Table 5 and Supplementary Figure S2). Ten of the samples turned out to be mixtures, which were confirmed for eight of the samples by STR analyses (Table 5). For the twelve samples, where DNA was extracted from two different areas of the sample material, concordant SNP profiles were obtained from the two DNA preparations (data not shown).

The samples were subsequently typed with the AmpFℓSTR® SEfiler Plus™ PCR amplification kit using the validated protocol for crime case samples [36] and the same amount of DNA used for the SNP typing. The typing success with the AmpFℓSTR® SEfiler Plus™ kit was improved compared to the older STR kits (Supplementary Table S1). Nevertheless, no STR allele was detected in four samples and only amelogenin was amplified in three samples (see Table 5 and Supplementary Figure S2 for example).

Table 4
Interpretation of 100 pg experiments.

Sample	Composite model			Relaxed consensus model			Strict consensus model		
	Typed loci	Allele drop-out	Allele drop-in	Typed loci	Allele drop-out	Allele drop-in	Typed loci	Allele drop-out	Allele drop-in
OO7	49/49	0	0	49/49	0	0	49/49	0	0
9947a	49/49	0	0	49/49	0	0	47/49	0	0
9948	49/49	0	0	48/49	0	0	47/49	0	0
K562	49/49	0	0	48/49	0	0	47/49	0	0
XX74	49/49	0	0	49/49	0	0	48/49	0	0
XY1	49/49	0	0	49/49	0	0	49/49	0	0
S1	49/49	0	0	49/49	0	0	48/49	0	0
S2	49/49	0	0	49/49	0	0	49/49	0	0
S3	49/49	0	0	49/49	0	0	49/49	0	0
S4	49/49	0	0	49/49	0	0	48/49	0	0
S5	49/49	0	0	49/49	0	0	49/49	0	0
S6	49/49	0	0	49/49	0	0	48/49	0	0
S7	49/49	0	0	49/49	0	0	49/49	0	0
S8	49/49	0	0	49/49	0	0	49/49	0	0
S9	49/49	0	0	49/49	0	0	49/49	0	0
S10	49/49	0	0	49/49	0	0	49/49	0	0
Total	784/784	0	0	782/784	0	0	774/784	0	0

Table 5
Crime case samples.

Sample number	Sample material	DNA used in the PCR	Match probability	
			AmpFℓSTR® SEfiler Plus™	SNP*for*ID multiplex
1	Hat[a]	1 μL of 10× dilution[b,c]	No alleles detected	Mixture
2	Hat[a]	40 pg	Mixture	Mixture
3	Nail[a]	4 μL of 10× dilution[b,c]	No alleles detected	3.6E−20
4	Nail	4 μL of 10× dilution[b,c]	5.0E−01	7.9E−23
5	Bone[a]	240 pg[c]	No alleles detected	Mixture
6	Bone	2 μL of 50× dilution[b,c]	No alleles detected	6.5E−15
7	Glove	100 pg[b,c]	5.0E−01	2.9E−18
8	FFPE[d] tissue[a]	2.4 ng[b]	5.0E−01	1.1E−15
9	FFPE[d] tissue[a]	2.8 ng	2.0E−07	8.3E−22
10	Hat	160 pg	Mixture	Mixture
11	Skirt	140 pg	Mixture	Mixture
12	Shoe	500 pg	Mixture	Mixture
13	Bone[a]	300 pg[b,c]	8.1E−03	1.1E−21
14	Cigarette end	200 pg	2.0E−14	1.1E−20
15	Nail	800 pg[c]	7.3E−06	1.6E−21
16	Muscle	120 pg[c]	7.7E−16	2.4E−21
17	Blood	500 pg	4.5E−13	8.1E−22
18	Glove[a]	4 μL of 10× dilution[b,c]	Mixture	Mixture
19	Hat[a]	600 pg	Mixture	Mixture
20	Bone[a]	50 pg	1.1E−14	2.3E−21
21	Hat	160 pg	Mixture	Mixture
22	Shirt	400 pg[c]	Mixture	Mixture
23	Cigarette end[a]	1.1 ng	4.3E−14	1.5E−21
24	Blood[a,e]	500 pg	1.2E−11	6.6E−21
25	Nail[e]	500 pg	4.7E−11	1.6E−21
26	FFPE[d] tissue[a,e]	1 ng	2.0E−04	3.6E−19
27	FFPE[d] tissue	500 pg	1.7E−10	4.0E−21
28	Muscle	1 ng	4.0E−08	4.6E−20

