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Human identification & forensic analyses of degraded or low level DNA Antoinette-Andrea Westen

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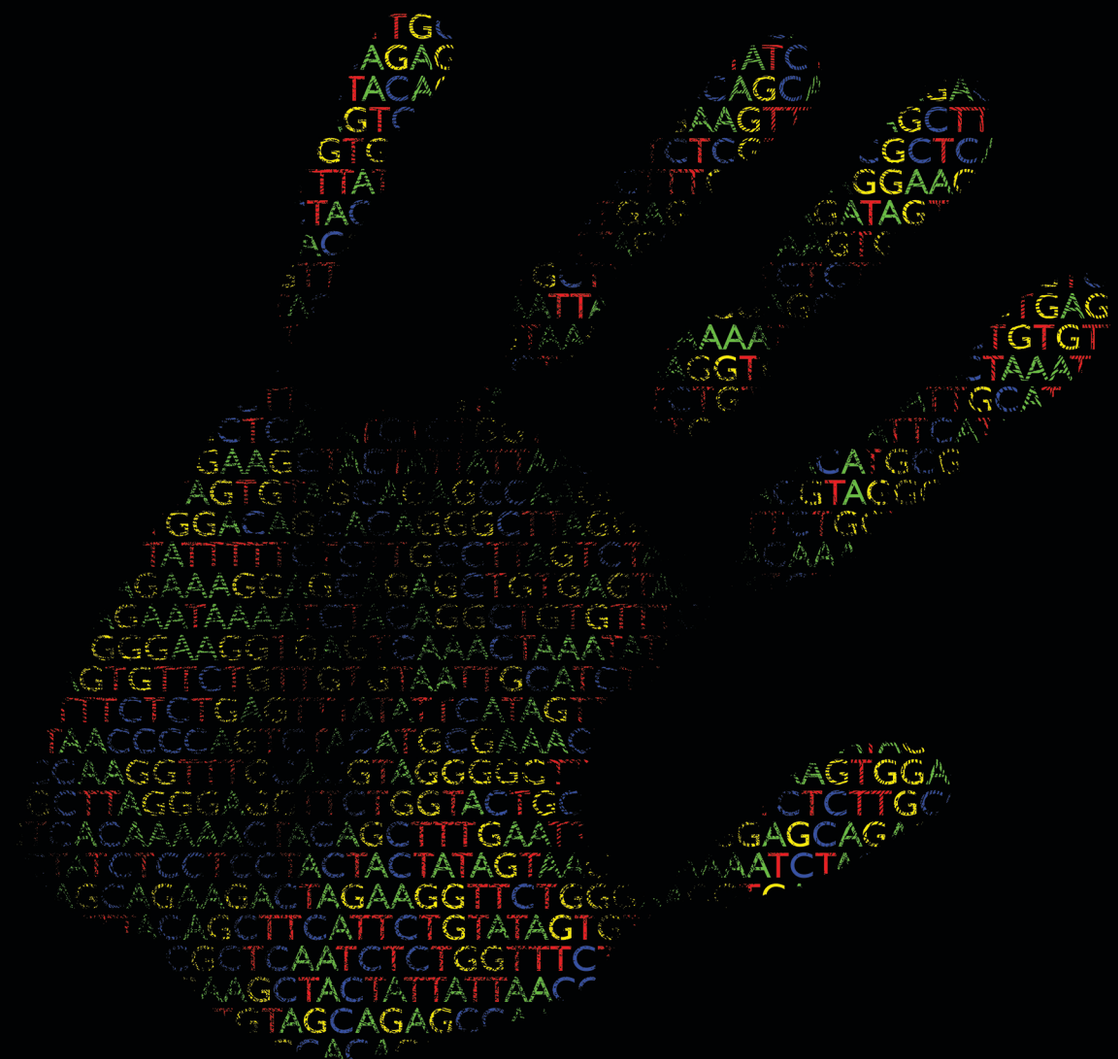
Antoinette-Andrea Westen



Human identification & forensic analyses of degraded or low level DNA

Antoinette-Andrea Westen

Colophon

Human identification & forensic analyses of degraded or low level DNA

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Life is what happens to us while we are making other plans.
Allen Saunders

Voor mijn ouders, die er altijd voor me zijn.
Voor Jasper, die zoveel vrolijkheid brengt.
Voor Hugo, met wie ik nog veel avonturen hoop te beleven.

Table of contents

Outline

of this thesis

Human identification is needed in situations such as mass disasters, terrorist attacks, missing person cases and forensic investigations. The identification can be based on body characteristics (for instance facial recognition, dactyloscopy or odontology) or on DNA-based evidence. Especially the latter method has proven to be useful when bodies are beyond recognition or incomplete.

In order to make a DNA profile of a person, the DNA has to be of sufficient quality and quantity. However, when the time between death and discovery of the body is long and/or the body has been exposed to harsh conditions (fire, submerged, warm humid air, acidic soil, chemical agents, etc.) the DNA may be severely degraded and/or available in minute amounts only.

The research described in this thesis has been directed towards method development for cases in which the DNA samples are compromised (i.e. low in quality and/or quantity). The aim of the research is to provide additional or alternative methods to extract information from a person's DNA when standard DNA typing methodology is not sufficient for human identification.

DNA profiles used for human identification are usually based on short tandem repeats (STRs) in the DNA sequence. The number of repeats per allele (depicted as a peak in the DNA profile) differs between persons, and a combination of alleles for several markers makes a DNA profile that is (virtually) unique per person when enough markers are used. To obtain a DNA profile of the best possible quality, it is of utmost importance that samples are excised, handled and stored under the most optimal conditions achievable, until their analysis in a specialised DNA laboratory. Especially in mass disaster conditions, bodies (and samples taken from those) are easily contaminated by DNA from other victims. **Chapter 1** describes a standard operating procedure for sample excision, contamination prevention and optimum sample storage conditions in a mass disaster environment. Sampling instructions are given for femur, rib and tooth (or molar) samples. In addition, practical advice is given on inexpensive and simple solutions for excision tools, decontamination fluid preparation and preservation of the samples.

In order to generate a DNA profile, specific marker regions within the DNA are amplified (by a polymerase chain reaction (PCR)). When DNA is low in quantity, DNA profiles have lower peak heights and may suffer from stochastic amplification effects, such as peak height imbalances, allele and/or locus drop-outs (which result in incomplete DNA profiles), allele drop-ins and elevated stutter peaks (which are artefacts that resemble real peaks). These effects impede DNA profile interpretation and may prevent the identification of a person. In order to obtain more information from the DNA donor, a technique was developed to sensitise the DNA detection method (i.e. capillary electrophoresis), which is described in **Chapter 2**. This technique is based on increased injection settings during electrophoresis to obtain higher peak

heights and more genotyping information from single donor and (unequally) mixed DNA samples. It can be used irrespective of the DNA marker system to amplify the DNA. Since this method makes use of the remaining portion of the PCR product mixture (that otherwise would be discarded after standard DNA analysis) no additional use of DNA extract is needed. Nevertheless, it is regarded as a low template DNA technique and it is therefore recommended to perform replicate analyses.

Amplicons (i.e. the DNA fragments that are multiplied during amplification) of the STR kits used in the first part of this thesis varied in size from 100 to around 400 nucleotides. When the quality of the DNA is low, due to (severe) degradation, the DNA fragments may become shorter than some of the STR amplicons. In these cases, the peaks that represent the longer STRs have low peak heights or are even absent from the DNA profile. Another type of DNA marker is a single nucleotide polymorphism (SNP). SNPs used in human identification typically have amplicons of 55 to around 115 nucleotides, which make them interesting for the analysis of degraded DNA. In **Chapter 3**, we analysed tri-allelic SNPs, a special class of SNPs that exhibits three different alleles (instead of the usual two). This characteristic makes them specifically interesting for use in human identification and forensic analyses, as the detection of mixtures (recognised by a third allele on a locus) is much easier for tri-allelic than for bi-allelic SNPs (for which only peak height differences can be used), thus diminishing the chance on incorrect genotyping results. We developed multiplex genotyping assays and determined allele frequencies for Dutch and Netherlands Antilles populations in order to analyse degraded DNA samples and to assess the power of discrimination of the tri-allelic SNPs.

Subsequently, these assays and several alternatives were evaluated for the analysis of degraded DNA in a comparative study, as described in **Chapter 4**. Next to standard DNA typing, which was performed with SGM Plus™ at that time, we tested DNA repair enzyme cocktails (PreCR™ and Restorase™) that aim to repair the DNA prior to amplification. In addition, an STR kit designed to carry all amplicons in small size (mini-STRs (MiniFiler)) was included, as well as bi-allelic SNPs (a semi-commercial system denoted as GenPlex™) and the tri-allelic SNP set described in Chapter 3. For each of these methods we determined the percentage detected alleles, and showed the required DNA input in perspective to the random match probability that could be obtained.

After the decision by the European Council to add five additional STR markers to the European standard set of STRs, new STR kits were developed by several companies containing 15 STR loci or more. In these kits, mini-STRs (with amplicon sizes from 70 base pairs and up) are incorporated as much as possible to obtain more information from degraded DNA, and the sensitivity is enhanced by using optimised buffers and (an) additional amplification cycle(s). The NFI decided to work with the AmpF*l*STR® NGM™ kit, which was subsequently validated in-house. Specific aspects

of this validation are described in **Chapter 5**. We determined the stochastic threshold (below which alleles are prone to drop out due to low template amplification effects), together with the stutter ratio filters and the optimal strategy to sensitise genotyping of low template DNA. These aspects will assist in the optimal interpretation of unequal mixtures and low template DNA samples.

Sometimes, the 15 STR loci in the current generation STR kits do not provide enough discrimination power. This can occur when the DNA is degraded to the extent of locus drop-out, but also in complex kinship analyses, for example. A relatively new kit to the forensic market (HDplex™) contains 9 STRs that are additional to the commonly used markers, and additional genotyping with this kit can increase the power of discrimination. In **Chapter 6** we assessed whether the 30 STRs present in NGM™, HDplex™ and Identifiler™ can be regarded as independent, which is particularly relevant for the markers that are present on the same chromosome (an occurrence that is inevitable when the number of markers increases). In addition, we calculated the (combined) match probabilities (under the assumption of independence) for the supplementary genotyping results of the three kits. This information will point out which markers can be combined within one calculation to assist complex kinship or degraded DNA analyses.

Information about the longer loci in an STR profile can be missing due to degradation of the DNA or low template amounts of the minor contributor in an unequal mixture. Sensitising the reactions (e.g. by increased capillary electrophoresis injection settings as described in Chapter 2) may result in over-amplified or overloaded DNA profiles for the shorter loci and/or the major component in the mixture. In **Chapter 7** we explored whether DNA profiling of such samples can be improved by preferential capturing of the longer amplified fragments. We developed a size-selective post-PCR purification protocol (based on AMPure™ XP beads) and compared it to an unselective post-PCR purification system (DTR gel filtration) and no purification of the PCR products. These methods have the advantage that the remaining portion of the PCR products is used (likewise the method described in Chapter 2), without usurping additional DNA extract.

In **Chapter 8**, different aspects of DNA-based human identification are discussed with emphasis on low quality and/or quantity of the DNA. In addition, some recent developments and future possibilities are considered that may aid human identification and forensic analyses.

Chapter 1

Femur, rib, and tooth sample collection for DNA analysis in disaster victim identification (DVI)

A method to minimise contamination risk

Forensic Science, Medicine, and Pathology (2008) 4:15–21

Antoinette A. Westen
Reza R. R. Gerretsen
George J. R. Maat

Abstract

Although much literature is available on DNA extraction from tissue samples to obtain the best possible genotyping results, to the best of our knowledge no written recommendations exist on how to excise or extract bone and tooth samples from a victim to facilitate this. Because the possibility of cross-contamination is high, especially when excising numerous samples under disaster conditions, it is important to minimise this risk and to keep samples in optimum condition. In this paper a standard operating procedure is proposed for collection of femur, rib, and tooth samples to aid victim identification both after mass disasters and in (single) forensic investigations.

Introduction

DNA identification is a tool used in a growing number of mass disasters [1–3] and forensic investigations [4–6]. When the identity of a person cannot be established with traditional identification methods, for example facial recognition, dactyloscopy, or odontology, DNA analysis may offer a solution. This is of particular interest for people who are unrecognizable, for example as a result of fire, natural decomposition, or deliberate mutilation. The two most important requirements for DNA-based identification are collection of representative, high quality tissue samples from the victim and the availability of reference samples, either from the suspected victim or from family members, with which to compare the tissue samples [7–9]. Many publications and protocols have been presented on methodology for isolating DNA from tissue samples for genotyping purposes in the laboratory [1, 5, 10, 11]. In contrast, limited information is available about the collection of bone and tooth samples, and advice on the prevention of contamination for these samples is often contradictory [1, 3, 5, 9, 11]. Because (cross-) contamination is one of the largest pitfalls during sample collection, appropriate measures should be taken to prevent this.

The South East Asian tsunami of December 2004 was an excellent example of a mass disaster incontrovertibly showing the importance of minimizing contamination risk during tissue sample collection for DNA analysis. Forensic investigators from 31 different countries arrived in Thailand to help with disaster victim identification (DVI). The DVI teams used many different protocols. To standardise protocols and procedures, on January the 20th, the Thai tsunami victim identification (TTVI) committee was initiated. This comprised many of the scientists that were present at the scene. Most protocols were based on the Interpol Disaster Victim Identification Guide [12]. This guidance did not provide a protocol for tissue-sample collection for DNA research, however. As a result the Dutch team created guidelines for the collection of bone and tooth samples based on obvious common sense and existing theoretical knowledge. These guidelines were approved and recommended by the TTVI. In this paper we describe a standard operating procedure (SOP) for bone and tooth sample collection based on our experience during the aftermath of the tsunami and the guidelines we developed then. This SOP can be used for human identification both during mass disasters and in forensic investigations.

Working conditions and methods

A temporary morgue was established on the premises of a Buddhist temple in Wat Yan Yao on the peninsula Khao Lak. The tsunami victims were transported to the morgue in trucks in which the bodies were lying on top of each other, which obviously

resulted in body-surface cross-contamination. Initially, the bodies were cooled with dry ice, but later all the deceased were placed in body bags, which were tagged with a tracking number and stored in containers that were cooled below 0°C. The Dutch DVI team advised placing the bodies on wooden scaffolding (in the containers) to facilitate the cooling and to prevent further putrefaction as a result of the heat of decomposition.

To identify the human remains, which were often highly putrefied and partially skeletonised, multiple methods were combined. Fingerprints and palmprints were taken and external body details, for example clothing, personal belongings, scars, and marks (e.g. tattoos) were photographed, described, and recorded [5, 13]. Autopsy was performed to expose the nature of any previous surgery. Odontology consisted of one or two periapical radiographs to assist determination of the age of children and two bitewings for adults. After exarticulation of the jaw, Polaroid images were taken of the maxillary and mandibular occlusal tables and the anterior edge-to-edge view of the incisors [14]. Also, bone and tooth samples were collected for DNA testing (as described below). All post-mortem (PM) data were written on (pink Interpol) DVI forms and, after completion, entered into a database (Plass Data Software A/S, Denmark, 2003).

Contamination

Under chaotic and often hectic conditions, large numbers of samples from many different individuals had to be collected, nonetheless, accurately and consistently. Because it was important that samples for DNA analysis were free from contamination, great care was taken to prevent exogenous contamination by examiners or microbes and, in particular, cross-contamination with DNA from other victims. The SOP was designed to minimise (cross-) contamination during the collection of the tissue samples for DNA-based identification.

Standard operating procedure for tissue sample collection for DNA analysis

Preconditions at all times

- The site of sample collection should be clean and separate from other sites of interference, for example autopsy, dental examination, etc.
- Personal protective equipment, for example an overall or a long sleeved coat, an extra plastic apron, a hair net, and a mouth or gas mask, should be worn, both

for protection of the examiner and to prevent shedding of contaminants, for example hair and saliva, from the examiner on to the samples.
– Double (surgical) gloves should be worn, so that the exterior gloves can be removed instantly if clean, dry hands are needed.
– DNA remover preparation comprises an aqueous solution of 1 mL L^{-1} liquid soap and at least 5 % bleach.
– If an instrument or hand inadvertently touches an unclean area (including cleaned skin) the procedure should be stopped and the instrument again cleaned meticulously with DNA remover before proceeding further.
– If an excised tissue sample may have touched an unclean area (including cleaned skin) a new clean sample must be taken.
– The SOP should be executed with care, as if in the operating theatre.

Femur

I. Preparations for femur sample excision

1. Prepare a large bucket and a rectangular tub containing DNA remover.
2. Fill a small container with absolute alcohol.
3. Place a fresh disposable cleaning cloth in the large bucket (for body surface cleaning) and a fresh disposable cleaning cloth and a brush in the tub (for instrument cleaning and storage).
4. Wipe the surface of the instrument table with the disposable cleaning cloth from the large bucket and then discard the cloth.
5. Clean all instruments (scalpel, surgical tweezers, small hacksaw, Freer periosteal elevator, scissors, etc.) with the disposable cleaning cloth and/or the brush from the tub and then store them at the bottom of the tub under the surface of the DNA remover.

II. Exposure of the femur before sample excision

6. Elevate the thigh of the victim slightly above the dissection/autopsy table, e.g. by tucking part of the body bag underneath it.
7. Clean the skin of the thigh with a fresh disposable cleaning cloth that has been soaked in the large bucket with DNA remover, then discard after use.
8. Discard the outer gloves and replace with new.
9. Use a clean disposable surgical blade (a number 22 blade is ideal).
10. Make a superficial H-incision a few millimetres deep only (Fig. 1), i.e.:
– a longitudinal incision over the topmost part of the thigh, extending from a little below the inguinal area to approximately 5 cm above the knee, plus

– transverse incisions crossing the proximal and the distal ends of the longitudinal cut. Both transverse cuts should extend a little more than half the circumference of the thigh.

11. Clean the scalpel in the rectangular tub, paying extra attention to its neck where a skin smear may be present.
12. Deepen the H-incision with the cleaned scalpel and the surgical tweezers until the femur is touched.

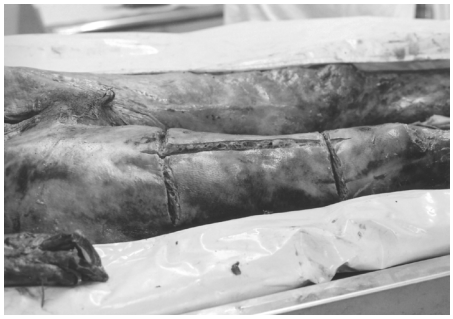

Fig. 1 H-incision of the thigh.

Reminder: the tweezers should be cleaned if the skin is accidentally touched.

13. The femoral shaft should be freed from muscle tissue in such a way that the medial and lateral muscle compartments fold back similar to doors opening (Fig. 2). This can be facilitated by means of a few longitudinal cuts in the muscles. The object is to expose the femoral shaft in such a way that it can be approached for sawing without touching anything else.

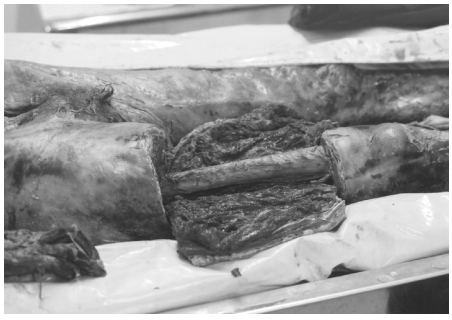

Fig. 2 Exposing the femur before excision of the bone sample.

III. Processing of an excised femur wedge

14. Remove the periosteum with the scalpel and the Freer periosteal elevator to facilitate sawing.
15. Place a clean disposable saw-blade in the hacksaw.
16. With the hacksaw saw a wedge from the midshaft of the femur (Fig. 3). If possible do NOT saw through the complete shaft, because the femur will become unstable for further sawing and transportation.

Fig. 3 Collecting excised femur sample.

17. Lift the wedge with DNA-free tweezers.
18. Rinse the sample in the small container with absolute alcohol to accelerate the drying process. Do not put the sample down in the meantime.
19. Once "dry", put the sample in a sample container (Fig. 3), sealing it with tape and marking it with the appropriate tracking number.
20. Store the sample container in a freezer.
21. Complete the inventory list and the chain-of-custody form.
22. Close and suture the excision wound.
23. Clean every instrument with the DNA remover in the rectangular tub and store the instruments at the bottom of the tub under the surface of the DNA remover.
24. Replace the contents of the small container with fresh absolute alcohol.

Rib

I. Preparations for rib sample excision

1. Repeat paragraphs 1–5 as described above.

II. Exposure of the rib before sample excision

2. Palpate a rib in the lower half of the chest.
3. Clean the chest area with a fresh disposable cleaning cloth that has been soaked in the large bucket with DNA remover and then discard after use.
4. Discard the outer gloves and replace with new.
5. Use a clean disposable surgical blade (a number 22 blade is ideal).
6. Make a superficial rectangular incision with a length of ca. 10 cm and only a few millimetres deep, well surrounding the osteochondral junction of the chosen rib.
7. Clean the scalpel in the rectangular tub, paying extra attention to its neck where a skin smear may be present.
8. Excise the skin and the underlying muscle tissue in one movement.
9. Clean the scalpel as described above.
10. Deepen the incision along the sides of the rib until you penetrate the thoracic cage.

Reminder: the tweezers should be cleaned if the skin is accidentally touched.

11. Expose the rib in such a way that it can be approached with scissors without touching anything else (Fig. 4).

III. Processing of an excised rib sample

12. Use the scissors to cut through the bone of the rib, approximately 3 cm from the osteochondral junction.
13. While holding the bone end with DNA-free tweezers, cut with the scissors through the cartilaginous part of the rib, also ca. 3 cm from the osteochondral junction (Fig. 5).
14. Repeat paragraphs 18–24 as described above.