[a] Two different sample preparations from the same sample material were typed.
[b] Successful typing with the SNP*for*ID multiplex was performed in AmpFℓSTR® SEfiler™ Plus Master Mix.
[c] The internal PCR control in the Quantifiler® kit was partially or completely inhibited.
[d] FFPE, formalin fixed paraffin embedded.
[e] Used in the GenPlexT HID system interlaboratory exercise [8].

4. Discussion

Two improvements to the SNP*for*ID assay were introduced: (1) 100 SBE cycles increased the overall sensitivity of the assay without affecting the heterozygote balance and (2) the AmpFℓSTR® SEfiler Plus™ Master Mix increased the typing success of poor sample materials.

Sensitivity was tested in a series of LtDNA experiments with 25, 50 and 100 pg DNA. The typing success and quality of the 100 pg experiments resembled the average results obtained from the past five years of routine case work investigations [29]. In contrast, heterozygote imbalances were frequent in the 50 pg and especially in the 25 pg experiments, which led to a high number of failed genotype calls and a dramatic increase in allele and locus drop-outs compared to the 100 pg experiments. Nevertheless, all samples were positively identified even from 25 pg of DNA. Three different interpretation models (one composite and two consensus models) were applied. Correct profiles with 42–49 SNPs were generated from the 50 and 100 pg experiments with all three models. In contrast, a few incorrect genotypes were included in the profiles in the 25 pg experiments. Allele drop-outs and drop-ins were included in some of the composite profiles, whereas allele drop-outs were the only errors observed in the consensus profiles. It was noteworthy, that these were observed in samples where the number of locus drop-outs was high. A locus drop-out may be considered as two independent allele drop-outs [16]. Thus, multiple locus drop-outs indicate that the allele drop-out rate is very high and that the generated consensus profile may be less reliable even though it is based on multiple LtDNA typings.

As expected, allele-drop-ins were very rare and they were only observed in the 25 pg experiments. The four allele drop-ins were reexamined, but it was not possible to deduce whether the drop-ins were caused by a contamination or unusual high background noise in the electropherograms. The low drop-in rate of the sensitized SNP*for*ID assay is in sharp contrast to the sensitized STR assays, where the drop-in rate may be 100 times higher [10–18]. Allele drop-ins are very problematic, because they may lead to false inclusions as well as false exclusions. Fortunately, allele drop-ins are rarely reproducible and most drop-ins will not be included in the generated consensus profiles. This is a strong argument in favor of the consensus interpretation models. On the other hand, if the sample profile is generated from a composite interpretation model, all accepted alleles are included, which may be considered a more veracious way of reporting. Also, more loci would be reported if complete reproducibility is not a requirement. The low drop-in rate of the sensitized SNP*for*ID assay makes the composite intrepretation model more tempting [37]. Nevertheless, we will not recommend the composite model, because we consider reproducibility pivotal in forensic genetic investigations. Furthermore, only a few extra SNPs were included in the composite profiles and the majority of the incorrect genotypes in the 25 pg experiments were excluded with the consensus interpretation models.

Four repetitions of 25 pg experiments seemed to be a reasonable number. Fewer than four would certainly increase the number of incorrect genotypes and if there were sufficient DNA to perform more than four, we would recommend to increase the amount of DNA in the PCR and reduce the number of repetitions. If the sample contained 200 pg in total, two 100 pg experiments would be sufficient.

The sensitised SNP*for*ID assay with 100 SBE cycles in combination with the AmpFℓSTR® SEfiler Plus™ Master Mix made it possible to type all the degraded crime case samples in this study. A minimum of 41 SNPs were typed and the match probabilities ranged from 1.1×10^{-15} to 7.9×10^{-23} for the single source samples. In comparison, four of the samples could not be typed with the commercial STR kits and the match probabilities were higher than 10^{-7} for six other samples. This confirmed the conclusions from previous studies [4,7–9,25,30,38] that SNPs and indels may be the preferred markers for typing of highly degraded DNA.

Eleven of the crime case samples used in this study could not be quantified with the Quantifiler™ Human DNA Quantification kit because of partial or complete inhibition of the PCR. Nevertheless, the samples were typed with the SNP*for*ID assay using the AmpFℓSTR® SEfiler Plus™ Master Mix. In the last couple of years, the buffer systems for the forensic STR kits, such as the AmpFℓSTR® SEfiler Plus™ kit, have been improved significantly, which allows amplification of samples with PCR inhibitors. This is illustrated by the comparison of the AmpFℓSTR® SGM Plus, AmpFℓSTR Identifiler and the AmpFℓSTR® ® SEfiler Plus kits in this work (see Supplementary Table S1). However, the buffer systems for the quantification kits have not been improved accordingly. Therefore, it is important not to rely entirely on the DNA quantification results when downstream reactions are being planned.

In conclusion, we introduced two important technical improvements to the SNP*for*ID assay that increased the typing success of poor sample materials and allowed successful typing of samples that could not be typed with the commercial STR kits. We demonstrated that LtDNA analyses of SNPs is possible and may even be preferred to LtDNA STR analyses because of the very low allele drop-in rate.

Acknowledgements

The work was supported by Ellen and Aage Andersen's Foundation. We thank Nadia Jochumsen for technical assistance.

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.2013.02.004.