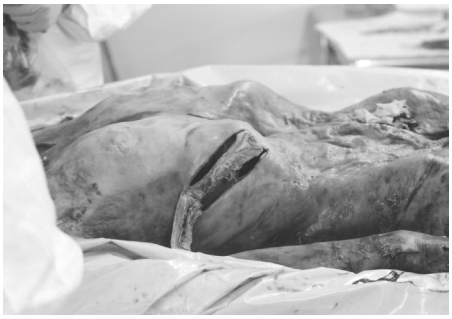

Fig. 4 Exposing the rib before excision of the bone sample.

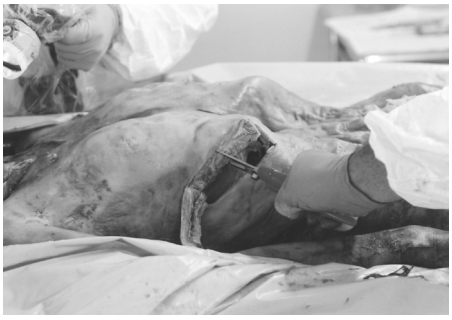

Fig. 5 Cutting the rib.

Tooth

I. Preparations for tooth sample extraction

1. Prepare a bowl with DNA remover and permanently keep a toothbrush and tweezers in the bowl.
2. Fill a small container with absolute alcohol and permanently keep a pair of DNA-free tweezers in this container.

II. Processing of an extracted tooth specimen

3. Extract a healthy intact tooth (i.e. without caries, fillings, or other artificial modifications), preferably a canine, an upper incisor, or a molar, with intact roots (see Recommendations) with extraction pliers.
4. Drop the extracted tooth, which is still dirty, in the bowl containing DNA remover.
5. Discard the outer gloves and replace with new.
6. Clean the tooth with the toothbrush from the bowl. You may use your gloved hands.
7. Lift the tooth with the tweezers from the bowl and drop it in the small container containing absolute alcohol; place the tweezers back in the bowl.
8. Lift the tooth with the tweezers from this small container after rinsing it with

absolute alcohol to facilitate the drying process. Do not put the tooth down in the meantime.

9. Repeat paragraphs 19–21 as described above.
10. Clean the toothbrush and the tweezers from the bowl with the DNA remover.
11. Replace the contents of the small container with fresh absolute alcohol.

Recommendations

Some additional recommendations are suggested with regard to this standard operating procedure. During earlier work on disaster victim identification in Kosovo [15, 16] it appeared to be difficult to keep a grip on a scalpel during autopsy of seriously decomposed corpses. Especially under disaster conditions, you do not want to "lose" the scalpel in the corpse and risk injury to yourself or others nearby. Thus, a large grip was designed that can hold a standard surgical blade (Fig. 6). The grip is ergonomically shaped to facilitate control. It is made of brass, which is bacteriocidal as a result of the regular formation of a layer of copper oxide on its surface.

Routine work at the "morgue" showed that even passive storage of amputation saws in the tub with DNA remover resulted in blunting within hours. This was probably because of erosion by the aggressive cleaning fluid solution. It appeared that a small tool shop hacksaw (Fig. 6) was of more practical use than the standard amputation saw. Sharpening of the blades was no longer needed, because they could be simply replaced by inexpensive disposable blades.

No electrical equipment, for example an electric saw, was used during the sample collection for excising bone samples. This was for two major reasons: first, the possibility of spreading aerosols or small particles of tissue that could cause contamination of other samples and, second, cleaning the blades with DNA remover causes blunting and replacing them is relatively expensive.

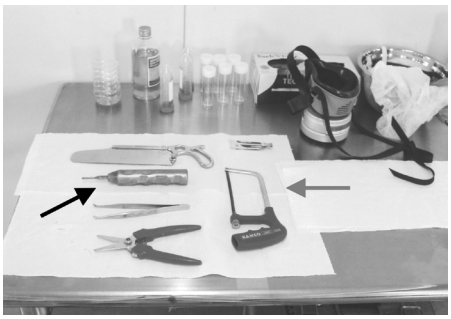

Fig. 6 Instrument table: brass grip (black arrow) and hacksaw (grey arrow).

When possible, it may be better for an odontologist to collect the tooth sample, because their training enables them to distinguish intact from artificially modified teeth and to extract without damaging the teeth. For DNA analysis the intact element with the largest pulp-cavity is preferred, because this should yield the largest amount of DNA. The dimension of the pulp-

cavity depends on the size of the tooth [17] and is age-dependent as a result of secondary dentine deposition. In children, open roots make the teeth much more susceptible to contamination and to destruction of DNA by the DNA remover.

We recommend using femur wedges instead of rib samples for DNA analysis. Because ribs have a very thin cortex and tend to protrude through the skin, the risk of contamination may be greater, especially for submerged corpses. After recovery of such samples, cleaning may be difficult or even impossible without damaging the endogenous DNA. We have, nevertheless, described the SOP for excision of a rib sample, because some countries insist on using rib samples for genotyping purposes. Their choice to use rib samples is probably because spongy or cancellous bone can be rich in DNA. Prinz et al. [9], however, report that preservation of cancellous bone is not reliable and dense cortical bone should always be the first choice, preferably from the weight-bearing long bones of the legs.

Finally, ensure that directly after excision or extraction, the bone and tooth samples are frozen. If no freezer is available, cooling the sample containers in a bath of water with melting ice will be effective as long as the ice melts, the temperature thus staying at 0°C.

Discussion

Because no special record was kept of the samples excised from the tsunami victims by means of the above described SOP, it is, unfortunately, very difficult (if not impossible) to track the samples and discover whether they provided adequate DNA profiles. A similar SOP for femur and tooth sample collection is used at our laboratory at the Netherlands Forensic Institute, which provides good genotyping results. Nevertheless, the hectic situation of a mass disaster contrasts sharply with the conditions during single forensic cases. Shortly after a mass disaster has occurred, especially, neither facilities nor trained people are immediately available for identification work. The absence of a cooling/freezer facility shortly after the tsunami, for example, led to on-going decomposition of the victims' bodies, which impeded identification. The number of victims to be identified was, in addition, so large that the identification teams had to work in multiple shifts and train extra people on site. Because the victims' bodies could easily cross-contaminate each other during transport and storage, it was also necessary to devote special attention to preventing cross-contamination of the bone and tooth samples from the body surface or unclean instruments. This contrasts markedly with a specialised forensic laboratory in which all the necessary facilities are available, the personnel is well-trained, and the section rooms and instruments are cleaned after each autopsy.

One of the assumptions made in the above-described protocol is that the tissue samples, assuming they are excised in a correct manner, are free from contamination and ready for DNA extraction. This is not always true. For example, during the WTC disaster the body parts were highly commingled, and during excavation of the mass graves in the former Yugoslavia the bodies were grossly putrefied or even skeletonised [5, 11]. In these situations, tissue samples can easily become contaminated. It is, therefore, necessary to clean the bones and bone fragments to remove contaminating DNA and potential polymerase chain reaction (PCR) inhibitors. Multiple cleaning methods are available. For body remains from the mass graves in the former Yugoslavia, Andelinovic et al. [5] report that all bone surfaces were cleaned from remnant soft tissue and traces of soil and were also brushed in warm water with mild detergent. Complete bones were then rinsed with distilled water several times and dried in air. Bone fragments were washed with commercial bleach, three times with deionised water, twice with 70 % ethanol and dried in air for 24 h. Alonso et al. [10] report that outer surfaces of tooth samples were extensively washed with distilled and sterile water before irradiation with UV light for 30 min on each side. Both Zehner [3] and Alonso et al. [10] advise physical removal of the external and internal surfaces of the bone. Several methods are described for this removal, for example rasping, sawing, or abrasion with sandpaper or a sanding machine [3, 10, 18–20]. Sanding, and grinding in a later stage of the research, can generate bone dust, which can lead to sample cross-contamination. Manual processing of single bone samples reduces the chance of sample cross-contamination compared with batch processing [8].

The effect of the environment on victims can vary greatly among different types of forensic cases and mass disasters. The tsunami victims were exposed to seawater and warm humid air whereas the victims of the WTC disaster were exposed to intense fire, heat, and subsequent extinguishing water. In contrast, the bones of people killed during the war in the former Yugoslavia were exposed to highly acidic soil and chemical agents that were used in deliberate attempts to degrade their DNA [1]. These different effects may cause different kinds of DNA damage. It would be interesting to develop a method to determine the type and extent of this damage and, where possible, to develop corresponding protocols for DNA extraction and the subsequent DNA analysis.

It is not always clear what effect environmental factors have had on tissues and whether these tissues can still provide good genotyping results. In general, blood or intact soft tissue samples are preferred for DNA analysis but when body putrefaction precludes DNA preservation or when much commingling of soft tissue is suspected, bone and tooth samples are preferred [7, 9]. During identification of the tsunami victims, not only bone and tooth samples were used for genotyping purposes. Steinlechner et al. [21] described the use of swabs from two, as intact as possible, internal organ or muscle surfaces at the disaster site in Sri Lanka. Because the quality

of DNA in soft tissue decreases rapidly with time, this method requires the swabs be taken from relatively fresh material. An advantage is that the analysis is less laborious and time-consuming than for bone and tooth samples. When the effect of precise environmental factors is uncertain, it seems sensible to collect different kinds of tissue samples from each victim. Another advantage of collecting several samples per victim from the outset is avoidance of laborious re-sampling and relabeling efforts when no DNA profile could be obtained from the first sample. Such sampling also gives rise to the possibility of a duplication policy, in which two specimens collected from the same body or body part are tested. This could help in identifying mislabeled or switched samples or extract-to-extract contamination, which could lead to incorrect identification when based on a single extraction [9].

DNA genotyping should not be problematical if the tissue samples are of high quality at the moment they reach the laboratory. Unfortunately, as a result of post-mortem processes, the DNA in forensic (mass disaster) tissue samples is often limited in quality and/or quantity, leading to difficulties in DNA analysis. In current forensic DNA practice the number of repeats of specific DNA fragments, called short tandem repeats (STRs), is counted at different loci in the genome and plotted per locus in a DNA profile. The DNA fragments to be analysed range in size between 114 and 353 base pairs. Degradation of the DNA may result in the inability to detect the larger DNA fragments, reducing the chance of victim identification. Although other genotyping methods are being developed, with the objective of using shorter DNA fragments, for example mini-STRs [22, 23] and single nucleotide polymorphisms (SNPs) [8, 24, 25], the results will be still determined by the quality of the tissue samples to be analysed. It is, therefore, of the greatest importance to collect tissue samples of the highest possible quality, to minimise the risk of contamination, and to keep the samples under optimum conditions until they can be genotyped in the laboratory.

Conclusion

This standard operating procedure for excision and extraction of bone and tooth samples to be used for genotyping purposes was developed under disaster conditions and is based on common sense, theoretical knowledge, and best practice. Because the materials used are inexpensive and easy to obtain, execution of the SOP should not cause problems. Further research and use of this SOP under controlled circumstances (e.g. in the laboratory or in single forensic cases) may reveal the possibility of improvement.

Educational message

1. Wear protective clothing and work in a clean separate area to minimise exogenous contamination risk.
2. Clean instruments thoroughly after touching the skin of a victim and before examining another victim to minimise the risk of cross-contamination.
3. Freeze, or cool when no freezer is available, the tissue samples directly after collection (and labelling) to keep the samples under optimum conditions until they can be genotyped.

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Chapter 2

Higher Capillary Electrophoresis Injection Settings as an Efficient Approach to Increase the Sensitivity of STR Typing

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Abstract

Evidentiary traces may contain low quantities of DNA, and regularly incomplete short tandem repeat (STR) profiles are obtained. In this study, higher capillary electrophoresis injection settings were used to efficiently improve incomplete STR profiles generated from low-level DNA samples under standard polymerase chain reaction (PCR) conditions. The method involves capillary electrophoresis with higher injection voltage and extended injection time. STR peak heights increased six-fold. Inherent to the analysis of low-level DNA samples, we observed stochastic amplification artefacts, mainly in the form of allele drop-out and heterozygous peak imbalance. Increased stutter ratios and allele drop-in were rarely seen. Upon STR typing of 10:1 admixed samples, the profile of the major component did not become overloaded when using higher injection settings as was observed upon elevated cycling. Thereby an improved profile of the minor component was obtained. For low-level DNA casework samples, we adhere to independent replication of the PCR amplification and boosted capillary electrophoresis.

Introduction

In forensic disputes, the evidentiary items can hold bodily fluids or contact traces. Especially from handled items, the DNA recovery is often low, resulting in incomplete DNA profiles in standard forensic procedures. Several options exist to enhance the sensitivity of DNA genotyping. First, the extract containing the DNA can be concentrated (1) by various methods after which fewer polymerase chain reaction (PCR) amplifications are possible, a disadvantage that can be met, in part, by using reduced-volume reactions (2,3). Second, the PCR method can be adjusted, and the techniques vary from nested PCR and whole genome amplification (4,5) to the use of an increased number of amplification cycles (6–8). Third, post-PCR purification has been put forward as a simplified approach for low-level DNA analysis (9), and in our laboratory ChargeSwitch™ magnetic beads (Invitrogen, Breda, The Netherlands) were successfully used to increase peak heights (PH) three-fold (C.C.G. Benschop and T. Sijen, unpublished results). The most popular method is to elevate the number of PCR cycles from 28 to 34, which is based on the observation that 34 cycles are sufficient to analyse the DNA present in a single diploid cell (6). The above-described methods effectively improve DNA profiling, but artefacts, such as allele drop-out, heterozygous peak imbalance, increased stutter peaks, and sporadic contamination (allele drop-in) (6–8,10) are observed. These issues are generally dealt with by generating a so-called consensus profile based on multiple independent PCRs (11).

In this study, we explored the effects of using higher electrokinetic injection settings to increase the sensitivity of genotyping (12). Short tandem repeat (STR) PH would increase with minimal handling, costs, and consumption of PCR product. We compared characteristics of low-level DNA genotyping data obtained using boosted injection and more amplification cycles. We examined the possibilities of improving the genotyping data of the minor component in 10:1 admixed samples (13–15).

Materials and Methods

DNA Samples and STR profiling

Three pristine DNAs were used: DNA 007, the reference DNA in the AmpF*l*STR® SGM Plus™ (SGM+) kit (Applied Biosystems [AB], Nieuwerkerk aan de IJssel, The Netherlands), human DNA (hDNA), the reference DNA in the Quantifiler™ kit (AB), and DNA9947A representing the reference DNA in the AmpF*l*STR® Profiler™ (Profiler) kit (AB). Mock casework samples consisted of plastic straws and cups, from which volunteers (with known STR profile) drank once, and of plastic tie wraps, which the volunteers pulled once fiercely. For negative controls unused items were sampled.

All items were UV irradiated for 3 h prior to usage. This procedure did not remove all DNA contamination (F. Beemster and T. Sijen, unpublished results). Samples were taken using standard cotton swabs and isolated with the QiaAmp 96 DNA Swab Biorobot Kit on a Qiagen Biorobot Universal System (Qiagen, Venlo, The Netherlands).

DNA quantification of DNA isolations was performed using the Quantifiler™ kit (AB), and analysis was performed on a 7900 real time PCR (AB). STR profiling was performed using the SGM+ kit (AB). For 28 + 6 amplifications, 10 µL of 28-cycling PCR product was transferred to a new tube, 0.5 µL of fresh AmpliTaq™ polymerase (AB) was added, and the SGM+ PCR protocol was applied for six cycles.

Performa DTR gel filtration cartridges (Edge BioSystems, Gaithersburg, MD) were used to remove residual dye molecules that cause the presence of dye-blobs. To remove all storage liquid, the pre-hydrated cartridges were centrifuged for 3 min at 1600 × *g*, placed on a new microtube, centrifuged for 2 min at 1600 × *g*, and transferred to a collection tube. An aliquot of 5 µL PCR product was added to the cartridge and collected by centrifugation for 2 min at 850 × *g*.

Capillary Electrophoresis

Electrophoresis was performed on a 3130XL Genetic Analyzer (AB). Injection settings varied as described in the experiments. Injection mixtures for standard 3 kV, 10 sec injections consisted of 1 µL PCR product, 0.4 µL Gene-Scan™-500 size marker (AB), and 8.6 µL HiDi-formamide (AB). For 9 kV/15 sec injections, 1 µL PCR product, 0.05 µL Gene-Scan™-500, and 8.95 µL Hi-Di™ formamide were mixed. For 9 kV/15 sec injections of Performa™ DTR gel filtration cartridges purified PCR mixtures, 2 µL product, 0.02 µL Gene-Scan™-500, and 7.98 µL Hi-Di™ formamide were mixed. The number of allelic ladder in runs with higher injection settings or purified PCR products was reduced 20-fold. Samples were denatured for 4 min at 98°C and rapid-cooled on ice blocks.

STR Typing Analysis

GeneMapper™ ID Version 3.2.1 software (AB) was used to analyse STR profiles and determine the PH. Heterozygote balance, calculated as lowest peak/highest peak, was determined when both concordant alleles in heterozygous loci were present.

All STR profiles that were based on 28 cycling PCR products were analysed using the marker specific stutter ratios provided by AB GeneMapper® ID software (D3S1358, 11 %; vWA, 11 %; D16S539, 13 %; D2S1338, 15 %; amelogenin (AMEL), 0 %; D8S119, 12 %; D21S11, 13 %; D18S51, 16 %; D19S433, 17 %; THO1, 6 %; FGA, 11 %). In profiles based on 34 or 28 + 6 cycling conditions, stutter percentages were 1.5 times of these marker-specific ratios.

Our casework interpretation guidelines aimed to prevent false homozygote calling by removing allele calls for all single peaks below 100 rfu and single peaks between 100 and 175 rfu when uncalled peaks were visible. These interpretation guidelines were applied to the mock casework samples. In profiles obtained by boosted injection or elevated cycling, all peaks above the allele calling threshold of 50 rfu were included in the genotyping result. To enable direct comparison of the sensitivity of allele detection in amplifications of pristine DNA samples and also for the STR profiles obtained by standard procedure (28 cycles, 3 kV / 10 sec injection), all alleles above 50 rfu were included in the genotyping data.

Baseline noise was determined as the maximum rfu value for the FAM, JOE, and NED channels in the region 250 to 295 nt of electropherograms of negative amplifications. This region was chosen because it is uninfluenced by dye-blobs or size marker pull-up peaks.

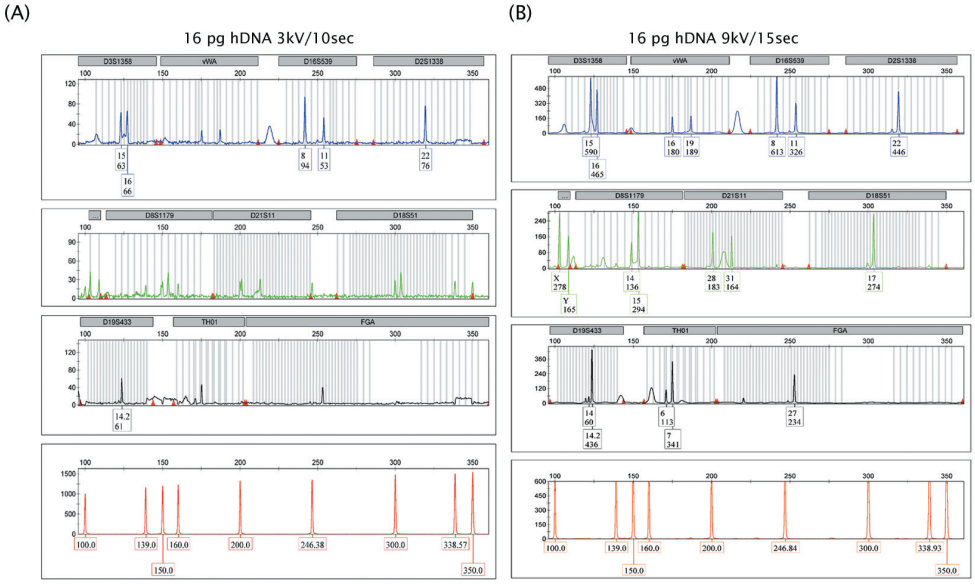
When determining the percentage of concordant alleles present in a SGM+ STR profile, homozygous alleles were counted as 2. Locus drop-out was calculated as 0 alleles detected for that locus. A drop-out allele refers to an undetected allele in a heterozygous locus where the other allele was called. Drop-in alleles refer detected non-concordant alleles. Drop-in alleles could occur due to polymerase slippage (such as stutters at -1 position that were 4 nt shorter PCR products or stutters at +1 position that were 4 nt longer PCR products), or due to sporadic DNA contamination of sampled items.

A consensus profile in our laboratory consisted of alleles called upon standard STR profiling and alleles detected in *n*-1 of the profiles obtained after high sensitivity analysis in which *n* stands for the number of PCR repetitions performed.