References

[1] D.J. Balding, J. Buckleton, Interpretating low template DNA profiles, Forensic Sci. Int. Genet. 4 (2009) 1–10.
[2] P. Gill, J. Buckleton, A universal strategy to interpret DNA profiles that does not require a definition of *low-copy-number*, Forensic Sci. Int. Genet. 4 (2010) 221–227.
[3] L.A. Dixon, A.E. Dobbins, H.K. Pulker, J.M. Butler, P.M. Vallone, M.D. Coble, W. Parson, B. Berger, P. Grubweiser, H.S. Mogensen, N. Morling, K. Nielsen, J.J. Sanchez, E. Petkovski, A. Carrecedo, P. Sanchez-Diz, E. Ramos-Luis, M. Brion, J.A. Irwin, R.S. Just, O. Loreille, T.J. Parsons, D. Syndercombe-Court, H. Schmitter, B. Stradmann-Bellinghausen, K. Bender, P. Gill, Analysis of artificially degraded DNA using STRs and SNPs – results of a collaborative European (EDNAP) exercise, Forensic Sci. Int. 164 (2006) 33–44.
[4] J.J. Sanchez, C. Phillips, C. Børsting, K. Balogh, M. Bogus, M. Fondevila, C.D. Harrison, E. Musgrave-Brown, A. Salas, D. Syndercombe-Court, P.M. Schneider, A. Carracedo, N. Morling, A multiplex assay with 52 single nucleotide polymorphisms for human identification, Electrophoresis 27 (2006) 1713–1724.
[5] T.J. Parsons, R. Huel, J. Davoren, C. Katzmarzyk, A. Milos, A. Selmanovic, L. Smajlovic, M.D. Coble, A. Rizvic, Application of novel "mini-amplicon" STR multiplexes to high volume casework on degraded skeletal remains, Forensic Sci. Int. Genet. 1 (2007) 175–279.
[6] J.J. Mulero, C.W. Chang, R.E. Legacé, D.Y. Wang, J.L. Bas, T.P. McMahon, L.K. Hennessy, Development and validation of the AmpFℓSTR® MiniFiler™ PCR amplification kit: a MiniSTR multiplex for the analysis of degraded and/or PCR inhibited DNA, J. Forensic Sci. 53 (2008) 838–852.
[7] R. Pereira, C. Phillips, C. Alves, A. Amorim, A. Carrecedo, L. Gusmão, A new multiplex for human identification using insertion/deletion polymorphisms, Electrophoresis 30 (2009) 3682–3690.
[8] C. Tomas, G. Axler-DiPerte, Z.M. Budimlija, C. Børsting, M.D. Coble, A.E. Decker, A. Eisenberg, R. Fang, M. Fondevila, S.F. Fredslund, S. Gonzalez, A.J. Hansen, P. Hoff-Olsen, C. Haas, P. Kohler, A.K. Kriegel, B. Lindblom, F. Manohar, O. Maroñas, H.S. Mogensen, K. Neureuther, H. Nilsson, M.K. Scheible, P.M. Schneider, M.L. Sonntag, M. Stangegaard, D. Syndercombe-Court, C.R. Thacker, P.M. Vallone, A.A. Westen, N. Morling, Autosomal SNP typing of forensic samples with the GenPlex™ HID System: results of a collaborative study, Forensic Sci. Int. Genet. 5 (2011) 369–375.
[9] S.L. Friis, C. Børsting, L. Poulsen, S.F. Fredslund, C. Tomas, N. Morling, Typing of 30 insertion/deletions in Danes using the first commercial Indel kit, Forensic Sci. Int. Genet. 6 (2012) e72–e74.
[10] P. Gill, J. Whitaker, C. Flaxman, N. Brown, J. Buckleton, An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA, Forensic Sci. Int. 112 (2000) 17–40.
[11] P.J. Smith, J. Ballantyne, Simplified low-copy-number DNA analysis by post-PCR purification, J. Forensic Sci. 52 (2007) 820–829.
[12] L. Forster, J. Thomson, S. Kutranov, Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle "low copy number" (LCN) method for analysis of trace forensic DNA samples, Forensic Sci. Int. Genet. 2 (2008) 318–328.
[13] A.A. Western, J.H.A. Nagel, C.C.G. Benschop, N.E.C. Weiler, B.J. de Jong, T. Sijen, Higher capillary electrophoresis injection settings as an efficient approach to increase the sensitivity of STR typing, J. Forensic Sci. 54 (2009) 591–598.
[14] S. Petricevic, J. Whitaker, J. Buckleton, S. Vintiner, J. Patel, P. Simon, H. Ferraby, W. Hermiz, A. Russell, Validation and development of interpretation guidelines for low copy number (LCN) DNA profiling in New Zealand using the AmpFℓSTR® SGM plus™ multiplex, Forensic Sci. Int. Genet. 4 (2010) 305–310.
[15] S. Cowen, P. Debenham, A. Dixon, S. Kutranov, J. Thomson, K. Way, An investigation of the robustness of the consensus method of interpreting low-template DNA profiles, Forensic Sci. Int. Genet. 5 (2011) 400–406.
[16] C.C.G. Benschop, C.P. van der Beek, H.C. Meiland, A.G.M. van Gorp, A.A. Western, T. Sijen, Low template STR typing: effect of replicate number and consensus method on genotyping reliability and DNA database search results, Forensic Sci. Int. Genet. 5 (2011) 316–328.
[17] A.A. Western, L.J.W. Grol, J. Harteveld, A.S. Matai, P. De Knijff, T. Sijen, Assessment of the stochastic threshold, back-, and forward stutter filters and low template techniques for NGM, Forensic Sci. Int. Genet. (2012), http://dx.doi.org/10.1016/j.fsigen.2012.05.001.
[18] C.M. Pfeifer, R. Klein-Unseld, M. Klintschar, P. Wiegand, Comparison of different interpretation strategies for low template DNA mixtures, Forensic Sci. Int. Genet. (2012), http://dx.doi.org/10.1016/j.fsigen.2012.06.006.
[19] T. Tvedebrink, P.S. Eriksen, H.S. Mogensen, N. Morling, Estimating the probability of allelic dropout in forensic genetics, Forensic Sci. Int. Genet. 3 (2009) 222–226.
[20] H. Haned, T. Egeland, D. Pontier, L. Pène, P. Gill, Estimating the drop-out probabilities in forensic DNA samples: a simulation approach to evaluate different models, Forensic Sci. Int. Genet. 5 (2011) 525–531.
[21] T. Tvedebrink, P.S. Eriksen, H.S. Mogensen, N. Morling, Statistical model for degraded DNA samples and adjusted probabilities for allelic drop-out, Forensic Sci. Int. Genet. 6 (2012) 97–101.
[22] T. Tvedebrink, P.S. Eriksen, H.S. Mogensen, N. Morling, Allelic drop-out probabilities estimated by logistic regression – further considerations and practical implementation, Forensic Sci. Int. Genet. 6 (2012) 263–267.
[23] C. Børsting, E. Rockenbauer, N. Morling, Validation of a single nucleotide polymorphism (SNP) typing assay with 49 SNPs for forensic genetic testing in a laboratory accredited according to the ISO 17025 standard, Forensic Sci. Int. Genet. 4 (2009) 34–42.
[24] C. Børsting, J.J. Sanchez, H.E. Hansen, A.J. Hansen, H.Q. Bruun, N. Morling, Performance of the SNP*for*ID 52 SNP-plex assay in paternity testing, Forensic Sci. Int. Genet. 2 (2008) 292–300.
[25] C. Phillips, M. Fondevila, M. García-Magarinos, A. Rodriguez, A. Salas, A. Carrecedo, M.V. Lareu, Resolving relationship tests that show ambiguous STR results using autosomal SNPs as supplementary markers, Forensic Sci. Int. Genetics 2 (2008) 198–204.
[26] C. Tomas, J.J. Sanchez, J.A. Castro, C. Børsting, N. Morling, Forensic usefulness of a 25 X-chromosome SNP marker set, Transfusion 50 (2010) 2258–2265.
[27] C. Børsting, N. Morling, Mutations and/or relatives? Six case work examples where 49 autosomal SNPs were used as supplementary markers, Forensic Sci. Int. Genet. 5 (2011) 236–241.
[28] C. Børsting, N. Morling, Re-investigations of six unusual paternity cases by typing of autosomal single nucleotide polymorphisms, Transfusion (Paris) 52 (2012) 425–430.
[29] C. Børsting, M. Mikkelsen, N. Morling, Kinship analysis with diallelic SNPs – experiences with the SNP*for*ID multiplex in an ISO17025 accreditated laboratory, Transfus. Med. Hemother. 39 (2012) 195–201.
[30] C. Phillips, M. Garcia-Magariños, A. Salas, A. Carrecedo, M.V. Lareu, SNPs as supplements in simple kinship analysis or as core markers in distant pairwise relationship tests: when do SNPs add value or replace well-established and powerful STR tests? Transfus. Med. Hemother. 39 (2012) 202–210.
[31] T. Schwark, P. Meyer, M. Harder, J. Modrow, N. von, Wurmb-Schwark, The SNP*for*ID assay as a supplementary method in kinship and trace analysis, Transfus. Med. Hemother. 39 (2012) 187–193.