Results and Discussion

Higher Injection Settings for Capillary Electrophoresis

For an ABI 3130xl Genetic Analyzer (AB), recommended injection settings resided between 1–3 kV and 3–22 sec, and the standard injection setting in our laboratory was 3 kV / 10 sec. However, the apparatus allowed injections up to 15 kV for 600 sec. Injection conditions affect peak shape; generally higher injection voltages sharpen peaks while longer injection times broaden peaks. We raised the injection voltage from 3 to 6, 9, 12, and 15 kV and the injection time from 10 to 15, 20, 25, 30, 40, 50, 60, 120, and 300 sec, and found that injection at 9 kV during 15 sec resulted in peak shapes that still allowed correct binning and discrimination from background structures like spikes and blobs, while improving sensitivity (Fig. 1A and B). Boosted injection did not result in an increase in baseline noise nor in the presence of contaminating alleles in



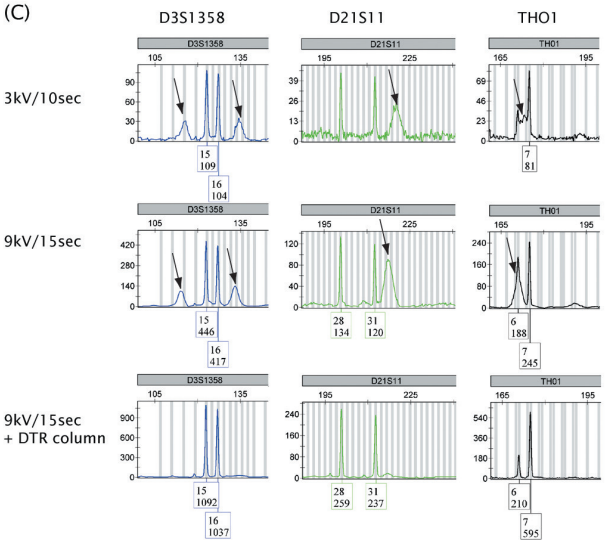


Fig. 1 Comparison of SGM+ profiles obtained using standard (A) and higher (B) injection settings. Polymerase chain reaction (PCR) was performed on 16 pg pristine DNA (hDNA). In the box underneath an allele, the upper number represents the allele call and the lower number, the peak height (in rfu). After boosted injection, all alleles except allele 19 in the FGA locus were detected. (C) Dye-blobs intensified upon boosted injection but could be removed using Performa™ DTR gel filtration cartridges. Dye-blobs in the loci D3S1358, D21S11, and THO1 are shown and indicated by arrows. The filtration cartridges removed dye molecules, nucleotides, and salts, which allowed the favored uptake of PCR products as was apparent from a further increase in peak heights.

30 negative amplification controls. Therefore, the detection threshold for standard STR analysis, which was at 50 rfu in our laboratory, was not changed. Dye-blobs that were present in profiles obtained using standard settings intensified upon boosted injection. They occurred at predictable positions, and could be discriminated from true alleles by peak shape. The residual dye molecules that caused these dye-blobs were efficiently removed by cleaning the PCR products over Performa DTR gel filtration cartridges (Fig. 1C) (16). These filtration cartridges removed dye molecules, nucleotides, and salts, after which the uptake of PCR products and a further increase in PH was observed (Fig. 1C).

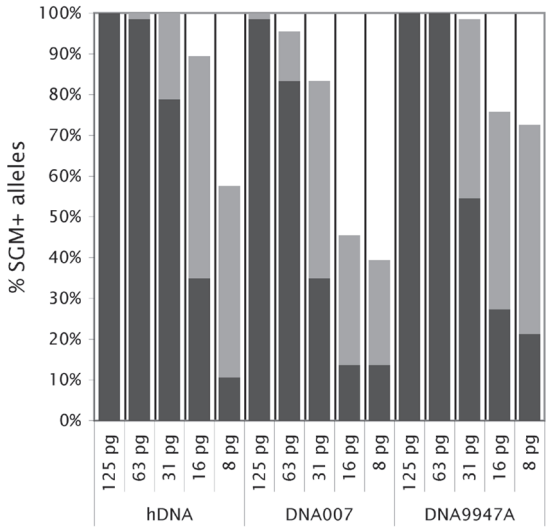


Fig. 2 Improved SGM+ genotyping data obtained by boosted capillary electrophoresis. Five DNA inputs (125, 63, 31, 16, and 8 pg) of three pristine DNAs (hDNA, DNA007, and DNA9947A) were used. Average percentages of detected alleles of triplicate polymerase chain reactions are presented. Dark grey bars indicate alleles called upon injection at 3 kV / 10 sec; light grey bars correspond to additional alleles called after injection at 9 kV / 15 sec. Missing alleles occurred predominantly at the larger amplicons (results not shown).

For three pristine DNA samples (DNA 9947A, DNA 007, and hDNA), triplicate SGM+ PCRs with five different DNA inputs (125, 63, 31, 16, and 8 pg) were analysed using standard 3 kV/10 sec injection and increased 9 kV/15 sec injection. While at normal injection settings, 125 pg DNA was required to obtain a full SGM+ profile, for one-third of the PCRs with 31 pg DNA input (five diploid cell equivalents), complete profiles were obtained. Signal strength (measured in rfu) increased 6.1-fold (average of 515 alleles). The genotyping results generated by boosted injection were based on amplifications from low-level DNA inputs and accompanied by allele drop-outs (mainly for lower DNA inputs) and heterozygous peak imbalance while allele drop-ins and increased -1 stutters occurred sporadically (mainly for higher DNA inputs) (Table 1). Therefore, we analysed STR profiles generated by boosted injection using standard marker-specific stutter ratios. As boosted injection aimed to obtain a maximum of genotyping information, we did not make use of an analysis threshold to prevent false homozygote calling. Figure 2 shows the average of the SGM+ genotyping results in the

triplicate PCRs of the various DNA inputs using standard and increased injection. Incomplete but informative profiles (genotyping data for at least 50 % of the SGM+ STR loci) were obtained for the amplifications using 8 or 16 pg DNA input (Figs. 1 and 2). Missing alleles occurred predominantly at the larger amplicons (results not shown).

We studied the effects of boosted injection in SGM+ profiles in mock casework samples of known single donor origin that consisted of 228 positive samples and 108 negative controls (Table 2). Almost all of the positive samples with an incomplete profile upon standard injection showed an increase of concordant alleles when analysed after boosted injection (on average 8.9 additional alleles per profile) (Table 2). Drop-out alleles were found in 78.6 % of the improved profiles (Table 2).

TABLE 1—*Occurrence of stochastic amplification artifacts in SGM+ genotyping data obtained by boosted injection.*

Method	No. Dropouts	No. Drop-ins	Heterozygote Balance
3 kV, 10 sec	0 (n = 45 profiles)	0 (n = 45 profiles)	0.69 ± 0.19 (n = 182 pairs)
9 kV, 15 sec	94 (n = 45 profiles)	13 (n = 45 profiles)*	0.52 ± 0.23 (n = 110 pairs)†
	0 (125 pg)	6 (125 pg)	
	3 (63 pg)	4 (63 pg)	
	13 (31 pg)	3 (31 pg)	
	29 (16 pg)	0 (16 pg)	
	49 (8 pg)	0 (8 pg)	

*8 at −1 stutter position; 3 at +1 stutter position.
†Only alleles added after 9 kV/15 sec analyses.
Five amounts of DNA input (125, 63, 31, 16, and 8 pg) of three pristine DNAs (hDNA, DNA007, and DNA9947A) were used in triplicate polymerase chain reactions.

TABLE 2—*Analysis of SGM+ genotyping data obtained by boosted injection of mock casework samples of single donor origin with known DNA profile.*

No. Profiles	No. Among Positive Samples	No. Among Negative Samples
Samples	228	108
Incomplete 3 kV/10 sec	204–6.0 detected alleles/profile	0
Improved 9 kV/15 sec	201–8.9 additional alleles/profile	0
With sporadic contamination 3 kV/10 sec	6 (2.6%)–1.7 alleles/profile	0
With sporadic contamination 9 kV/15 sec	45 (19.7%)–1.7 alleles/profile*	24 (22.2%)–2.3 alleles/profile
With dropout 3 kV/10 sec	0	0
With dropout 9 kV/15 sec	158 (78.6%)–2.1 alleles/profile	0

*Average number of alleles determined among those profiles in which sporadic contamination was observed.

Sporadic contamination was found in 2.6 % of the standard DNA profiles and in 19.7 % of the improved DNA profiles. Also in 22.2 % of the negative samples sporadic contamination was observed when using boosted injection, and we inferred that low amounts of unrelated DNA were present on the items before usage by the volunteers (Table 2). Boosted injection effectively increased the sensitivity of STR typing in mock casework samples. Improved profiles were obtained for various profiling methods like SGM+, Profiler, and Y-Filer and for samples of various origins (blood, saliva, sperm, skin epithelial, vaginal epithelial, and hair roots) that were collected by various sampling methods (cotton swap, nylon swap, tape lift, and laser micro dissection) (results not shown).

Higher Injection Settings Versus Increased PCR Cycle Number

The most frequently used low-level DNA profiling approach was increased PCR cycling by either performing 34 cycles on a fresh PCR or taking a portion of the 28 cycles PCR mixture, adding fresh polymerase, and amplifying for an additional six cycles (17). The 28 + 6 cycling approach had the advantage that both standard and high sensitivity genotyping data were obtained from a single DNA input. We compared triplicate PCRs of various DNA inputs (125, 63, 31, 16, and 8 pg) of three pristine DNA samples for standard 3 kV/10 sec injection, boosted 9 kV/15 sec injection, 34 PCR cycles, and 28 + 6 PCR cycles. Standard injection, boosted injection, and 28 + 6 cycling were performed on the same sample. Overloaded profiles with many pull-up peaks occurred for several DNA inputs especially when using elevated cycle number, and only the amplifications with DNA inputs of 16 and 8 pg could be analysed using all three low-level DNA techniques. The average percentage of SGM+ alleles detected in the profiles generated

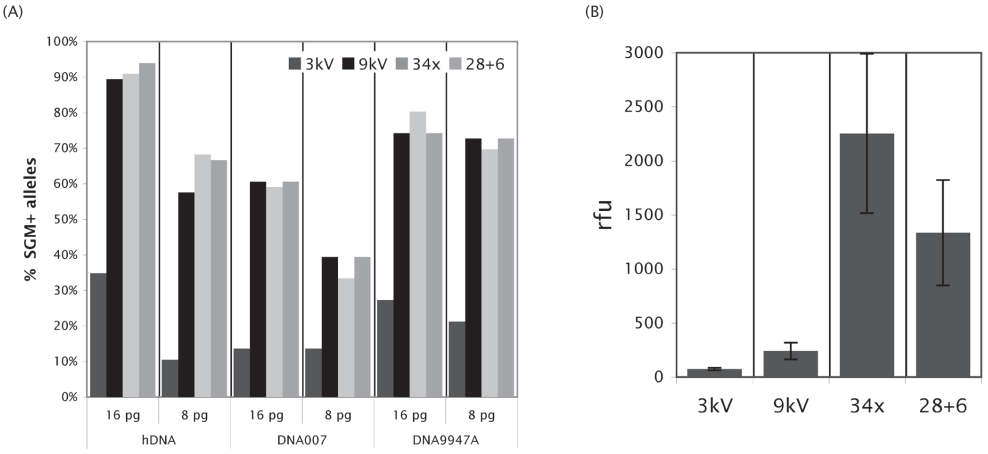


Fig. 3 (A) Boosted injection and increased polymerase chain reactions (PCR) cycle number resulted in a similar level of improvement of SGM+ genotyping. Average percentages of detected alleles of triplicate PCRs are presented. Dark gray bar, 3 kV / 10 sec injection; black bar, 9 kV / 15 sec injection; light gray bar, 34 amplification cycles; middle gray bar, 28 + 6 amplification cycles. Three pristine DNA samples (hDNA, DNA007, and DNA9947A) and two DNA inputs (16 and 8 pg) were used. (B) The average peak height (in rfu) was several fold higher when performing increased cycling. The data represented all alleles called in triplicate PCRs of both 16 and 8 pg DNA inputs. For each method, different numbers of allele calls were obtained: 3 kV / 10 sec injection 61 alleles; 9 kV / 15 sec injection 234 alleles; 34 amplification cycles 241 alleles, 28 + 6 amplification cycles 243 alleles. For boosted injection, peak heights increased 6.1-fold and for 28 + 6 cycling 35.0-fold (average of 61 alleles).

by these three different techniques was quite similar (Fig. 3A). However, the average peak height (in rfu) was several folds lower with boosted injection than upon increased cycling (Fig. 3B). Hyper-amplification was known to be accompanied by increased -1 stutter ratios, which was why elevated cycling genotyping data were analysed using 1.5-fold locus-specific stutter ratios (boosted injection profiles are analysed using the standard stutter ratios). All three low-level DNA methods were accompanied by a similar level of heterozygous peak imbalance (Table 3) and a similar frequency of allele drop-outs (Table 3). However, allele drop-ins occurred more frequently in the profiles generated by increased cycling (Table 3). Most drop-in alleles represented +1 stutters (Table 3), which was most likely due to the higher amplification levels in the 34 and 28 + 6 cycling PCRs (14).

TABLE 3—*Occurrence of stochastic amplification artifacts in SGM+ genotyping data obtained by boosted injection and increased cycling.*

Method	Heterozygote Balance	No. Dropouts	No. Drop-ins
3 kV, 10 sec	0.74 ± 0.08 (n = 6 pairs)	0	0
9 kV, 15 sec	0.58 ± 0.11 (n = 58 pairs)*	77 (n = 18 profiles)	0
34x	0.54 ± 0.12 (n = 71 pairs)	60 (n = 18 profiles)	5 (n = 18 profiles)†
28 + 6	0.55 ± 0.13 (n = 63 pairs)*	72 (n = 18 profiles)	5 (n = 18 profiles)‡

*Only alleles added after 9 kV/15 sec or 28 + 6 cycling.
†1 at −1 stutter position; 3 at +1 stutter position; 1 at other position.
‡1 at −1 stutter position; 4 at +1 stutter position.
Two amounts of DNA input (16 and 8 pg) of three pristine DNAs (hDNA, DNA007, and DNA9947A) were used in triplicate polymerase chain reactions.

Higher Injection Settings to Enhance Detection of the Minor Component in Mixtures

We studied the effect of boosted injection versus more amplification rounds for the ability to improve genotyping of the minor component in mixtures. We used triplicate PCRs of 10:1 admixed samples of DNA9947A and hDNA with DNA inputs of 630 pg + 63 pg, 310 pg + 31 pg, 160 pg + 16 pg, and 80 pg + 8 pg. Eighteen of the 22 alleles in a SGM+ profile were distinct for hDNA in comparison to DNA9947A. Upon boosted injection, the major contributor induced overloaded profiles for the 630 pg + 63 pg and 310 pg + 31 pg DNA inputs. With more PCR cycles, all mixtures became over-amplified as was apparent from the occurrence of many pull-up peaks, but we analysed the 80 pg + 8 pg mixtures notwithstanding the presence

TABLE 4—*Genotyping results of 10:1 admixed samples using standard injection, boosted injection, and increased cycling.*

Method	Input DNA9947A + hDNA	Alleles Major*	Alleles Minor†	Drop-Ins/ Per Profile
3 kV	160 pg + 16 pg	22/22	8.3/18	0
3 kV	80 pg + 8 pg	21/22	2.7/18	0
9 kV	160 pg + 16 pg	22/22	14/18	1
9 kV	80 pg + 8 pg	22/22	11/18	0
34x	80 pg + 8 pg	22/22	14.7/18‡	4.7§
28 + 6	80 pg + 8 pg	22/22	11/18‡	2.7¶

Average of triplicate polymerase chain reaction is presented.
*Calculated over all 22 SGM+ alleles.
†Calculated over 18 alleles for which minor component differs from major component.
‡Occasionally alleles of minor contributor at stutter position removed manually upon using *c.* 15% stutter ratios.
§2.0 at −1 stutter position; 1.3 at +1 stutter position; 1.3 at other positions.
¶0.3 at −1 stutter position; 1.3 at +1 stutter position; 0.3 at other positions.

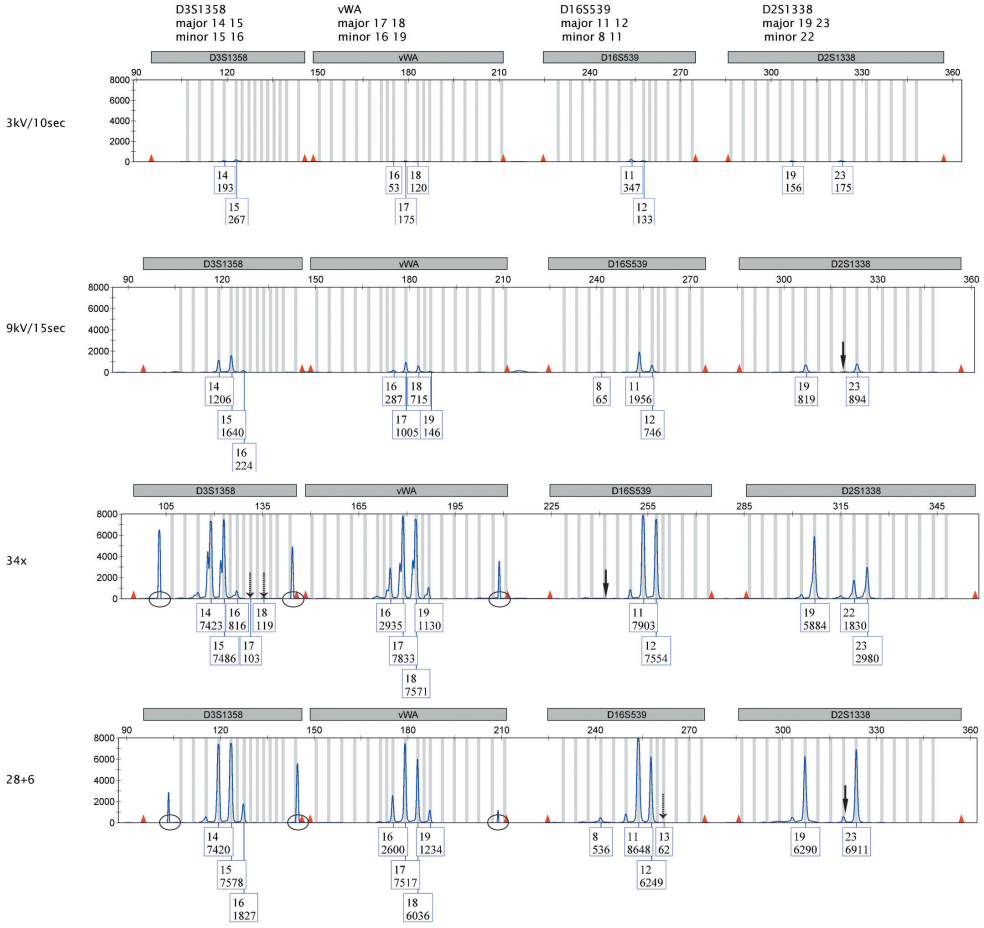


Fig. 4 Comparison of SGM+ genotyping results obtained for a 10:1 admixed sample consisting of 80 pg DNA9947A and 8 pg hDNA for standard DNA analysis and three low-level DNA techniques. Genotyping data are shown for the loci D3S1358, vWA, D16S539, and D2S1338 present in the FAM channel. Full arrows indicate the position of a drop-out allele of the minor component. Dashed arrows indicate drop-in alleles. Circles indicate pull-up peaks. In the box underneath an allele, the upper number represents the allele call and the lower number the peak height (in rfu).

of these pull-up peaks (Fig. 4). Full profiles were obtained for the major contributor (DNA9947A, input 80 pg) for all three low-level DNA methods. Boosted injection revealed a similar number of detected alleles of the minor component as 34 and 28 + 6 cycling did (Table 4, Fig. 4). Boosted injection was found to result in less allele drop-ins

TABLE 5—*Consensus profiles of minor component in 10:1 mixtures generated by standard and boosted injection.*

Sample	D3	vWA	D16	D2	AMEL	D8	D21	D18	D19	THO1	FGA
Major	14 15	17 18	11 12	19 23	X	13	30	15 19	14 15	8 9.3	23 24
Minor	15 16	16 19	8 11	22	XY	14 15	28 31	17	14 14.2	6 7	19 27
Specific*	16	16 19	8	22	Y	14 15	28 31	17	14.2	6 7	19 27
16 pg 3 kV, #1	–	16 19	8	–	–	15	–	17	–	6	19
16 pg 3 kV, #2	–	–	–	22	Y	15	28	17	14.2	–	19 27
16 pg 3 kV, #3	–	16 19	–	22	–	–	28	17	–	–	–
16 pg 9 kV, #1	16	16 19 *15*†	8	–	–	14 15	28 31 *26.2*	17	14.2 *12*	6 7	19
16 pg 9 kV, #2	–	19 *15*	8	22 *21*	Y	14 15	28 31	17	14.2	6	19 27
16 pg 9 kV, #3	–	16 19	–	22	Y	14	28	17	–	6 7	19 27
Consensus	–	16 19 *15*	8	22	Y	14 15	28 31	17	14.2	6 7	19 27
8 pg 3 kV, #1	–	–	–	22	–	–	–	17	–	–	19
8 pg 3 kV, #2	–	16	–	–	–	–	–	–	–	–	–
8 pg 3 kV, #3	–	–	–	–	–	–	–	17	–	–	–
8 pg 9 kV, #1	–	19	–	22	Y	14 15	28 31	17	–	7	19
8 pg 9 kV, #2	16	16 19	8	–	Y	14 15	31	–	–	6 7	19 27
8 pg 9 kV, #3	–	19	–	22	–	14 15	29	17	–	7	
Consensus	–	16 19	–	22	Y	14 15	31	17	–	7	19

AMEL, amelogenin.
*Specific alleles for minor contributor.
†Drop-in alleles are shown in italics.
Polymerase chain reactions were performed in triplicate. Two DNA inputs (160 pg + 16 pg and 80 pg + 8 pg of DNA9947A + hDNA) were used.

(Table 4, Fig. 4). Three alleles of the minor component were at stutter position of an allele of the major component (Table 5), and may therefore be masked. This was only observed in profiles obtained after elevated cycling.

We generated consensus profiles for the 160 pg + 16 pg and 80 pg + 8 pg mixtures after boosted injection (Table 5). The 160 pg + 16 pg consensus profile contained 17 of the 18 distinct alleles of the minor component plus one drop-in allele and the 80 pg + 8 pg consensus profile comprised 11 of the 18 distinct alleles (Table 4). We inferred that boosted injection could be of use to obtain more genotyping data on a minor component consisting of 8 or 16 pg DNA in 10:1 admixed samples, which was not achieved by using 28 + 6 or 34 amplification rounds as the samples get over-amplified. We expected more robust improvements from the use of higher injection settings when analysing more evenly balanced mixtures (e.g., 5:1 or 2:1 mixture ratios, with a maximum DNA input of 100 pg).

Concluding Remarks

In this study we have shown that incomplete STR profiles can be efficiently improved by increasing the capillary electrophoresis injection settings on an ABI3130XL from 3 kV / 10 sec to 9 kV / 15 sec. Peak heights (in rfu) increased on average 6-fold. Amplification using six additional PCR cycles increased the peak height on average 35-fold. Interestingly, the percentage of alleles detected in samples with 16 or 8 pg DNA input was similar upon boosted injection or more PCR cycles, although the average peak height was much lower in the 9 kV/15 sec injection profiles than in those generated with an elevated cycle number. As dye-blobs were also enhanced upon boosted injection, effort might be needed to distinguish true peaks from dye-blobs or remove dye molecules from PCR mixtures, which was hardly an issue in 34-cycle or 28 + 6 cycle profiles. On the other hand, the use of 34 or 28 + 6 amplification rounds could result in overloaded profiles with many pull-up peaks, a feature hardly observed upon boosted injection. As with other low-level DNA methods, artefacts occurred due to stochastic effects during amplification. These included heterozygous peak imbalance, allele drop-outs, and allele drop-ins. Occurrence of increased stutter peaks was rare which implied that boosted injection (unlike elevated cycling) did not necessitate the use of increased stutter ratios. Amplification of pristine DNAs showed that boosted injection and more PCR cycles resulted in a similar level of heterozygous peak imbalance and number of drop-outs in the genotyping data. The main difference between the two approaches was the presence of more allele drop-ins (like +1 stutters) when using additional amplification rounds. The occurrence of allele drop-ins in boosted injection profiles seemed to depend on the type of sample because for pristine DNAs no drop-ins were found while in the majority of our mock casework samples a few alleles due to sporadic contamination were detected. We inferred that the sampled items were not completely free of sporadic DNA contamination before usage by our volunteers.

We were able to improve the genotyping data of the minor component in 10:1 admixed samples by boosted injection, while amplification of these mixtures for 34 or 28 + 6 cycles resulted in over-amplified samples and overloaded profiles that would normally not be analysed. We did not observe masking of alleles of the minor component in the 10:1 admixed samples that were at stutter position of an allele of the major component.

Clearly, boosted injection had to be considered a low-level DNA technique that was accompanied by a substantial number of allele drop-outs. We recommend generating a consensus profile from multiple independent amplifications. In our laboratory, a consensus profile consisted of all alleles detected with the standard procedure plus those alleles that were detected in at least two out of three profiles generated by a low-level DNA procedure. The low-level DNA technique of 9 kV / 15 sec capillary

electrophoresis injection had the advantage that for each separate PCR, both a standard and an enhanced STR profile were easily obtained. In case where higher peaks were required, one could decide to perform 28 + 6 cycling subsequently (17). This could be carried out on the same PCR product without using more DNA extract.

In summary, boosted capillary electrophoresis is a simple method to increase the sensitivity of STR typing. It is accompanied by the occurrence of allele drop-outs and heterozygous peak imbalance, but does not require the use of increased stutter ratios. Boosted injection is suited to improve not only single donor profiles but also the genotyping data of the minor component in mixtures. The method has been accredited for casework in our laboratory.

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Chapter 3

Tri-allelic SNP markers enable analysis of mixed and degraded DNA samples

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Abstract

For the analysis of degraded DNA in disaster victim identification (DVI) and criminal investigations, single nucleotide polymorphisms (SNPs) have been recognised as promising markers mainly because they can be analysed in short sized amplicons. Most SNPs are bi-allelic and are thereby ineffective to detect mixtures, which may lead to incorrect genotyping. We developed an algorithm to find non-binary (i.e. tri-allelic or tetra-allelic) SNPs in the NCBI dbSNP database. We selected 31 potential tri-allelic SNPs with a minor allele frequency of at least 10 %. The tri-allelic nature was confirmed for 15 SNPs residing on 14 different chromosomes. Multiplex SNaPshot™ assays were developed, and the allele frequencies of 16 SNPs were determined among 153 Dutch and 111 Netherlands Antilles reference samples. Using these multiplex SNP assays, the presence of a mixture of two DNA samples in a ratio up to 1:8 could be recognised reliably. Furthermore, we compared the genotyping efficiency of the tri-allelic SNP markers and short tandem repeat (STR) markers by analysing artificially degraded DNA and DNA from 30 approximately 500-year-old bone and molar samples. In both types of degraded DNA samples, the larger sized STR amplicons failed to amplify whereas the tri-allelic SNP markers still provided valuable information. In conclusion, tri-allelic SNP markers are suited for the analysis of degraded DNA and enable the detection of a second DNA source in a sample.

Introduction

DNA used in disaster victim identification (DVI) and forensic human genotyping is often degraded. In DNA profiling, this results in the loss of the higher molecular weight short tandem repeat (STR) markers and, consequently, in lower discrimination power of the obtained partial DNA profiles [1–4]. STR amplicons vary in length between 100 and 450 base pairs (bp). Two different strategies have been proposed to decrease the target region [5–7]: (1) the use of so-called mini-STRs for which the primer binding sites are moved closer to the repeat region resulting in amplicons usually <150 bp [8–10] and (2) single nucleotide polymorphism (SNP) markers that involve the analysis of only one nucleotide resulting in amplicons that can be designed to be as small as 50 bp [11,12]. These very small amplicons make SNPs particularly promising markers for forensic analysis of degraded DNA [13,14].

SNPs have several other advantageous characteristics. One of these advantages is a low mutation rate (10^{-8} versus 10^{-3} for STRs), which makes them useful for paternity testing and complex kinship analysis [15,16]. In addition, SNPs can be analysed using high throughput systems, and are not accompanied by the occurrence of stutter peaks, which simplifies the interpretation of the SNP based profiles [17–19]. The vast majority of SNPs are bi-allelic and these binary SNPs are unable to reliably detect the presence of a second DNA source in a sample [5,17]. However, Phillips et al. have described that non-binary SNPs can detect the presence of a DNA mixture [20]. This is important to recognise, for example, contamination by soft tissue or bodily fluids from other victims during a mass disaster. With computer simulations is estimated that 45–65 bi-allelic SNPs are needed to reach a discrimination power that is equal to 12–16 STRs [21–23]. In theory, less tri-allelic SNPs would be needed, since they have an increased discrimination power per SNP.

In this study we apply non-binary SNPs to forensic relevant samples. We developed an algorithm to search for non-binary SNPs in the NCBI SNP database (dbSNP). For a selection of the tri-allelic SNP candidates that were found, SNaPshot™ multiplex assays were set up, and over 250 reference samples from the Netherlands and the Netherlands Antilles were analysed. A web-based application was written to calculate allele frequencies from the SNP genotyping data. Furthermore, two-donor mixtures in various ratios were studied. Artificially degraded DNA and DNA from approximately 500-year-old bone samples were genotyped both by the tri-allelic SNP assays and standard STR profiling in order to compare the genotyping efficiency of both methods.

Materials and methods

Samples

The reference set for verification of the non-binary nature of the SNPs consisted of 153 Dutch and 111 Netherlands Antilles samples obtained from employees of the Netherlands Forensic Institute, anonymous Dutch blood donors and policemen from the Antilles. The Netherlands Antilles population has an admixed origin of Native Americans, Europeans and Africans with an undetermined mixture ratio. Y chromosomal research indicates that approximately half of the males from the reference population displays Y chromosomes of African origin (PdK, unpublished results). From the YCC panel that consists of cell lines from males representing worldwide populations, 59 samples were analysed: 5 European, 12 Russian/Siberian, 8 Asian/Pakistan, 9 African, 14 South African and 11 Native American [24]. The sensitivity of the SNPs was determined using pristine DNA (Quantifiler™ Human DNA standard denoted as hDNA) with a wide range of PCR inputs for both the SNP and STR analyses of 5 pg, 10 pg, 20 pg, 30 pg, 40 pg, 50 pg, 60 pg, 70 pg, 80 pg, 90 pg, 100 pg, 200 pg, 300 pg, 400 pg, 500 pg, 750 pg, 1 ng, 10 ng and 50 ng. For mixture analysis, DNA from several pairs of reference donors was mixed in various ratios: 1:8, 1:4, 1:2, 1:1, 2:1, 4:1 and 8:1.

In order to obtain artificially degraded DNA, pristine hDNA of 200 ng/μL was irradiated with 254 nm UV light in a CL-1000 UV CrossLinker (UVP, Inc.) at 0.9 J/cm2 for 0, 5, 10, 15, 30, 60, 90 and 120 min. Two series of hDNA were used: hDNA irradiated at room temperature or hDNA denatured for 5 min at 95 °C and placed and irradiated on ice. Furthermore, pristine hDNA samples were degraded by different concentrations of TURBO™ DNase (Ambion™ TURBO DNA-free™ Kit). DNA fragments of specific size ranges were isolated from agarose gel with the QIAquick Gel Extraction Kit (Qiagen), and diluted to 1 ng/μL after DNA concentration measurement with a NanoDrop™ 1000 spectrophotometer (Thermo Scientific). Genotyping efficiency was determined using the artificially degraded DNA samples and DNA of thirty 450–550-year-old bone and molar samples excavated in Delft (the Netherlands).

SNP selection

To find non-binary SNP candidates, the NCBI database dbSNP (build 126) was searched with a custom-made algorithm, which can be found on http://www.liacs.nl/rvjlaros/projects/snp/. This algorithm specifically searches for non-binary SNPs with variation allele: V (A, C or G), H (A, C or T), D (A, G or T), B (C, G or T) or N (G, A, T or C) and SNP class: snp. It filters out any unconfirmed allele calls from opposite strands (N) and non-existent data (-). SNPs with a minor allele frequency (i.e. the lowest frequency of the three alleles) above 10 % in at least one population in dbSNP were selected for further analysis. SNPs can erroneously be assigned non-binary due

to a lack of clarity regarding the direction of the sequence reads entered into dbSNP. Therefore, the non-binary character and allele frequencies of the SNP candidates were redetermined after manual entering in dbSNP. The test set of SNPs was selected on the following criteria: (1) a high minor allele frequency, (2) a high number of populations that showed three alleles for that SNP, (3) an equal distribution of the other two alleles, and (4) the opportunity to develop suitable primers. To diminish the chance of linkage between the SNPs, one SNP per chromosome was selected from the test set for the development of the SNaPshot™ multiplexes.

PCR

The web-based version of Primer3 was used to design PCR primers (supplementary table S1) resulting in amplicon sizes between 40 and 100 bp, with a primer length between 18 and 24 bases, a primer Tm between 55 and 61 °C and a primer GC percentage between 30 and 70 % [25]. All primers were checked for the absence of primer–dimer formation, hairpin structures and complementarity to other primers in the multiplex with the program Autodimer [26]. The primers were all HPLC purified after synthesis (Biolegio BV or Isogen Life Science).

A 12.5 µL PCR was set up using 1× PCR Gold buffer, 9 mM MgCl2, 2 mM dNTPs, 0.5 µL Taq Gold, 100 nM of each primer and 1 ng DNA. The PCR program consisted of an initial hot start of 10 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and a final hold at 72 °C for 5 min. To remove unused primers and nucleotides 2.5 µL ExoSAP-IT™ (USB Corporation) was added to the PCR products. This enzyme mixture was incubated for 30 min at 37 °C and inactivated by incubation at 80 °C for 15 min.

All measurements were performed in dedicated laboratories (ISO 17025 accredited), while wearing protective clothing. For comparison of the SNP markers with STR markers, the AmpF*l*STR® SGM Plus™ kit from Applied Biosystems was used according to their protocol, but with half the volumes (25 µL PCR).

SNaPshot™ (single base extension)

Extension primers were designed immediately upstream of the SNP position [11,27]. Primer3 was used to design the primers with a primer size between 15 and 23 bases, a Tm between 48 and 52 °C, and a GC percentage between 25 and 60 % [25]. Since the SNPs are analysed in multiplex they need to be spatially separated during capillary electrophoresis; therefore the extension primers were tailed at the 5' end with a non-human DNA sequence resulting in primer sizes between 23 and 50 nucleotides.

Terminator ddNTPs, labeled with four different fluorescent dyes, were used to extend the primers at the SNP position. The extension reaction was set up with

2.5 µL SNaPshot™ Ready Reaction Mix (Applied Biosystems), extension primer concentrations between 5 and 75 nM (supplementary table S1), 1.0 µL ExoSAP-IT™ treated PCR product and added up to a total volume of 5 µL. The extension program has an initial denaturation step of 2 min at 96.0 °C, followed by 40 cycles of 96.0 °C for 10 s, 50.0 °C for 5 s and 60.0 °C for 30 s. To remove unincorporated nucleotides 1.5 µL Shrimp Alkaline Phosphatase (USB Corporation) was added to the extension products, incubated for 60 min at 37 °C and inactivated for 15 min at 72 °C.

SNP detection and analysis

The fluorescently labeled SNaPshot™ extension products were detected by capillary electrophoresis with an ABI Prism 3130xl Genetic Analyzer with a 36 cm capillary array and POP-4 polymer (Applied Biosystems). Data Collection software v3.0 with the default run module SNP36_POP4_1 and dye set E5 were used to analyse 1.0 µL SAP-treated extension product mixed with 8.75 µL Hi-Di™ formamide and 0.25 µL GeneScan-120LIZ™ size standard (Applied Biosystems) after 5 min of denaturation at 95 °C and 5 min of cooling on ice.

Alleles were automatically called with GeneMapper® ID v3.2.1. Since the ratio of the fluorescent signals for G, A, T and C differ per nucleotide dye and between SNPs, the allele balance cut-off value in the SNaPshot™ default analysis method was adjusted from 0.30 to 0.125 in order to call both G and C in a heterozygous locus. For the analysis of the dilution series and the artificially degraded DNA, reference DNA samples with a known SNP and STR profile were used. For these samples, the percentage of detected alleles could be calculated, in which homozygous alleles were counted as two alleles. Since the SNP and STR profiles of the analysed bone and molar samples were unknown, homozygous alleles could not be discriminated from a single heterozygous allele without the second allele. For these samples only the percentage of detected loci is determined.

Allele frequencies and statistics

To calculate the allele frequency distribution of the Dutch and Netherlands Antilles samples, genotype tables are exported from the GeneMapper® plot display to a .csv-file and copied into SNPstat, a custom-made program that can be found on http:// www.liacs.nl/rvhmeiland/projects/snpstat/. Expected and observed heterozygosity values and PIC values are calculated using the Excel Microsatellite Toolkit [28]. Genepop v4.0.7 is used to determine the p-value for Hardy–Weinberg (HW) equilibrium testing, for HW testing when H1 = heterozygote deficit, when H1 = heterozygote excess and for deviation from independence between or across loci [29]. The power of discrimination and the power of exclusion were calculated with the Excel spreadsheet Genetic Identity PowerStats v12 (Promega) [30].

Sequencing

Sanger sequencing was used to confirm the different alleles found with the SNaPshot™ method in the Dutch and Netherlands Antilles reference samples. Monoplex PCRs were performed under the same conditions and with the same primers as described above, and the PCR products were cleaned with 1.5 µL ExoSAP-IT™ (USB Corporation). The sequencing reaction was performed in a volume of 20 µL with 1x Sequencing Buffer, 1.0 µL BigDye™ Terminator v1.1 Ready Reaction Mix (Applied Biosystems), 0.16 mM SNP specific primer and 1.0 µL ExoSAP-IT™ treated PCR product. To remove unincorporated nucleotides and salts the 20 µL sequencing product was mixed with 20 µL XTerminator™ Solution and 90 µL SAM™ Solution in a MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems). The plate was vortexed for 30 min at 2000 rpm, centrifuged for 2 min at 1000 × *g*, placed directly in an ABI Prism 3130xl Genetic Analyzer and analysed with the BDx_UltraSeq36_POP4_1 run module and dye set E-BigDyeV1. The sequences were analysed with Sequencing Analysis 5.2 (Applied Biosystems).

Results and discussion

SNP selection and multiplexing

Using our custom-made algorithm, dbSNP was searched for non-binary SNPs with a minor allele frequency above 10 % in at least one population. This search yielded 74 SNP candidates. After manual entry in dbSNP to correct for inconsistencies in direction of sequencing, 63 tri-allelic SNP candidates remained, which are listed per chromosome in Table 1 and supplementary table S2. To obtain a tri-allelic SNP for chromosome 3, the minor allele frequency had to be lowered to 6 % (Table 1). Since the first candidate on chromosome 22 (rs3859849) yielded no useful primers, a second tri-allelic SNP candidate was found by lowering the minor allele frequency to 8 % (Table 1). Thereby a total of 65 tri-allelic SNP candidates was obtained. For chromosome 13, 15, X and Y no suitable SNPs were found. In contrast, the number of tri-allelic SNPs found on chromosome 6 is considerable compared to the other chromosomes, probably due to extensive research to a particular part of this chromosome.

The 65 tri-allelic SNP candidates are distributed over 20 chromosomes. The chance of linkage between the SNPs is minimised when they reside on different chromosomes. In order to find the best SNP per chromosome, PCR and extension primers were tested in monoplex for the 31 SNPs that are shown in Table 1. To reduce the number of reactions and the amount of DNA needed, SNaPshot™ multiplex assays were designed. The primer sets that were examined for the SNPs on chromosome 16, 17, 19 and 21 proved unsuited for multiplexing, probably due to interactions with other PCR

Table 1
Examined potential non-binary SNPs found in dbSNP with a custom-made algorithm.

Chromosome	rs-number	dbSNP maximum minor allele frequency[a]	SNP name in multiplex	Allele frequencies for SNPs examined in multiplex							
				NL (*n* alleles = 306)				ANT (*n* alleles = 222)			
				A	C	T	G	A	C	T	G
1[b]	**3091244**	**26.1**	01a	31.37		6.21	62.42	31.08		25.68	43.24
1	1630312	20.3									
1	951416	19.3									
2	**727241**	**21.4**	02a		26.47	73.53			21.17	78.83	
2	1047883	11.5									
3	**35528968**	**6.2**	03a			100.00				100.00	
4	4540055	27.1									
4	**356167**	**17.5**	04b	17.65	11.11		71.24	4.05	24.77		71.17
5	**9329104**	**20.8**	05a	87.91			12.09	58.11	12.61		29.28
6	**9274701**	**28.6**	06a		+[d]	+	+		+	+	+
7	**2032582**	**15.2**	07a	0.98		43.79	55.23	1.35		10.36	88.29
8	**433342**	**18.8**	08a		70.59	27.78	1.63		36.04	35.59	28.38
8	4532634	14.4	[c]								
9	**1112534**	**12.5**	09c	22.22			77.78	26.13			73.87
9	3818367	10.1									
10	**17287498**	**24.0**	10e	23.86	51.63	24.51		43.69	46.40	9.91	
10	2803554	14.6									
10	9333212	11.9									
11	**5030240**	**14.8**	11a		26.80	7.84	65.36		29.28	32.88	37.84
11	11042874	12.5									
12	**2307223**	**26.8**	12a	81.05		16.67	2.29	28.38		9.91	61.71
12	7133606	15.3									
14	**1008686**	**10.0**	14a	43.79		56.21		63.51		36.49	
16	2278489	13.0									
16	11865501	10.0	[c]								
17	1050528	27.1	[c]								
18	**2853525**	**10.4**	18a		32.03	67.97		8.11	39.19	52.70	
19	385780	26.1	[c]								
20	**2069945**	**15.9**	20a		45.75	11.11	43.14		71.62	5.86	22.52
21	471010	10.0									
22	**34741930**	**8.3**	22b		100.00				100.00		

[a] The population with the highest minor allele frequency in dbSNP (when allele frequencies were determined for more than one population).
[b] SNPs shown in bold are tested in multiplex SNaPshot™ assays.
[c] Not suited for multiplex, but three alleles detected in monoplex SNaPshot™ pilot experiments.
[d] Allele frequencies could not be determined due to ambiguous SNaPshot™ results.

or extension primers. The SNPs that were chosen for the remaining 16 chromosomes are shown in bold in Table 1. These 16 SNPs were combined in multiplex A, B, and C with seven, four, and five SNP markers, respectively (supplementary table S1).

In order to test whether these 16 SNPs were non-binary, 153 Dutch and 111 Netherlands Antilles reference samples were analysed using the three multiplexes (supplementary table S3). Nine SNPs were found to be tri-allelic in both populations, and two SNPs were tri-allelic in the Antilles samples but appeared to be bi-allelic in the Dutch samples. Three SNPs were bi-allelic in both populations and two SNPs were fixed. The analyses were extended with 59 samples from the Y Chromosome Consortium dispersed over six genetically distinct populations, but no additional alleles were detected (supplementary table S3). Thereby, 11 of the 16 SNPs in the multiplexes were found to be truly tri-allelic. Monoplex assays on a limited number of samples from the reference set revealed four additional tri-allelic SNPs: a second one on chromosome 8 and three SNPs on chromosome 16, 17 and 19 for which the primers were unsuited for multiplexing (Table 1). Thus, in total 15 SNPs on 14 different chromosomes were confirmed to be tri-allelic.

For the 16 SNPs in the multiplex SNaPshot™ assays 41 different alleles were observed. To confirm the occurrence of these alleles, per SNP up to 8 samples were analysed by Sanger sequencing using the same primers as for the PCRs preceding the SNaPshot™ assays. The alleles that were observed by Sanger sequencing were consistent with the SNaPshot™ results. Eight of the 41 alleles could not be confirmed, which is probably due to ineffective sequencing within the very short sized amplicons. Sanger sequencing was very useful for the interpretation of ambivalent SNaPshot™ results obtained for SNP 06a. While in samples 1 and 2 (Fig. 1A and B, and C and D) a heterozygous GC and a homozygous T are detected by both methods, in sample 3 (Fig. 1E and F) Sanger sequencing clearly detects a homozygous G, while the SNaPshot™ shows a large G-peak and a small additional T-peak. This additional small T-peak is neither observed in the PCR and extension negative controls (data not shown) nor in Fig. 1B. Due to these ambiguous SNaPshot™ results, SNP 06a was left out of further analyses.