[32] C. Børsting, C. Tomas, N. Morling, in: A. Alonso (Ed.), DNA Electrophoresis Protocols for Forensic Genetics – Typing of 49 Autosomal SNPs by Single Base Extention and Capillary Electrophoresis for Forensic Genetic Testing, Methods in Molecular Biology Series, vol. 830, Humana Press, New Jersey, USA, 2012 pp. 87–108Chapter 7.
[33] C. Børsting, C. Tomas, N. Morling, SNP typing of the reference materials SRM 2391b 1-10, K562, XY1, XX74, and 007 with the SNP*for*ID multiplex, Forensic Sci. Int. Genet. 5 (2011) e81–e82.
[34] F.M. Ausubel, R. Brent, R.E. Kingston, et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., 1995.
[35] C. Børsting, J.J. Sanchez, N. Morling, Forensic genetic DNA typing with PCR-based methods, in: S. Hughes, A Moody (Eds.), PCR, Method Express Series, Scion Publishing Ltd., Bloxham, UK, 2007, pp. 123–142.
[36] S.F. Fredslund, H.S. Mogensen, N. Morling, Validation of the AmpF*ℓ*STR® SEfiler Plus amplification kit for forensic STR analysis, Forensic Sci. Int. Genetic. Suppl. (2) (2009) 27–28.
[37] J.-A. Bright, P. Gill, J. Buckleton, Composite profiles in DNA analysis, Forensic Sci. Int. Genet. 6 (2012) 317–321.
[38] T. Senge, B. Madea, A. Junge, M.A. Rothschild, P.M. Schneider, STRs, mini STRs and SNPs – a comparative study for typing degraded DNA, Legal Med. 13 (2011) 68–74.