The SNaPshot™ was chosen as analysis platform since this method is not dependent on SNP specific probes and the possibility of a third allele does not complicate the analyses. Furthermore, multiplexing is possible, thereby reducing the amount of input DNA required. In addition, most forensic laboratories possess the instruments needed. A disadvantage of the SNaPshot™ is that the amount of fluorescent signal differs per nucleotide dye. The ratio G:A:T:C was estimated to be 3:2:1:1 after measuring the average allele peak heights per SNP in the 153 Dutch samples with SNPstat. This ratio showed some variation both between individual SNPs and between samples for the same SNP. For SNP 11a, the ratio G:C sometimes rose to 8:1, and the allele balance cut-off value in the analysis method was set to 1/8 = 0.125. Due to this difference in signal and to

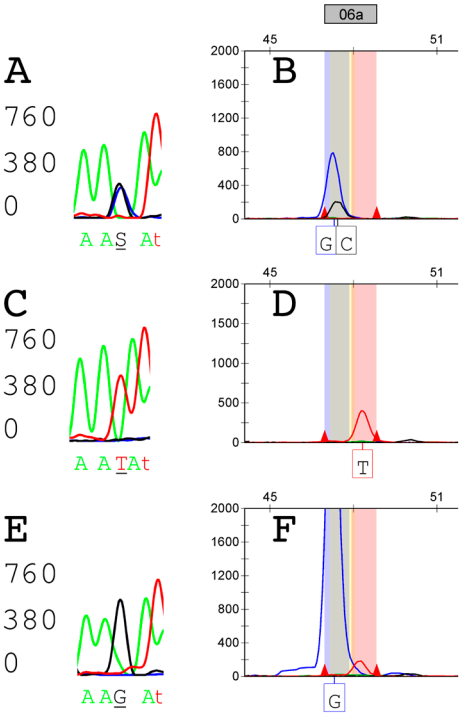

Fig. 1 Sanger sequencing (A, C, E) and SNaPshot™ (B, D, F) results for SNP 06a_rs9275142 for three individuals. (A and B) Heterozygous GC for both methods. (C and D) Homozygous T for both methods. (E and F) Sanger sequencing shows a clear homozygous G, but the SNaPshot™ results show a large G-peak and a small additional T-peak.

the interactions of the many PCR and extension primers present, SNaPshot™ multiplexes require several optimization steps. Phillips and co-workers also encountered these problems and compared four forensically relevant SNP typing techniques: SNaPshot™ genotyping, TaqMan™ real-time PCR assays, Sequenom™ iPLEX™ MALDI-TOF spectrometry and Genplex™ oligo-ligation assays (a modification of the SNPlex™ chemistry), of which the Genplex™ system seemed the most promising alternative [31].

Allele frequencies and statistics

Genotyping data, allele frequency distributions and a summary of the statistics for the SNP markers in the Dutch and Netherlands Antilles reference samples are shown in Table 1 and supplementary tables S3, S4 and S5. A few p-values are below the threshold of 0.05, but after Bonferroni correction, no significant deviation from Hardy–Weinberg equilibrium or linkage was observed. The number of tri-allelic SNP markers that we examined does not suffice to reach a discrimination power that equals 10–15 STR markers, and further research is needed (for discrimination and exclusion powers per SNP see supplementary table S4). Unfortunately, the amount of population data available in dbSNP is rather limited and does not enable an efficient pre-selection of non-binary SNPs with promising allele distributions at the moment. For the 15 SNP markers analysed, the allele distribution per population is visualised in Fig. 2. It is clear that the allele distribution of the tri-allelic SNPs can differ greatly between the two populations. For example, SNPs 08a and 12a both have an allele that is rare in the Dutch samples, while common in the Netherlands Antilles samples, and SNPs 05a and 18a show only two alleles in the Dutch while three alleles in the Netherlands

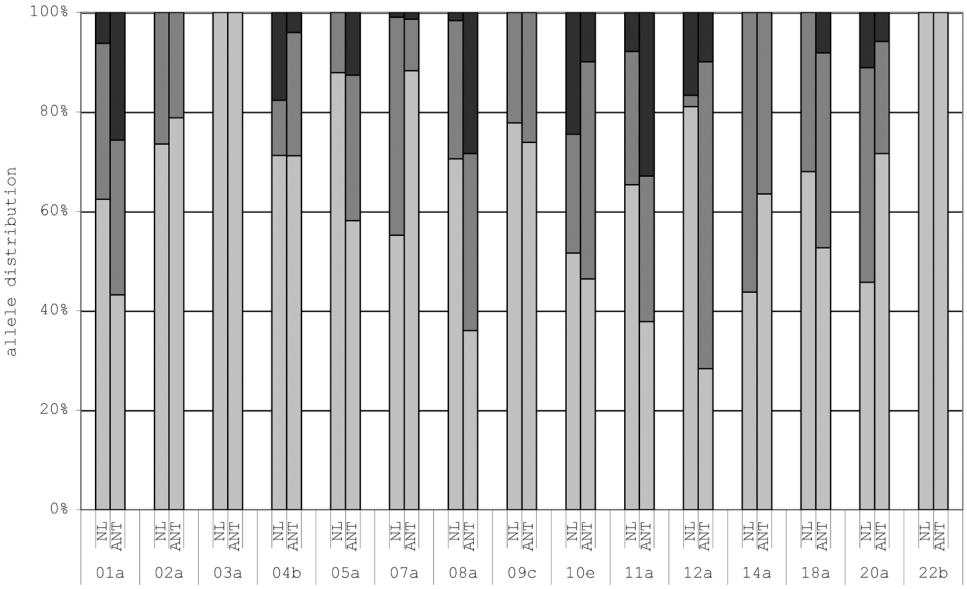

Fig. 2 Allele distributions for the SNPs that were analysed in the Dutch (NL, *n* = 306 alleles) and Netherlands Antilles (ANT, *n* = 222 alleles) samples.

Antilles samples (Fig. 2 and Table 1). As some of the alleles in these SNP markers seem to be determined geographically, these SNPs might not only be interesting for identification, but also as ancestry informative markers (AIMs) [32]. When the source individual of a DNA sample is unknown, AIMs can point out the most likely population of origin [32]. For this purpose, it is important to keep in mind that the examined Dutch DNA samples represent a cross-section of the Dutch population and that the donors may not all have a European background. The allele frequencies of the six YCC population groups are summarised in supplementary table S6 (notwithstanding the small sample sizes). These findings support the suggestion that SNPs 05a and 18a might be interesting AIMs, since the third allele is only detected in the African and South African populations.

Dilution series and mixtures

To assess the sensitivity of the three SNaPshot™ multiplex assays in relation to the STR profiling system AmpF*l*STR® SGM Plus™ a range of pristine hDNA PCR inputs between 5 pg and 50 ng was analysed. Using an input of 5 pg DNA, 43, 67, 40, and 9 % of the genotypes was obtained for multiplex A, B, C and SGM Plus™, respectively. With an input of 10 or 50 ng DNA, the SNaPshot™ multiplex assays were overloaded but still interpretable, while the SGM Plus™ resulted in strongly overloaded profiles or "no

sizing data". The minimal amount of input DNA with which full profiles were obtained are 300, 200, 100 and 50 pg for multiplex A, B, C and SGM Plus™, respectively (data not shown). Thus, although SGM Plus™ is better capable of generating full profiles, the SNaPshot™ multiplex assays provide a higher percentage of detected alleles using very minute amounts of DNA and give genotyping data when very high DNA inputs are used.

Next, we investigated whether the mixing of samples can be detected using the tri-allelic SNP assays. Two-donor mixtures in various ratios between 1:8 and 8:1 were analysed in which the total amount of input DNA was 2 ng per reaction. Two individuals were selected that differ for five of the seven SNP markers that are present in multiplex A (Fig. 3A and B). When the DNA of these two individuals is mixed, it is expected that (1) three alleles are visible for SNPs 05a and 08a, (2) altered peak height ratios are visible for SNPs with overlapping alleles like SNPs 07a and 02a and (3) SNPs with no overlapping alleles look like a heterozygous when mixed in a 1:1 ratio or have an altered peak height ratio with other mixture ratios like SNP 04b. In a 1:1 mixed sample (Fig. 3C), clearly three alleles are detected for SNPs 05a and 08a pointing to the presence of a second DNA source. This finding is supported by the detection of altered peak height ratios for SNPs 07a and 02a. Normally the ratio G:A:T:C is around 3:2:1:1. For SNP 02a the C:T ratio in the 1:1 mixed sample is around 1:7 and thereby distinct from the normal 1:1 ratio. For SNP 07a the G:T ratio is far above the normal 3:1 ratio resulting in an uncalled T-peak because this peak (that is clearly above the allele calling threshold of 50 rfu) falls below the allele balance cut-off value of 0.125 (corresponding to a G:T ratio of 8:1). In a 1:8 mixture (Fig. 3D), three alleles are detected for SNP 05a only. In addition, an altered peak height ratio is seen for SNP 04b: the normal G:C ratio of 3:1 has lowered to 1:4. A 8:1 mixture from the same donors and two-donor mixtures from other individuals show similar results (data not shown). In conclusion, in 1:8 to 8:1 mixed samples the presence of a second DNA source is recognised in the SNaPshot™ assays for the tri-allelic SNP markers. The indicators are the presence of three alleles on one locus, unexpected peak height ratios and uncalled peaks above the detection threshold.

The presence of three alleles on one locus is the clearest sign for the occurrence of a mixture and does not depend on quantification of the fluorescent signal. This quantification is complicated for the analysis of SNaPshot™ assays since the fluorescent signal differs per dye, but is possible in other SNP typing technologies such as pyrosequencing and mass spectrometry [33]. However, compared to SNaPshot™ assays these methods have other limitations such as less multiplexing capability or the need for a higher amount of input DNA [27,34].

In order to estimate the utility of the tri-allelic SNPs for mixture detection we determined the theoretical occurrence of a third allele on at least one locus by evaluating all possible two-person mixtures in the Dutch and Netherlands Antilles

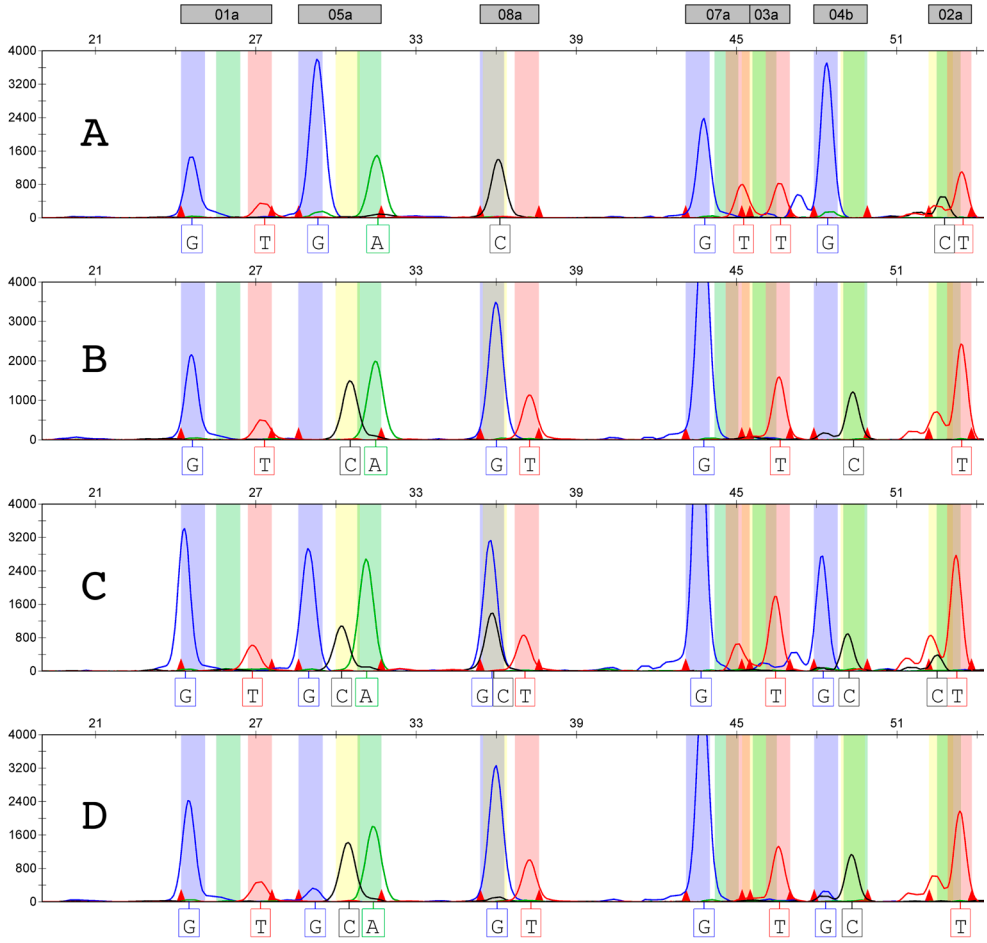

Fig. 3 Detection of a mixture of two individuals using tri-allelic SNP SNaPshot™ multiplex A that analyses 7 SNP markers. The horizontal grey bars on top label the SNP markers. (A) SNP profile for person 1. (B) SNP profile for person 2. (C) 1:1 mixture for person 1:person 2. (D) 1:8 mixture for person 1:person 2. These individuals differ for markers 05a, 08a, 07a, 04b and 02a.

reference samples. The percentage of detected mixtures was determined by two approaches: automated counting (Table 2) and a statistical approximation based on the allele frequencies (supplementary table S7). The Dutch and Netherlands Antilles populations have different allele frequencies, and therefore we determined the percentage of detected mixtures both separately and combined (Table 2). 75 % of the two-person mixtures within the Dutch population is detected (based on 8 tri-allelic SNPs), while 95 % of the mixtures is detected for the Netherlands Antilles samples

(based on 10 tri-allelic SNPs). The counting and the statistical approximation show similar results (Table 2). Even this limited number of tri-allelic SNP markers effectively detects the majority of the mixtures.

Degraded samples

In order to obtain information on the performance of the tri-allelic SNP assays to analyse degraded DNA, pristine DNA was artificially damaged. Native and denatured hDNA samples of 200 ng/μL were irradiated for increasing time with UV light in a cross-linker. By denaturing the DNA prior to UV irradiation, we intended to induce the formation of single-stranded breaks, which are the most common type of post-mortem DNA degradation [35]. Analysis of the samples on ethidium bromide stained, 0.8 % agarose gels showed that the UV treatment had resulted in DNA degradation rather than inter-strand cross-linking since reduced sized DNA smears were visible for both the native and the denatured DNA samples. In addition, longer UV treatment resulted in smears of reduced fragment length (results not shown). The denatured samples were selected to test the performance of the tri-allelic SNP assays on artificially degraded DNA. The samples of 200 ng/μL were diluted 200-fold and 1 μL was used as PCR input for the SNaPshot™ and SGM Plus™ analyses. Fig. 4A shows that SGM Plus™ STR profiling fails for the higher molecular weight STR markers when DNA is treated by 5 min of UV irradiation, and that only 14 % of the alleles is called when DNA is treated by UV irradiation for 120 min. In contrast, the SNaPshot™ multiplex assays show their first loss of marker detection when using DNA treated by UV irradiation for 60 min, and 73 % of the alleles are still called when using DNA treated for 120 min of UV irradiation.

Furthermore, pristine hDNA samples were degraded using increasing TURBO™ DNase concentrations. Reduced sized DNA smears were visible after running these samples on an ethidium bromide stained, 2 % agarose gel. DNA fragments were isolated in size ranges of approximately 400–350 bp, 300–250 bp, 200–150 bp and <100 bp by gel extraction. One nanogram DNA was used in both the SNaPshot™ and SGM Plus™ analyses. Fig. 4B shows that the percentage of detected STR alleles reduces with decreasing fragment length, and that no

Table 2
Two-person mixture detection by three alleles on at least one locus.

Population	*n* samples	*n* profile comparisons	*n* tri-allelic SNPs/population	% detected mixtures	Statistical approximation (%)
NL	153	11628	8	75.3	74.9
ANT	111	6105	10	95.7	94.7
NL and ANT	264	34716	10	87.5	n.a.[a]

[a] The different background of the populations does not allow the use of a combined allele frequency.

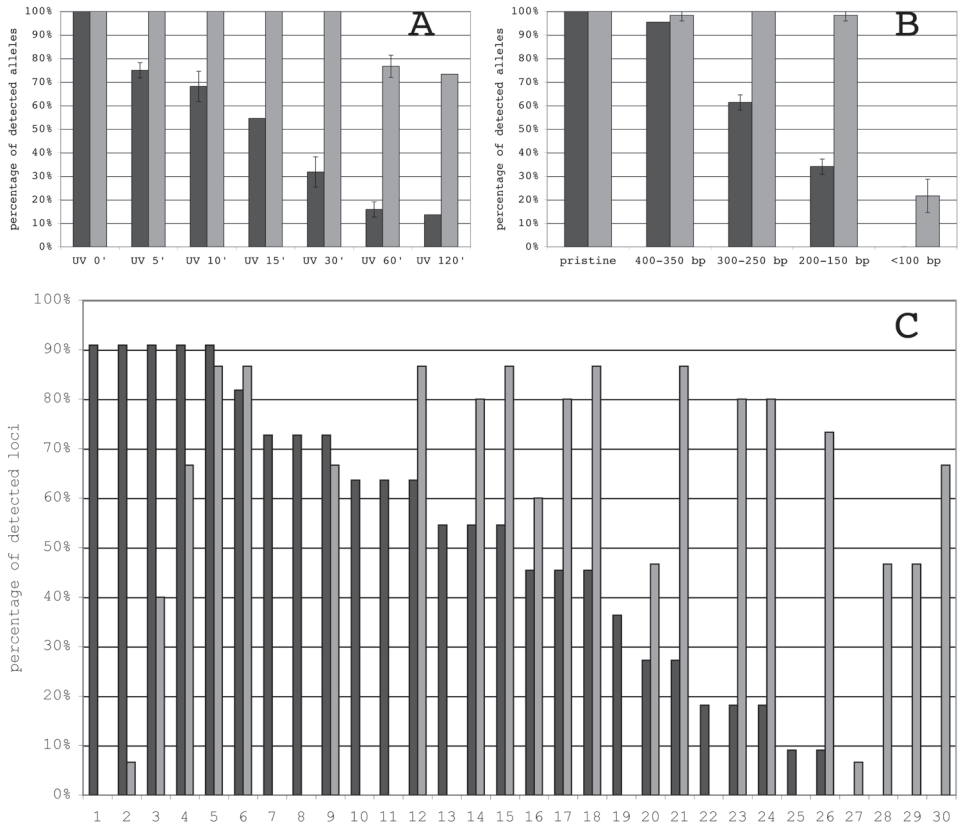

Fig. 4 Genotyping results for degraded DNA samples. Dark grey bars represent SGM Plus™ data (11 loci) and middle grey bars represent the SNaPshot™ results for multiplexes A, B, and C together (15 loci). The experiments shown in (A) and (B) were performed in duplo and the error bars represent the standard deviation; when no error bars are displayed, both measurements were equal. (A) UV irradiation time is plotted against the average percentage of detected alleles. (B) TURBO™ DNase degraded DNA fragments of decreasing length are plotted against the average percentage of detected alleles. (C) Results for 30 approximately 500-year-old bone and molar samples are plotted against the percentage of detected loci.

STR alleles are found when using DNA fragments smaller than 100 bp. For the latter DNA fragments, 22 % of the SNP alleles are still detected, and the SNP profiles are nearly complete when using DNA fragmented to 150–400 bp. These results show that the tri-allelic SNP markers are better capable of analysing artificially degraded DNA than SGM Plus™ STR profiling, which is most likely due to the use of smaller sized amplicons in the SNaPshot™ assays.

In addition to the artificially degraded DNA samples, thirty 450–550-year-old bone and molar samples were analysed using both the SNaPshot™ multiplex assays and SGM Plus™ STR profiling with a constant input of 3 µL DNA extract. Sixteen out of the 30 samples show an increase in the percentage of loci that were called for the SNP markers compared to the STR markers (Fig. 4C). The finding that for some samples STR data but no SNP data are obtained may have various reasons: (1) limiting sensitivity of SNP assays with low quantities of DNA, (2) differences between the two PCR assays in susceptibility for PCR inhibitors, and (3) level of optimization of the multiplex PCR. The individual SNP markers vary in robustness and the assays could be improved by further balancing of the multiplexes or development of a more sensitive assay, which would aid the analysis of both degraded and low quantities of DNA. STR locus drop-out mainly occurs for the larger sized amplicons, which is in accordance with DNA degradation in the samples. Four samples provided SNaPshot™ results, while no SGM Plus™ data are obtained. This is probably due to a high level of DNA degradation in these samples. Thus, when the higher molecular weight STR markers fail to amplify, tri-allelic SNP markers may provide additional information.

Conclusion

In this study 15 tri-allelic SNPs on 14 different chromosomes are detected in DNA samples from Dutch and Netherlands Antilles donors. We showed that such non-binary SNPs have the ability to reveal the presence of a second DNA donor in mixed samples with a ratio up to 1:8. Indications for a mixture are the presence of a third allele on one locus, unexpected peak height ratios and uncalled peaks above the detection threshold. Several of the tri-allelic SNP markers may not only be interesting for identification purposes, but also as ancestry informative markers. Furthermore, degraded (UV irradiated, TURBO™ DNase treated and 500-year-old bone and molar) DNA samples show that when the higher molecular weight STR markers fail to amplify, tri-allelic SNP markers can still provide valuable information.

Acknowledgements

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Supplementary data

Table S1
Primers for the non-binary SNP candidates in multiplex A, B, and C

multiplex	SNP name	F-primer	R-primer	F&R conc (nM)	amplicon size	B-primer	B conc (nM)	direction	SNP
A	01a_rs3091244	GCGAAAATAATGGGAAATGG	GGCTGAAGTAGGTGTTGGAG	100	86	aaGGAAATGGTAACATATTAAAC	30	F	D
A	02a_rs727241	TGCTTTCAATCATTCCTTGTC	ACTCACAGCTCTGCTCCTTAC	100	100	actaggtgccacgtctgtaaagtcttgacaaTGCTCCTTACTAATTCAAAG	40	R	D
A	03a_rs35528968	TCCATTTGATGGTGCTGAC	TTCAATAGCCAGATCCTTTG	100	86	ccacgtcgtaaagtcttgacaaTGGTGCTGACTCTTTATTAT	30	F	H
A	04b_rs356167	AAAACCATCACCACAGTTCC	CCAAATGTGCCAGATACAAAC	100	91	gtgccacgtcgtgaaagtcttgacaaCTGTTGTTGTTCTTCTTCTT	5	F	V
A	05a_rs9329104	AATCTCTAACGTAAGCAACTGC	GCTGGAAACACAGCCTGTAG	100	96	ctgacaaCTTTTAGAACTATCTCCTCCT	10	F	V
A	07a_rs2032582	CATATTTAGTTTGACTCACCTTCC	TGTTGTCTGGACAAGCACTG	100	72	cgtcgtgaaagtcttgacaaAAGATTAAGAAAGAACTAGAAGGT	15	R	H
A	08a_rs433342	AATCTGTGAGCATCCACCTC	AGAACAGGCAAAAATGATGG	100	58	tgaaagtcttgacaaGATGGGAAAAGAGATGTG	20	R	V
B	06a_rs9275142	AATCCCAGAAGGGTTTATGC	GACCTCAAGACCAAATTAGATG	100	97	taggtgccacgtcgtgaaagtcttgacaaTACTCCATCAAGGAGAA	75	R	V
B	09c_rs1112534	GTTTGCTGATCCTTTGGGTC	TTCAGACCCCTTCCTAAGTTC	100	97	agtcttgacaaTTTTAACTCTAGAATGGGTAGTA	5	F	V
B	11a_rs5030240	CAAAGTGCCAGGATCACAG	TTCAGCCCTAGAAAATGTGG	100	67	ccacgtcgtgaaagtcttgacaaGGAAAGTAGGCCAGG	15	R	V
B	12a_rs2307223	CACCTGTAGCAGATAGGAACTG	GAGCGGAACGAGAAGATG	100	96	tgacaaCAATTACACAACAGAACACTAT	5	R	H
C	10e_rs17287498	CAGGGAGGGTGCAAAGTG	GGCTGGACAGGAGAACAAC	100	69	cacgtcgtgaaagtcttgacaaTTAACTGCATTTGTTTTTAC	20	F	H
C	14a_rs1008686	AGCTTCCTGCAACAAGAAC	GCCCTCAAAACACTATCAAGC	100	73	caaATCTCTGGTTTCTCTGAGTT	10	R	D
C	18a_rs2853525	GCTGTTGGATTTTAGTAGGAAAG	TTCCTGTGAATCTGTAAAACAA	100	68	tgccacgtcgtgaaagtcttgacaaTTGGATTTTAGTAGGAAAGTT	15	F	H
C	20a_rs2069945	CCTACGTGCAAACCTTGG	TTTCCCCAGTGGCTTAATG	100	100	caaTATTAACCCCAGTCTACATG	20	R	V
C	22b_rs34741930	ACCAGAACCTCCCTAAGCAC	TCCTTGAAGAAGACGACAGG	100	68	actaggtgccacgtcgtgaaagtcttgacaaTTAGCTTTCAAGTGGACAG	40	R	D

Table S2

Unexamined potential non-binary SNPs found in dbSNP with a custom-made algorithm

chromosome	rs-number	dbSNP maximum minor allele frequency*
1	2184030	11,9
6	668871	11,8
6	865577	10,4
6	956297	14,3
6	1059553	21,4
6	1694115	10,7
6	2072899	15,1
6	2246068	19,2
6	2523610	10,7
6	2621363	12,5
6	2647086	21,4
6	3095299	12,5
6	3104368	10,3
6	3129158	12,9
6	4993691	17,9
6	6925893	17,9
6	9264962	15,9
6	9274703	28,6
6	9274723	21,1
6	9275142	27,1
6	9276016	14,3
6	17203067	10,4
6	17203741	12,0
6	17210062	12,0
8	353721	12,5
8	36031660	10,0
9	6560007	18,2
10	1047111	16,7
12	720578	20,0
14	28909974	12,5
17	2642157	27,6
17	5819132	16,7
19	6508976	16,7
22	3859849	19,6

* The population with the highest minor allele frequency in dbSNP (when allele frequencies were determined for more than one population).

Table S3
Genotyping data of Dutch, Netherlands Antilles and YCC samples analyzed with multiplex A, B and C

	01a	02a	03a	04b	05a	07a	08a	09c	10e	11a	12a	14a	18a	20a	22b
NL_001	G G	C C	T T	G C	A A	G T	C C	G G	A C	G G	A A	T T	C C	T T	C C
NL_002	G G	T T	T T	G G	A A	G T	C T	G G	C C	G G	A A	A A	T T	G G	C C
NL_003	G A	T T	T T	G G	A A	G T	C C	G A	C C	G C	A A	A T	T T	G C	C C
NL_004	G A	C T	T T	G G	A A	G T	C C	G G	C T	G C	A T	A T	T T	G C	C C
NL_005	G A	C T	T T	G G	A A	G T	T T	G A	C T	G C	A T	T T	C C	G G	C C
NL_006	G G	C T	T T	G G	A A	G T	C T	A A	C T	G G	A T	A T	C T	C T	C C
NL_007	A T	T T	T T	G G	G A	G T	C T	G G	A C	C C	A A	A T	T T	G T	C C
NL_008	G G	T T	T T	G G	A A	G T	C T	G A	C C	G T	G A	A A	T T	G T	C C
NL_009	G A	T T	T T	G G	A A	G T	C C	G G	C C	G G	A A	A T	T T	G T	C C
NL_010	G A	C T	T T	G A	G A	G T	C C	G A	C C	C C	A A	A T	T T	G T	C C
NL_011	A A	T T	T T	G A	A A	G T	C C	G A	C T	A A	T T	C T	C T	G C	C C
NL_012	G A	C C	T T	G G	A A	G T	C T	G G	A T	G G	A A	A T	T T	G G	C C
NL_013	G A	C C	T T	G G	G A	G T	C T	G G	C T	G T	A A	A T	T T	G G	C C
NL_014	G A	C T	T T	G G	A A	G T	T T	G G	C C	G C	A A	T T	T T	G G	C C
NL_015	G G	T T	T T	G G	A A	G T	C T	G G	C C	G G	A T	T T	C T	G G	C C
NL_016	G T	T T	T T	G A	A A	G T	C T	G A	C C	G C	A A	A T	T T	G G	C C
NL_017	G A	C T	T T	G G	A A	G T	G G	G A	C C	G C	A A	T T	T T	G C	C C
NL_018	G G	C T	T T	G C	A A	G G	C T	G A	C T	G C	A A	A T	T T	G G	C C
NL_019	A A	C T	T T	G A	A A	G G	C T	G A	C C	G C	A A	T T	C T	G C	C C
NL_020	A A	T T	T T	G A	G A	G T	C T	G A	A A	G G	A A	A T	C T	G G	C C
NL_021	G G	C C	T T	G G	G A	G T	C T	G A	A A	G G	A A	T T	C T	C T	C C
NL_022	G G	T T	T T	A A	A A	G T	T C	G A	C C	C C	A A	A T	C T	C T	C C
NL_023	G G	T T	T T	G A	A A	G T	C T	A A	A T	G G	A A	A T	T T	G C	C C
NL_024	G G	C C	T T	G C	A A	T G	T C	G G	C T	G G	A A	T T	T T	C C	C C
NL_025	G A	C T	T T	G C	A A	G T	C T	G G	C C	G G	A A	T T	T T	G C	C C
NL_026	G G	C T	T T	G C	G A	G T	C T	G G	C C	G C	A T	T T	T T	C C	C C
NL_027	G G	C T	T T	G C	A A	G T	C T	G G	C T	G G	A A	A T	C T	G G	C C
NL_028	G A	C T	T T	G C	A A	G G	C T	G G	A A	C C	A A	A A	T T	C C	C C
NL_029	G A	T T	T T	G G	A A	G T	C T	G A	A T	C T	A A	A T	C T	C C	C C
NL_030	G G	T T	T T	G A	A A	G G	C T	G A	C C	G C	A T	A T	C T	C C	C C
NL_031	G G	T T	T T	G C	A A	G T	C T	G A	C C	G C	A T	T T	C T	G C	C C
NL_032	G G	T T	T T	G C	G A	G T	C T	G G	C C	G C	A A	A A	C T	G G	C C
NL_033	G A	T T	T T	G G	A A	G T	C T	G G	C C	G G	A A	T T	C T	G G	C C
NL_034	A A	T T	T T	G C	A A	G T	C T	G G	A C	G T	A A	A T	C T	G G	C C
NL_035	G A	T T	T T	G A	A A	T G	T G	G G	A C	G C	A A	A A	C T	T T	C C
NL_036	A A	C C	T T	G G	A A	T G	C T	G G	A C	G T	A A	A T	C T	G G	C C
NL_037	A A	C C	T T	G A	A A	T T	C T	G A	C T	C G	A A	A T	T T	G C	C C
NL_038	G G	C T	T T	G A	A A	T G	C T	G A	C C	G G	A A	T T	C C	G C	C C
NL_039	G A	C T	T T	G C	A A	T G	C C	G A	C T	G G	A A	T T	C C	C C	C C
NL_040	G G	C T	T T	G G	A A	G T	C T	G G	C T	G T	A A	T T	C T	C C	C C
NL_041	G A	C C	T T	G G	A A	G T	C T	G G	T C	G T	A A	A T	C T	G G	C C
NL_042	G G	C T	T T	G A	G A	T T	C T	G G	A C	G G	A A	A T	C T	T G	C C
NL_043	G A	T T	T T	G G	A A	T G	C C	G G	A C	G G	A A	A T	C T	G G	C C
NL_044	G G	C T	T T	G A	A A	G G	C C	G G	A C	G T	A A	A T	C T	T C	C C
NL_045	G G	C T	T T	G G	A A	G G	C T	G G	A C	G G	A T	A T	C T	G C	C C
NL_046	A A	T T	T T	G G	G A	G A	C T	G G	A T	G C	A A	A T	T T	G C	C C
NL_047	G A	T T	T T	A A	A A	G T	C T	G A	A C	G G	A A	A T	C T	G C	C C
NL_048	G G	T T	T T	C C	A A	G T	C C	G A	C T	G G	A A	T T	C T	G C	C C
NL_049	G A	T T	T T	G G	A A	G G	C C	G A	C T	G T	A T	T T	C T	G C	C C
NL_050	A A	T T	T T	G G	A A	G G	C C	G G	A C	G C	A T	A T	C T	G C	C C
NL_051	G T	T T	T T	G A	A A	G G	C C	G G	A C	G G	A T	A A	T C	T C	C C
NL_052	G A	T T	T T	G A	A A	G T	C C	G G	A T	G C	A A	A T	C T	C T	C C
NL_053	G A	T T	T T	G G	G A	G T	C C	G G	C T	G C	A A	A T	C T	T T	C C
NL_054	G G	T T	T T	G G	A A	G T	C C	G G	A C	G C	A A	A T	C T	G T	C C
NL_055	G G	C T	T T	G G	A A	T G	C T	G G	T C	G C	A T	A T	T T	C C	C C
NL_056	G G	C T	T T	G G	G A	A G	C T	G A	C C	G C	A A	A T	C T	T T	C C
NL_057	G A	T T	T T	G A	A A	T G	C C	G G	A C	G C	A A	T T	C T	G C	C C
NL_058	G G	T T	T T	G A	A A	T G	C T	G G	C A	G C	A A	A T	T T	G C	C C
NL_059	G A	C T	T T	G G	A A	G T	C T	G A	C T	G T	A A	A T	T T	C T	C C
NL_060	G A	C T	T T	G G	A A	G T	A C	G G	A A	G G	A T	A T	C T	C C	C C
NL_061	G T	C T	T T	G G	G A	G A	C C	G A	C C	G C	A T	T T	C T	G C	C C
NL_062	G G	C T	T T	G A	A A	G G	C C	G A	C C	G T	A A	A T	C T	T C	C C
NL_063	G G	C T	T T	G A	A A	G T	C C	A G	C T	G G	A A	A T	C T	G G	C C
NL_064	G A	T T	T T	G G	G A	G T	C C	G G	A A	G G	A T	A T	T T	G C	C C
NL_065	A A	C C	T T	G A	A A	T G	C C	G G	A T	G G	A A	T T	C T	G C	C C
NL_066	G T	C T	T T	G A	A A	G T	T C	G G	C C	G G	A A	T T	C T	G C	C C
NL_067	A A	C T	T T	C C	A A	G T	C T	G G	C C	G G	A T	T T	C T	C T	C C
NL_068	G G	T T	T T	G C	A A	G T	C T	G G	A C	G C	T T	A T	T T	C C	C C
NL_069	G A	C T	T T	G C	A A	G G	C T	G A	A C	G C	G T	A T	C T	C C	C C
NL_070	G A	C C	T T	A A	A A	G G	C T	G A	C T	G C	A T	T T	C T	G C	C C
NL_071	G G	C T	T T	G C	G A	G T	C T	G A	C T	G C	A A	T T	C T	C C	C C
NL_072	G G	T T	T T	G G	A A	G T	C T	G G	A C	G C	A A	T T	C T	G C	C C
NL_073	G A	T T	T T	G G	G A	G A	C T	G A	C T	G C	A A	A T	C T	G C	C C
NL_074	G G	T T	T T	G G	A A	G G	C T	G A	A C	G G	A T	T T	C C	T C	C C
NL_075	G A	C T	T T	G G	A A	G G	C T	G G	T C	G G	A A	A T	C C	G C	C C
NL_076	G T	C T	T T	G G	A A	G G	C C	G G	C C	G G	A A	A T	C T	T C	C C
NL_077	G A	C T	T T	G G	A A	G T	C C	G G	A C	C T	A A	A A	T T	G G	C C
NL_078	G A	C T	T T	G C	A A	G T	C T	G G	C C	C T	A A	A T	T T	G G	C C
NL_079	A A	C T	T T	G A	A A	G T	T C	G G	C T	G C	A A	A A	T T	G C	C C
NL_080	G G	C T	T T	A A	A A	G T	C C	G G	C C	G G	A A	A T	C T	G C	C C
NL_081	G A	C T	T T	G G	A A	G T	C T	G G	A C	G C	A T	A T	T T	C C	C C
NL_082	G G	T T	T T	G A	G A	G T	T T	G G	C C	G C	A A	A A	T T	G C	C C
NL_083	G G	C T	T T	G G	A A	G G	C T	G G	C C	G C	A A	A T	T T	G C	C C
NL_084	G T	C T	T T	G C	A A	G G	C T	G G	A A	G C	A A	A A	C C	G G	C C
NL_085	G G	T T	T T	G G	A A	G T	C T	G G	C T	G C	T T	A T	C T	G C	C C

	01a	02a	03a	04b	05a	07a	08a	09c	10e	11a	12a	14a	18a	20a	22b
NL_086	A T	T T	T T	G G	A A	G T	C T	G A	A C	G C	A A	A T	T T	G C	C C
NL_087	G G	C T	T T	G A	A A	G T	G T	G A	A C	G C	A T	A A	T C	G C	C C
NL_088	G G	C T	T T	G G	A A	G T	C T	G G	A T	G C	A A	A T	C T	C T	C C
NL_089	G A	C T	T T	G G	A A	G T	C T	G G	C T	G G	A A	A T	C C	T C	C C
NL_090	G A	C T	T T	G G	A A	G T	C T	G G	C C	G C	A T	A T	C T	C T	C C
NL_091	G A	C T	T T	G G	G A	G T	C T	G A	C C	G G	A A	T T	C C	G C	C C
NL_092	G G	C T	T T	G G	A A	G T	C T	G A	C C	G G	A T	A A	C T	G C	C C
NL_093	G A	C T	T T	G C	A A	G T	C C	G A	C C	G G	T T	A A	C T	C G	C C
NL_094	G T	C T	T T	G A	G A	G T	C C	A A	C C	G T	A A	T T	C T	G G	C C
NL_095	G A	T T	T T	G A	A A	G T	C C	G A	C A	G C	A T	A T	C T	G G	C C
NL_096	A A	T T	T T	G C	A A	G T	C C	G A	C C	G C	A A	A A	C T	C C	C C
NL_097	G A	C T	T T	G A	A A	G T	C C	G G	A C	G C	A T	A T	C T	G C	C C
NL_098	G A	C T	T T	G A	A A	G T	C C	G G	C T	G T	A A	A T	C T	C C	C C
NL_099	G A	T T	T T	G C	A A	G G	C C	G G	C T	G G	A A	A A	C T	G G	C C
NL_100	G G	T T	T T	G A	A A	G G	C T	G G	A T	G G	A A	A A	C T	G C	C C
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NL_103	G G	C T	T T	G A	A A	G T	C C	G G	A T	G G	A A	T T	C T	C C	C C
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NL_105	G A	C T	T T	G G	G A	A A	C C	G G	C C	G G	G A	T T	C T	T C	C C
NL_106	A T	C T	T T	G G	A A	G T	C T	G G	A C	G C	A A	A T	C T	C T	C C
NL_107	G G	C T	T T	G A	A A	G T	C T	G G	A T	G C	A A	A T	C T	C T	C C
NL_108	G G	C T	T T	G G	A A	T T	C C	G G	A T	G G	G T	A T	C T	C G	C C
NL_109	G A	C T	T T	G G	A A	G G	C C	G G	A C	G C	A A	T T	C T	G G	C C
NL_110	G T	C T	T T	G G	A A	G T	C T	G G	A C	G C	A T	A T	C T	C T	C C
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NL_129	G G	T T	T T	G A	G A	G T	C T	G G	C C	G G	A T	A T	T T	C G	C C
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NL_136	G A	C T	T T	G A	A A	G G	C C	G G	A A	G G	A A	A T	T T	G C	C C
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NL_139	G G	C T	T T	G G	A A	G G	C C	G G	C C	G C	G T	T T	C T	C C	C C
NL_140	A T	T T	T T	G G	A A	G T	C T	G G	A C	G C	A T	A T	C T	G G	C C
NL_141	A A	T T	T T	G C	A A	G T	C T	G G	C C	G T	A A	A A	C T	T G	C C
NL_142	G G	C T	T T	G C	A A	G T	G C	G G	C C	G G	A A	A T	C T	G G	C C
NL_143	G G	C T	T T	G A	A A	G T	C C	G G	A C	G G	A T	A A	C T	G G	C C
NL_144	G A	C T	T T	G G	A A	G T	C C	G G	A C	G T	A A	A T	C T	G C	C C
NL_145	G A	C T	T T	A C	A A	G T	C C	G G	C T	G G	C A	A A	C C	T C	C C
NL_146	G G	C T	T T	G G	A A	G T	C T	G G	A T	G G	A A	T T	C C	T C	C C
NL_147	G G	C T	T T	G A	A A	G T	G C	G A	C C	G G	C A	A T	C T	C T	C C
NL_148	A A	C T	T T	G A	A A	G T	C T	G A	C C	G G	A T	A A	T T	C C	C C
NL_149	G A	C T	T T	G G	G A	G T	C T	A G	A C	G G	T A	A T	C T	G C	C C
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NL_151	G A	C T	T T	G A	A G	T T	C T	G G	A C	G G	A A	A T	C T	G C	C C
NL_152	G T	C T	T T	G A	A A	G T	C T	G G	C T	G G	A A	A T	C C	C C	C C
NL_153	G A	C T	T T	G G	A A	G T	C C	G G	C A	G G	A T	T T	C T	G T	C C
ANT_001	G G	C T	T T	G G	A A	G T	C T	G G	A C	G C	A A	A T	T T	C C	C C
ANT_002	G A	C T	T T	G G	G A	G T	G T	G A	A C	G C	A A	A T	A C	C C	C C
ANT_003	G G	C T	T T	G G	G A	G T	C T	G A	A C	G C	A A	A T	G C	C C	C C
ANT_004	G T	C T	T T	C C	G A	C T	G T	G A	C C	C T	G G	A A	C T	C C	C C
ANT_005	G T	T T	T T	G G	A C	G G	G C	G A	C T	C G	G G	A T	C T	C C	C C
ANT_006	A T	C T	T T	G G	G A	G T	G C	G A	C A	G G	A A	A A	C T	C C	C C
ANT_007	G A	C T	T T	G C	G A	G T	G C	G G	A T	G T	G G	A A	C C	C T	C C
ANT_008	G G	C T	T T	G G	G A	G T	G C	G G	C C	G C	A A	A T	C A	T C	C C
ANT_009	G T	C C	T T	G G	G A	G T	G C	G G	C T	G C	A A	A T	C A	T C	C C
ANT_010	T T	C C	T T	G C	A A	G G	G C	G G	A A	C T	G A	A A	C T	C C	C C
ANT_011	A T	C T	T T	G C	A A	G G	G C	G G	A C	G T	A A	A A	C T	C C	C C
ANT_012	A T	C T	T T	G C	G A	G G	C T	G G	A C	G T	G A	A A	C T	C C	C C
ANT_013	T T	C T	T T	G C	G A	G T	G C	G A	A C	G T	A A	T T	T T	G C	C C
ANT_014	G T	C T	T T	G C	A A	G T	C C	G A	A T	G G	T A	A T	T T	G C	C C
ANT_015	T T	C T	T T	G C	G C	A T	G C	G G	A C	G G	G A	A A	T T	G C	C C
ANT_016	A T	C T	T T	G C	G A	G T	C T	G G	A C	G G	G A	A T	A C	C C	C C
ANT_017	T T	C T	T T	G G	G A	G T	G C	A G	A A	G G	G A	A T	C T	C C	C C
ANT_018	A T	T T	T T	C C	A C	G G	C T	G G	A C	G G	G A	T T	C T	C C	C C
ANT_019	G A	C T	T T	G C	G A	G T	C C	G A	C C	C G	G A	A T	T T	G G	C C
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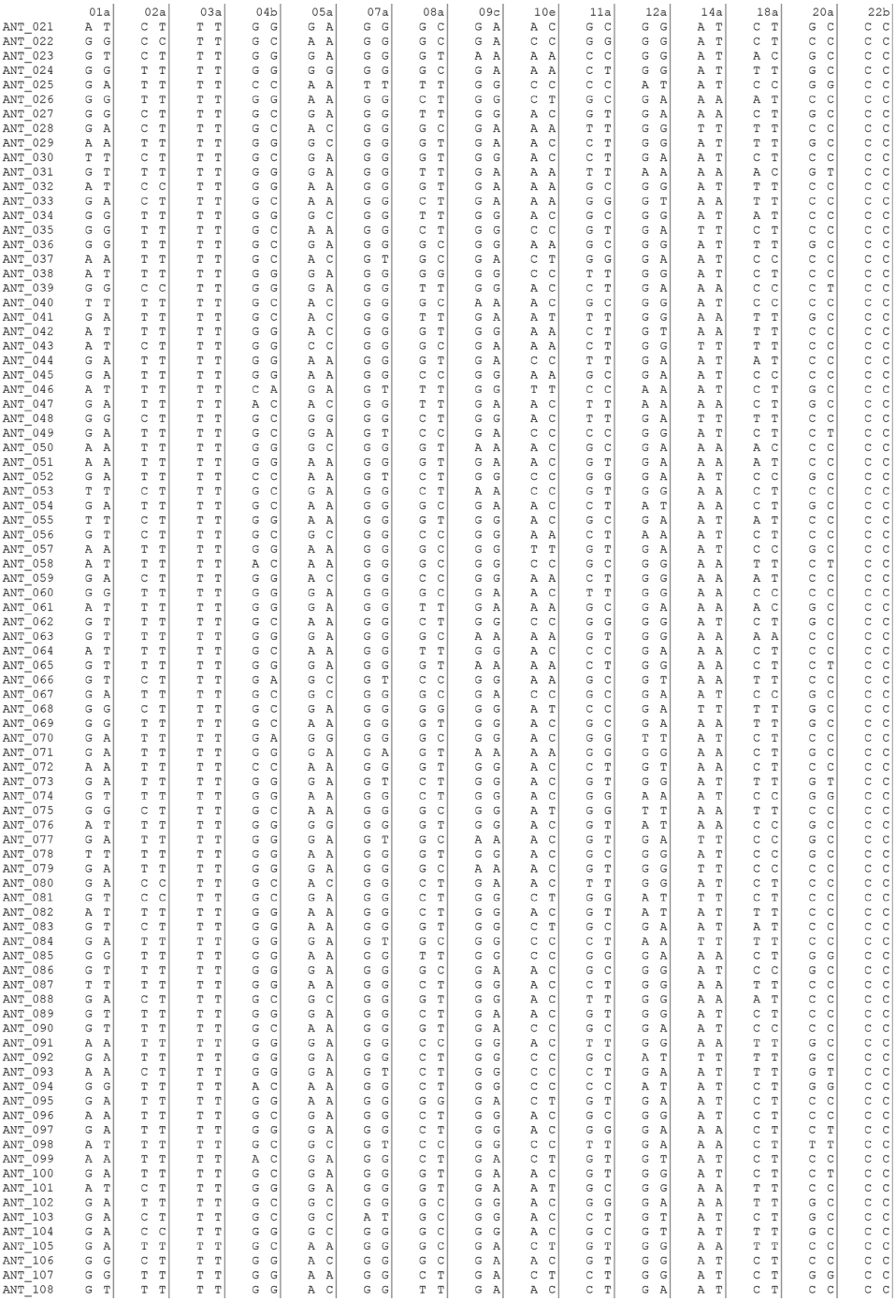
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ANT_109	G G	T T	T T	G A	A A	G G	C C	G G	A A	G C	G A	A A	C C	C C	C C
ANT_110	G G	T T	T T	G A	A A	G G	G T	G A	A T	C T	G G	A T	C C	G C	C C
ANT_111	G T	T T	T T	C C	A C	G G	G T	G A	A C	G T	G G	T T	A C	C C	C C
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AFR_YCC04	G G	C T	T T	G G	G C	G G	G T	G G	A C	G T	A A	A A	T T	C T	C C
AFR_YCC05	T T	T T	T T	G C	A A	G G	G G	G A	A A	C C	G G	A T	A C	G C	C C
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AFR_YCC55	G A	C T	T T	G G	G A	G G	G T	G G	A A	G T	G G	A A	A T	C C	C C
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NAM_YCC06	G G	T T	T T	G C	A A	G T	T T	G A	A C	G G	A T	A A	T T	C C	C C
NAM_YCC07	G G	T T	T T	G C	A A	G G	T T	A A	C C	G C	G A	A A	C C	G G	C C
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NAM_YCC22	G A	T T	T T	G C	G A	G A	T T	G G	C C	C C	A A	A A	C T	G C	C C
NAM_YCC23	G G	T T	T T	G C	G A	G G	T T	A A	C C	C C	A A	A A	C T	G G	C C
NAM_YCC24	G A	T T	T T	G C	A A	G T	T T	G A	C C	C C	A A	A A	C T	G G	C C
NAM_YCC25	G G	T T	T T	G C	G A	G G	T T	A A	C C	C C	G A	A A	C T	G G	C C
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ASI_YCC10	G G	T T	T T	G A	G A	G T	C T	G G	C T	G C	G A	A T	C T	G G	C C
ASI_YCC11	G T	T T	T T	C C	G A	G G	C T	G G	C T	G C	G G	A T	C T	G G	C C
ASI_YCC12	G G	T T	T T	C C	A A	G T	T T	G A	C C	G C	A T	A A	C T	G C	C C
ASI_YCC16	G A	T T	T T	G G	G A	G A	C C	A A	C C	C T	G T	A T	C T	C C	C C
ASI_YCC17	G A	T T	T T	C C	G A	G A	C C	G A	C C	G T	A A	A A	C T	C T	C C
ASI_YCC65	G G	C T	T T	G G	G A	G T	C T	G G	A C	G G	G A	A T	C C	C C	C C
ASI_YCC72	G T	T T	T T	G C	A A	T T	T T	G A	C C	G C	A A	A A	C C	G C	C C
ASI_YCC73	G G	T T	T T	G C	A A	A T	C T	G A	A C	C C	T T	A T	C C	G G	C C
SAF_YCC13	G G	T T	T T	G C	G A	G G	T T	G G	A A	G C	G G	A A	T T	C C	C C
SAF_YCC14	G T	T T	T T	G C	G A	G G	G C	G G	A A	G G	G A	A A	C T	C C	C C
SAF_YCC15	T T	T T	T T	C C	G C	G G	G T	G G	A C	G T	G G	A A	A T	C T	C C
SAF_YCC18	T T	C T	T T	G G	G A	G G	G T	G G	C C	G G	G G	A A	T T	C T	C C
SAF_YCC19	G A	T T	T T	G G	C C	G G	G T	G A	C C	G G	G G	T T	C C	C T	C C
SAF_YCC20	G T	T T	T T	G G	A C	G G	T T	G G	A C	G G	G G	T T	C C	C C	C C
SAF_YCC42	G T	T T	T T	G G	G C	G G	T T	G G	A C	G C	G G	A T	C T	G C	C C
SAF_YCC43	G T	C T	T T	G G	G C	G G	T T	G A	C C	T T	G A	A T	C T	C C	C C
SAF_YCC46	G G	C T	T T	G G	A A	G G	T T	G A	C C	G C	G G	A A	C T	C T	C C
SAF_YCC47	G T	C T	T T	G G	A A	G G	C T	G G	C C	C T	G G	A A	C T	C T	C C
SAF_YCC48	G A	C C	T T	G G	A A	G G	C T	G A	A C	G G	A A	T T	T T	G T	C C
SAF_YCC49	G G	T T	T T	G C	G A	G G	C T	G A	A C	T T	G A	A A	T T	C C	C C
SAF_YCC50	G G	T T	T T	C C	G A	G G	C T	G G	A A	G T	G G	A A	T T	C C	C C
SAF_YCC51	G G	T T	T T	G G	A A	G G	G T	G G	C C	G T	G G	A A	T T	C C	C C
RUS_YCC28	G G	T T	T T	G C	A A	A T	C C	G G	C T	G C	G A	T T	C C	C C	C C
RUS_YCC29	G G	T T	T T	G C	A A	G T	C T	G A	C C	G G	A A	T T	T T	G T	C C
RUS_YCC30	G G	T T	T T	G G	G A	G T	C T	G A	C C	G C	A T	A T	T T	G C	C C
RUS_YCC31	G A	T T	T T	G C	A A	G T	T T	A A	A T	G C	G A	A A	T T	G C	C C
RUS_YCC32	G G	T T	T T	G C	A A	T T	C T	G G	A C	G C	G A	A T	C T	G G	C C
RUS_YCC33	G G	T T	T T	G G	A A	G T	C T	G G	A T	G C	A A	A T	C T	G T	C C
RUS_YCC35	G T	T T	T T	G G	G A	G T	C T	G G	C T	G T	A A	T T	C T	G C	C C
RUS_YCC37	G G	T T	T T	A C	G A	G T	C T	G A	A C	G C	A A	A T	C T	G C	C C
RUS_YCC38	G G	T T	T T	G G	A A	T T	C T	G G	C T	G C	A A	A T	C T	G C	C C
RUS_YCC39	G G	C C	T T	G G	G A	G T	C T	G G	C C	G G	A A	A A	C T	G C	C C
RUS_YCC40	G G	C T	T T	G A	A A	G G	C C	G A	C T	G G	A A	T T	C T	G C	C C
RUS_YCC41	A A	T T	T T	G C	A A	G G	C T	G G	C C	G T	A A	A T	T T	G G	C C

Table S4
Summary statistics

						NL									
SNP name	01a	02a	03a	04b	05a	07a	08a	09c	10e	11a	12a	14a	18a	20a	22b
frequency allele A	31,37			17,65	87,91	0,98		22,22	23,86		81,05	43,79			
frequency allele C		26,47		11,11			70,59		51,63	26,80			32,03	45,75	100,00
frequency allele T	6,21	73,53	100,00			43,79	27,78		24,51	7,84	16,67	56,21	67,97	11,11	
frequency allele G	62,42			71,24	12,09	55,23	1,63	77,78		65,36	2,29			43,14	
n alleles	306	306	306	306	306	306	306	306	306	306	306	306	306	306	306
expected heterozygosity*	0,5098	0,3905	0,0000	0,4504	0,2133	0,5048	0,4257	0,3468	0,6184	0,4965	0,3159	0,4939	0,4368	0,5942	0,0000
observed heterozygosity*	0,4967	0,3856	0,0000	0,4706	0,2288	0,4641	0,4314	0,3660	0,5752	0,4902	0,3072	0,5490	0,3529	0,5817	0,0000
PIC-value*	0,4277	0,3135	0,0000	0,4041	0,1900	0,3860	0,3471	0,2859	0,5472	0,4274	0,2776	0,3711	0,3406	0,5046	0,0000
Hardy-Weinberg equilibrium*	0,8188	1,0000	n.a.	0,1586	0,6992	0,2334	0,7885	0,6397	0,6216	0,4770	0,0068	0,1899	0,0249	0,8872	n.a.
HW H1=heterozygote deficit*	0,5259	0,3525	n.a.	0,4453	0,9162	0,1411	0,5644	0,8215	0,1030	0,2333	0,6643	0,9399	0,0146	0,3605	n.a.
HW H1=heterozygote excess*	0,4854	0,6475	n.a.	0,5547	0,0838	0,8589	0,4356	0,3321	0,8989	0,7667	0,3362	0,0601	0,9854	0,6449	n.a.
power of discrimination	0,686	0,552	0,000	0,653	0,363	0,653	0,589	0,511	0,789	0,671	0,477	0,589	0,601	0,749	0,000
power of exclusion	0,185	0,105	0,000	0,163	0,038	0,158	0,134	0,095	0,262	0,179	0,067	0,234	0,088	0,269	0,000

						ANT									
SNP name	01a	02a	03a	04b	05a	07a	08a	09c	10e	11a	12a	14a	18a	20a	22b
frequency allele A	31,08			4,05	58,11	1,35		26,13	43,69		28,38	63,51	8,11		
frequency allele C		21,17		24,77	12,61		36,04		46,40	29,28			39,19	71,62	100,00
frequency allele T	25,68	78,83	100,00			10,36	35,59		9,91	32,88	9,91	36,49	52,70	5,86	
frequency allele G	43,24			71,17	29,28	88,29	28,38	73,87		37,84	61,71			22,52	
n alleles	222	222	222	222	222	222	222	222	222	222	222	222	222	222	222
expected heterozygosity*	0,6534	0,3353	0,0000	0,4324	0,5632	0,2105	0,6660	0,3878	0,5866	0,6660	0,5312	0,4656	0,5646	0,4348	0,0000
observed heterozygosity*	0,6126	0,2793	0,0000	0,4234	0,6126	0,1712	0,7658	0,3604	0,5946	0,6306	0,4505	0,4955	0,4955	0,3874	0,0000
PIC-value*	0,5770	0,2781	0,0000	0,3664	0,4893	0,1926	0,5888	0,3115	0,4938	0,5889	0,4584	0,3561	0,4711	0,3770	0,0000
Hardy-Weinberg equilibrium*	0,4185	0,0897	n.a.	0,2138	0,1022	0,0733	0,0218	0,4665	0,4731	0,5550	0,0659	0,5428	0,3485	0,2332	n.a.
HW H1=heterozygote deficit*	0,1705	0,0714	n.a.	0,5680	0,9284	0,0456	0,9925	0,3023	0,3366	0,2237	0,0145	0,8122	0,0925	0,0766	n.a.
HW H1=heterozygote excess*	0,8310	0,9770	n.a.	0,4395	0,0716	0,9544	0,0076	0,8411	0,6677	0,7783	0,9861	0,1878	0,9113	0,9234	n.a.
power of discrimination	0,807	0,496	0,000	0,604	0,716	0,335	0,780	0,552	0,724	0,817	0,706	0,591	0,736	0,615	0,000
power of exclusion	0,306	0,055	0,000	0,129	0,306	0,022	0,537	0,092	0,284	0,329	0,148	0,184	0,184	0,106	0,000

* *p*-values
p-value < 0.05

Table S5
Genotypic linkage disequilibrium *p*-values

ANT \ NL	01a	02a	03a	04b	05a	07a	08a	09c	10e	11a	12a	14a	18a	20a	22b
01a	x	0.776864	n.a.	0.768094	0.554408	0.449084	0.906388	0.386674	0.666754	0.522774	0.910890	0.686982	0.525956	0.604220	n.a.
02a	0.693724	x	n.a.	0.541810	0.717092	0.584350	0.062954	0.390606	0.434530	0.318558	0.932134	0.516262	0.122974	0.617360	n.a.
03a	n.a.	n.a.	x	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
04b	0.809268	0.055774	n.a.	x	0.582870	0.307594	0.051810	0.102298	0.659548	0.336222	0.468554	0.371268	0.085544	0.018580	n.a.
05a	0.986294	0.640120	n.a.	0.200956	x	0.009324	0.620296	0.539510	0.962066	0.463736	0.177510	0.380892	0.948088	0.970650	n.a.
07a	0.230586	0.911522	n.a.	0.506320	0.202782	x	0.668608	0.874236	0.006062	0.474902	0.333948	0.209784	0.728714	0.473842	n.a.
08a	0.907792	0.761834	n.a.	0.452190	0.416768	0.561336	x	0.034968	0.843162	0.903742	0.999132	0.926062	0.208814	0.640604	n.a.
09c	0.036390	0.800150	n.a.	0.868634	0.012470	0.646698	0.463700	x	0.974860	0.036878	0.324318	0.600836	0.811638	0.959972	n.a.
10e	0.470148	0.419254	n.a.	0.065620	0.945624	0.369076	0.176734	0.378790	x	0.759494	0.870230	0.863910	0.090670	0.442952	n.a.
11a	0.924560	0.878178	n.a.	0.347226	0.427664	0.577676	0.323374	0.509954	0.443796	x	0.237430	0.844922	0.884320	0.880672	n.a.
12a	0.607886	0.740100	n.a.	0.462906	0.287044	0.161766	0.604688	0.071070	0.417078	0.962820	x	0.804282	0.888624	0.565182	n.a.
14a	0.790824	0.853852	n.a.	0.790394	0.163306	0.870160	0.049378	0.415234	0.624794	0.127598	0.688134	x	0.002640	0.991248	n.a.
18a	0.745114	0.542452	n.a.	0.320042	0.804058	0.731918	0.086346	0.037428	0.105320	0.155318	0.680152	0.116036	x	0.212866	n.a.
20a	0.800878	0.873784	n.a.	0.698248	0.029354	0.176908	0.388732	0.984974	0.050990	0.572468	0.370824	0.426766	0.121558	x	n.a.
22b	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	x

p-value < 0.05

Table S6
YCC allele frequencies

European

SNP name	01a	02a	03a	04b	05a	07a	08a	09c	10e	11a	12a	14a	18a	20a	22b
frequency allele A	40,00			20,00	90,00				10,00		90,00	50,00			
frequency allele C		30,00					60,00		80,00	10,00			30,00	70,00	100,00
frequency allele T		70,00	100,00			50,00	30,00		10,00	20,00	10,00	50,00	70,00		
frequency allele G	60,00			80,00	10,00	50,00	10,00	100,00		70,00				30,00	
n alleles	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Russian / Siberian

SNP name	01a	02a	03a	04b	05a	07a	08a	09c	10e	11a	12a	14a	18a	20a	22b
frequency allele A	12,50			8,33	83,33	4,17		25,00	16,67		83,33	41,67			
frequency allele C		12,50		25,00			62,50		58,33	33,33			37,50	41,67	100,00
frequency allele T	4,17	87,50	100,00			50,00	37,50		25,00	8,33	4,17	58,33	62,50	8,33	
frequency allele G	83,33			66,67	16,67	45,83		75,00		58,33	12,50			50,00	
n alleles	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24

Asian / Pakistan

SNP name	01a	02a	03a	04b	05a	07a	08a	09c	10e	11a	12a	14a	18a	20a	22b
frequency allele A	12,50			6,25	68,75	18,75		37,50	12,50		43,75	68,75			
frequency allele C		6,25		50,00			50,00		75,00	43,75			62,50	43,75	100,00
frequency allele T	12,50	93,75	100,00			37,50	50,00		12,50	12,50	25,00	31,25	37,50	6,25	
frequency allele G	75,00			43,75	31,25	43,75		62,50		43,75	31,25			50,00	
n alleles	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16

African

SNP name	01a	02a	03a	04b	05a	07a	08a	09c	10e	11a	12a	14a	18a	20a	22b
frequency allele A	16,67				72,22			44,44	55,56		22,22	83,33	22,22		
frequency allele C		27,78		27,78	5,56		11,11		44,44	22,22			27,78	72,22	100,00
frequency allele T	22,22	72,22	100,00				50,00			33,33		16,67	50,00	16,67	
frequency allele G	61,11			72,22	22,22	100,00	38,89	55,56		44,44	77,78			11,11	
n alleles	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18

South African

SNP name	01a	02a	03a	04b	05a	07a	08a	09c	10e	11a	12a	14a	18a	20a	22b
frequency allele A	7,14				50,00			17,86	39,29		17,86	71,43	3,57		
frequency allele C		17,86		25,00	21,43		21,43		60,71	14,29			28,57	78,57	100,00
frequency allele T	32,14	82,14	100,00				60,71			28,57		28,57	67,86	14,29	
frequency allele G	60,71			75,00	28,57	100,00	17,86	82,14		57,14	82,14			7,14	
n alleles	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28

Native American

SNP name	01a	02a	03a	04b	05a	07a	08a	09c	10e	11a	12a	14a	18a	20a	22b
frequency allele A	31,82			4,55	77,27	13,64		59,09	9,09		72,73	86,36			
frequency allele C		9,09		36,36			4,55		86,36	59,09			36,36	22,73	100,00
frequency allele T		90,91	100,00			18,18	95,45		4,55		13,64	13,64	63,64	4,55	
frequency allele G	68,18			59,09	22,73	68,18		40,91		40,91	13,64			72,73	
n alleles	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22

Table S7
Statistical approximation of mixture detection probability by three alleles on at least one locus

If all three allele variants are present in a random sample of four alleles, then one variant must be realized twice and the other two once. The number of distinct permutations of four objects, two of which are indistinguishable from each other, is 4!/(2!1!1!)=12. Hence, the probability of obtaining all three allele variants in a random sample of four alleles equals 12*p1*p2*p3(p1+p2+p3)

For $p_{L,1}$ = estimated allele frequency of allele 1 on locus L
$p_{L,2}$ = estimated allele frequency of allele 2 on locus L
$p_{L,3}$ = estimated allele frequency of allele 3 on locus L

The probability of detecting three alleles on locus L in a two-person mixture = $12*p_{L,1}*p_{L,2}*p_{L,3}(p_{L,1}+p_{L,2}+p_{L,3}) = 12*p_{L,1}*p_{L,2}*p_{L,3}(1) = 12*p_{L,1}*p_{L,2}*p_{L,3}$

The probability of not detecting three alleles (i.e. one or two) on locus L in a two-person mixture =
$1 - 12*p_{L,1}*p_{L,2}*p_{L,3}$

The probability of not detecting three alleles on all loci in a two-person mixture =
$\Pi_L(1 - 12*p_{L,1}*p_{L,2}*p_{L,3})$

The probability of detecting three alleles on at least one of all loci in a two-person mixture =
$1 - \Pi_L(1 - 12*p_{L,1}*p_{L,2}*p_{L,3})$

NL (n alleles = 306)				
L	$p_{L,1}$	$p_{L,2}$	$p_{L,3}$	$1 - 12*p_{L,1}*p_{L,2}*p_{L,3}$
01a	0,3137	0,0621	0,6242	0,8541
04b	0,1765	0,1111	0,7124	0,8324
05a	0,8791	0,1209	0	1
07a	0,0098	0,4379	0,5523	0,9716
08a	0,7059	0,2778	0,0163	0,9616
10e	0,2386	0,5163	0,2451	0,6377
11a	0,2680	0,0784	0,6536	0,8352
12a	0,8105	0,1667	0,0229	0,9629
18a	0,3203	0,6797	0	1
20a	0,4575	0,1111	0,4314	0,7369
$1 - \Pi_L(1 - 12*p_{L,1}*p_{L,2}*p_{L,3})$				0,7490

ANT (n alleles = 222)				
L	$p_{L,1}$	$p_{L,2}$	$p_{L,3}$	$1 - 12*p_{L,1}*p_{L,2}*p_{L,3}$
01a	0,3108	0,2568	0,4324	0,5859
04b	0,0405	0,2477	0,7117	0,9143
05a	0,5811	0,1261	0,2928	0,7425
07a	0,0135	0,1036	0,8829	0,9852
08a	0,3604	0,3559	0,2838	0,5632
10e	0,4369	0,464	0,0991	0,7589
11a	0,2928	0,3288	0,3784	0,5628
12a	0,2838	0,0991	0,6171	0,7917
18a	0,0811	0,3919	0,5270	0,7990
20a	0,7162	0,0586	0,2252	0,8866
$1 - \Pi_L(1 - 12*p_{L,1}*p_{L,2}*p_{L,3})$				0,9471

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Chapter 4

Degraded DNA sample analysis using DNA repair enzymes, mini-STRs and (tri-allelic) SNPs

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Abstract

DNA degradation may cause the loss of the longer short tandem repeat (STR) markers, resulting in DNA profiles with lower discrimination power. We compared standard STR profiling with DNA repair enzyme incubation, and genotyping with mini-STRs or (tri-allelic) single nucleotide polymorphisms (SNPs) in progressively degraded, UV-irradiated DNA samples. In highly degraded DNA samples, most of the standard STR markers fail to amplify, while mini-STRs and especially (tri-allelic) SNPs still provide valuable information.

Introduction

The degradation of DNA may hinder successful human identification. The amplicon size of the STR markers that are used for DNA profiling usually ranges between 100 and 450 base pairs (bp). Due to DNA degradation, the longer fragments often cannot be amplified resulting in partial DNA profiles with lower discrimination power. To cope with degraded DNA, most strategies aim at shorter amplicon sizes, like with mini-STRs or SNPs [1,2]. Another possibility is to repair the DNA before amplification by the means of DNA repair enzymes [3,4].

In this study we compare these alternatives to standard STR typing (AmpF*l*STR® SGM Plus™, Applied Biosystems (AB)) for progressively UV-degraded DNA samples. We evaluated two commercially available DNA repair enzyme cocktails, PreCR™ (New England Biolabs) and Restorase™ (Sigma), against mini-STRs (AmpF*l*STR® MiniFiler™, AB), bi-allelic SNPs (GenPlex™, AB) and tri-allelic SNPs (as described by Westen et al. [5]).

Material and methods

All measurements were performed in dedicated laboratories (ISO 17025 accredited). In order to obtain artificially degraded DNA, pristine DNA (Quantifiler™ human DNA standard, AB) of 200 ng/µL was denatured for 5 min at 95 °C, placed on ice and irradiated with 254 nm UV-light in a CL-1000 UV CrossLinker (UVP, Inc.) at 0.9 J/cm2 for 0, 10, 30 and 120 min.

The incubations with DNA repair enzymes, PreCR™ and Restorase™, were performed according to the manufacturer's protocols in a 50 µL volume with a DNA input of 5 µL 200-fold diluted sample. 10 µL of the enzyme-incubated sample was used as input for the SGM Plus™ reactions.

Genotyping with the MiniFiler™ and SGM Plus™ kits was performed according to the manufacturer's protocols in a volume of 25 µL. GenPlex™ SNP genotyping was performed according to protocol v2.0.3 and the tri-allelic SNPs were analysed as described by Westen et al. [5] using SNaPshot™ single base extension (AB). The UV-irradiated samples of 200 ng/µL were diluted 200-fold and 1 µL was used as input for the SGM Plus™, GenPlex™ and tri-allelic SNP PCRs. For MiniFiler™ 1 µL of 800-fold diluted sample was used, since this kit uses 30 cycles PCR instead of 28 cycles.

All samples were analysed on an ABI Prism 3130xl Genetic Analyzer (AB). Analysis of the results was done with GeneMapper® v4.0 for GenPlex™ and GeneMapper® ID v3.2.1 for the other methods (AB).

Table 1
Comparison of analysis methods for degraded DNA samples.

	0 min UV	10 min UV	30 min UV	120 min UV	# Loci tested	RMP[a]	DNA input (ng)
SGM+							
Avg.[b]	100.00%	65.15%	27.27%	13.07%	11	3.0×10^{-13}	1
s.d.[c]	0.00%	3.71%	11.34%	8.22%			
n[d]	10	6	10	8			
PreCR & SGM+							
Avg.	100.00%	–	32.95%	14.77%	11	3.0×10^{-13}	5
s.d.	0.00%	–	25.27%	17.16%			
n	4	0	4	4			
Restorase & SGM+							
Avg.	100.00%	–	32.95%	22.73%	11	3.0×10^{-13}	5
s.d.	0.00%	–	28.11%	26.24%			
n	4	0	4	4			
MiniFiler							
Avg.	100.00%	95.83%	76.39%	59.72%	9	8.2×10^{-11}	0.25
s.d.	0.00%	5.32%	8.33%	5.32%			
n	4	4	4	4			
GenPlex							
Avg.	100.00%	98.40%	98.40%	87.77%	47[e]	9.6×10^{-18}	1
s.d.	0.00%	0.75%	0.75%	3.76%			
n	2	2	2	2			
Tri-allelic SNPs							
Avg.	100.00%	100.00%	100.00%	73.33%	15[f]	3.2×10^{-6}	3[g]
s.d.	0.00%	0.00%	0.00%	0.00%			
n	2	2	2	2			

[a] Random match probability for all loci in the kit determined in US Caucasians for (mini-)STRs and in Europeans for (tri-allelic) SNPs.
[b] Average percentage of detected alleles per profile.
[c] Standard deviation over the average percentage of detected alleles per profile.
[d] Number of measurements per method and treatment.
[e] Only 47 of the 49 GenPlex™ SNPs are analyzed due to background peaks.
[f] Determined in 153 Dutch samples, the 15 loci contain 8 tri-allelic and 5 bi-allelic SNPs; 2 presumed SNPs appeared to be fixed.
[g] The tri-allelic SNPs are measured in three multiplexes with 1 ng DNA input each.

Results and discussion

In order to determine which method performs best in examining degraded DNA, a series of UV-irradiated DNA samples was analysed with SGM Plus™ and evaluated against PreCR™, Restorase™, MiniFiler™, GenPlex™ and tri-allelic SNPs. Increasing UV-irradiation time results in progressive DNA degradation as demonstrated by detection of about 13 % of the alleles for SGM Plus™ after 120 min (Table 1, Fig. 1).

The SGM Plus™ results after incubation with PreCR™ or Restorase™ seem to show a slight enhancement in the average percentage of detected alleles compared to standard SGM Plus™ analysis (Table 1, Fig. 1). However, they also show very large standard deviations due to non-consistent results. In contrast, the mini-STRs show reproducible results with an average of about 60 % of detected alleles after 120 min of UV-irradiation (Table 1, Fig. 1). In addition, MiniFiler™ uses only 250 pg of DNA, while the PreCR™ and Restorase™ incubation reactions were performed with 5 ng DNA, whereas at least 50 ng DNA was recommended by the manufacturers. Furthermore, the hands-on and total processing time is the shortest for MiniFiler™, especially when compared to GenPlex™ and the tri-allelic SNPs.

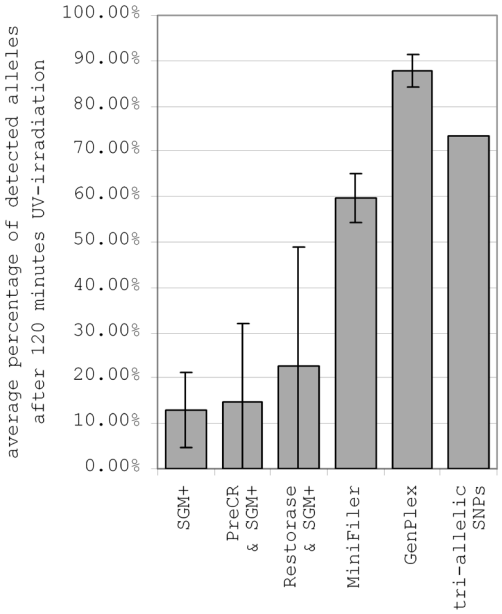

Fig. 1 The results for six analysis methods are plotted against the average percentage of detected alleles per profile after 120 min UV-irradiation. The error bars represent the standard deviation; when no error bars are displayed, all measurements were equal.

Both GenPlex™ and the tri-allelic SNPs showed an average percentage of detected alleles that was higher than for MiniFiler™; namely 88 % and 73 % respectively after 120 min UV-irradiation (Table 1, Fig. 1). This is probably due to the smaller amplicon sizes, being 59–115 bp for GenPlex™ and 58–100 bp for tri-allelic SNPs, compared to 70–283 bp for MiniFiler™. GenPlex™ has a very small random match probability compared to the other methods (Table 1). On the other hand, with GenPlex™ it is much more difficult to detect mixtures or contamination than with (mini-)STRs or tri-allelic SNPs.

The best choice for a certain method depends on the degradation level of the DNA sample and the type of investigation. When the DNA is highly degraded, SNPs perform better than (mini-)STRs. Unfortunately, no SNP information is stored in the (Dutch) national DNA databases and SNPs can therefore only be used in one-to-one comparisons, like for example with ante-mortem and post-mortem DNA evidence. In contrast, with a very small chance on discordance [6], MiniFiler™ results can be hold against the national DNA databases.

Conclusion

When the larger amplicons from a standard STR kit fail to amplify due to DNA degradation, MiniFiler™ can be used to complement the STR results, since it comprises of the longer amplicons from the AmpF*l*STR® SGM Plus™, Profiler™ and Identifiler™ kits. In our study, MiniFiler™ shows more reproducible results and a higher average percentage of detected alleles than standard STR analysis after incubation with DNA repair enzymes from PreCR™ or Restorase™. The SNP genotyping results

from GenPlex™ and the tri-allelic SNPs showed an even higher percentage of detected alleles than MiniFiler™ and are very suitable for one-to-one comparisons, like in human identification cases.

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Conflict of interest

None.

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Chapter 5

Assessment of the stochastic threshold, back- and forward stutter filters and low template techniques for NGM

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Abstract

The AmpF*l*STR® NGM™ kit shows an increased sensitivity compared to previous AmpF*l*STR® kits, and the addition of a 29th PCR cycle was found to be the major cause for this. During in-house validation, we evaluated whether the increased sensitivity requires elevation of the stochastic threshold (below which alleles are prone to drop out due to low template amplification effects). To determine the stochastic threshold, over 500 false homozygotes were examined and the threshold was set at the rfu value where 99 % of the alleles had a peak height below this value. Using 2085 Dutch reference samples, locus-specific stutter ratios were empirically determined and compared with the ones provided by Applied Biosystems. Application of sharp stutter filters is especially important for the analysis of unequal mixtures. To prevent allele calling of 99 % of the -1 repeat unit stutters, thirteen stutter ratio filters could be lowered by up to 1.79 % and for two loci the stutter ratio filters had to be elevated slightly with a maximum of 0.06 %. At all loci +1 repeat stutters were visible for the higher DNA inputs and for lower inputs at the tri-nucleotide repeat locus D22S1045 as well. The overall +1 stutter ratio filter was set to 2.50 % and for D22S1045 it was determined to be 7.27 %. To find the optimal strategy to sensitise genotyping for low template DNA samples, a comparison was made between enhancing the capillary electrophoresis settings (9 kV for 10 s) and increasing the number of PCR cycles (29 + 5 cycles).

Introduction

The AmpF*l*STR® Next Generation Multiplex (NGM™; Applied Biosystems (AB), Foster City, CA, USA) incorporates several new features when compared to SGM Plus™ (AB), which contains the core markers used in Europe until recently. To increase the discriminatory power, NGM™ combines the ten SGM Plus™ loci together with the five new European standard set short tandem repeat (STR) markers: D10S1248, D22S1045, D2S441, D1S1656 and D12S391 [1–3]. A 29th PCR cycle is added to enhance the sensitivity, and the buffer is improved to reduce the influence of inhibitory substances during PCR amplification [4]. When working under ISO 17025 guidelines, in-house validation of each new kit is mandatory. In addition to studying standard performance parameters as precision, repeatability, reproducibility, sensitivity, robustness, and suitability for mixture analysis [5] (and results not shown), we paid special attention to assessment of the stochastic thresholds, -1 and +1 repeat unit stutter filters and low template DNA techniques during our in-house validation of the NGM™, which will be described here.

When a (relatively) low single peak on a locus is analysed, it is compared against the stochastic threshold; when this peak is above the threshold, it is designated as a homozygous allele {a,a}, and when it is below the threshold, it is regarded as a potential heterozygous allele with allele drop-out of the sister allele and called {a,F} (where F stands for 'fail' and represents a 'wild card'). The stochastic threshold influences the number of false inclusions or exclusions during DNA database searches [6]; if it is set too high, homozygotes {a,a} will be called {a,F} and may result in false inclusions, and when it is set too low, heterozygotes {a,b} for which one allele has dropped out may be misdesignated as homozygotes {a,a} resulting in false exclusions [7]. The stochastic threshold is independent of DNA input; with higher inputs less allelic drop-outs occur than with lower inputs, and therefore comparison against the stochastic threshold is less often needed [6]. To assess the effect of the stochastic threshold in a NGM™ dataset, single allele peak heights were compared for heterozygous loci (after drop-out of the sister allele) and homozygous loci.

Stutter peaks are amplification artefacts that are usually ascribed to slipped strand displacement during PCR [8]. STRs, such as the tri-, tetra- and pentanucleotide repeats most widely used in forensic genotyping, predominantly form stutter products of one repeat length shorter (-1 stutter) than the parent allele. However, stutter products of one repeat unit longer (+1 stutter) or two repeat lengths shorter (-2 stutter) also occur [9]. Stutter peaks can greatly complicate the analysis of mixed stains, especially when, with unequal mixtures, the minor donor peaks are in the same peak height range as the stutter peaks of the major donor(s). The height of a stutter peak is affected by several aspects, like the number of nucleotides in the repeat, the AT-content of the repeat and the number of (uninterrupted) repeats [8,10]. As a result, different stutter

ratio thresholds are used for different STR loci in order to prevent calling of peaks on stutter position that are lower than the stutter ratio filter. We determined the -1 stutter ratio thresholds for in-house amplified samples and compared these to the AB stutter ratio thresholds. 2085 Dutch reference DNA profiles were evaluated and we found that the stutter ratio data were not normally distributed. Hence, the stutter ratio thresholds could not be calculated using the average plus 2 or 3 standard deviations from the mean without normalising the data, and we determined them empirically. In addition, we analysed whether +1 stutter ratio filters were needed for the analysis of NGM™ DNA profiles.

Within a locus the average -1 repeat stutter ratio is not the same for all alleles. Longer alleles tend to have higher stutter rates than shorter ones, and this is largely dependent on the length of the longest homogenous repeat stretch [8,11]. When analysing unequal mixtures and deciding on whether a peak is a stutter artefact or a minor donor allele, it might be important to know whether the stutter ratio for a specific allele is expected to be above or below the locus-specific stutter ratio threshold. Therefore, we explored the possibility to use allele-specific stutter ratio thresholds, by assessing the relation between stutter ratio and allele-specific repeat length.

The capillary electrophoresis (CE) injection settings used to analyse STR fragments differ between forensic laboratories, influenced by differences in analysts' opinions and differences between individual machines [12]. Therefore, we tested the effect of CE injection time on the stochastic threshold, percentage detected alleles, average peak height and the peak height ratio for low template (LT) DNA samples. These samples are prone to stochastic amplification effects that result in allele or locus drop-out, allele drop-in, or increased stutter peaks, especially when methods are applied that sensitise LT DNA typing. For these sensitising methods, several strategies have been proposed such as increasing the number of PCR cycles or changing the CE injection settings [13,14]. The use of replicate PCR amplifications in combination with a consensus method is advised to deal with uncertainties of LT DNA typing [6]. To determine what LT DNA technique performs best in combination with NGM™, we increased either the number of PCR cycles or the CE injection voltage.

Materials and methods

DNA samples

The reference DNA samples used for this validation study were kindly provided by the Forensic Laboratory for DNA Research (Leiden University Medical Centre, the Netherlands). They were extracted from blood samples of 2085 randomly sampled Dutch male blood donors coming from different regions in the Netherlands, who gave

their informed consent. These samples were genotyped using NGM™ and the allele frequencies will be described elsewhere (de Knijff and Sijen, in preparation). Pristine DNA007 (positive control DNA in NGM™ kit) and DNA9947a (positive control DNA in Profiler™ kit, AB) were used for determination of the stochastic threshold and LT DNA analyses.

PCR amplification and detection

The DNA was amplified in a multiplex PCR using the AmpF*l*STR® NGM™ kit (AB; early version without primer adjustments for amelogenin, D2S441 and D22S1045 [15]). Amplifications were performed with 29 PCR cycles, according to the protocol of the manufacturer. PCR products were detected by CE with an ABI Prism 3130xl Genetic Analyzer (AB) following the manufacturer's instructions. 1 µL sample or allelic ladder was analysed in combination with 8.7 µL Hi-Di™ Formamide and 0.3 µL GeneScan™ 500 LIZ™ Internal Size Standard (AB). CE injection settings were 3 kV for 15 s (3 kV/15 s), unless stated otherwise.

STR profile analysis

STR profiles were analysed using GeneMapper® ID-X software v. 1.1.1 (AB) with a peak detection threshold of 50 rfu (other settings were used for determination of the stutter ratios, see Stutter ratios section). The -1 stutter distance for D22S1045 was changed from "-4.75 to -3.25" to "-3.75 to -2.25", since it has a tri- and not a tetra-nucleotide repeat unit. The -1 repeat unit stutter filters provided by AB were used in combination with a general +1 stutter filter of 2.50 % and a 7.36 % +1 stutter filter for locus D22S1045, which shows high stutters due to its tri-nucleotide repeat structure. This initial percentage of 7.36 % was based on a subset (the first 528) of the 2085 reference samples. The 2.50 % +1 stutter filter follows from the reasoning that unequal mixtures with a minor to major ratio of 1:20 or higher, are regarded as too complex to interpret; when the major donor is homozygous and the minor donor is heterozygous, a 1:20 mixture results in a minor (heterozygous) donor peak that is 2.50 % (1:40) of the major (homozygous) donor peak.

Description of experiments

Stochastic threshold

For determination of the stochastic threshold, single alleles from heterozygous loci (representing drop-out of the sister allele) and homozygous loci were compared. Data for this comparison were derived from approximately 150 low template DNA samples, including inputs between 60 and 6 pg of pristine DNA007 and DNA9947a. In total,

511 single alleles at heterozygous loci and 138 homozygous peaks were analysed. The stochastic threshold was chosen at a relative fluorescence unit (rfu) value for which 99 % of the single alleles on heterozygous loci were below it.

Stutter ratios

Stutter ratios were obtained using the 2085 reference DNA profiles, which had a PCR input of 250–500 pg DNA. During analysis in GeneMapper® ID-X no stutter filters were applied and a detection threshold of 25 rfu (Supplementary Table S1) was used. Stutter ratios were calculated based on peak height: (stutter peak / parent allele) × 100 %. To determine the -1 stutter ratio thresholds for all loci and the +1 stutter ratio threshold for D22S1045, we used only the stutter percentage of parent alleles that were above the stochastic threshold of 400 rfu (Supplementary Table S1). Stutter peaks that were in-between two alleles on +1/-1 stutter position were regarded as -1 stutters of the longer allele. The -1 stutter ratio data were grouped into categories that comprised one percent (e.g. 2.00 – 2.99 %) and plotted against the number of observations per category. The distribution of the data was compared to a normal distribution using a Kolmogorov–Smirnov (K–S), a Lilliefors and a Shapiro–Wilk W test in Statistica v. 7.1 (StatSoft, Inc., Tulsa, OK, USA). Subsequently, all -1 stutter ratios were sorted per locus (using Excel (Microsoft Corporation, Redmond, WA, USA)), and after exclusion of the 1 % highest stutter ratios, the maximum value was chosen as the empirical locus-specific stutter ratio threshold.

When determining the stutter ratio thresholds, we noted that they are largely dependent on the number of data points that are evaluated; with more data points, more values will be in the 1 % highest stutter ratios, which facilitates a better fine-tuning of the stutter ratio threshold. GeneMapper® ID-X uses stutter ratio filter values with two decimals; although it could be more appropriate to use only one decimal for the stutter ratio thresholds, GeneMapper® ID-X would interpret a stutter ratio filter of for instance 13.3 as 13.30, and therefore we decided to present all our results with two decimals (similar to the AB stutter filters).

Low template DNA analysis

Different laboratories may choose different CE injection settings (e.g. lower settings for reference samples than for trace samples, or to minimise baseline artefacts, or higher settings to increase sensitivity). For use in routine casework, a comparison was made between 3 kV/15 s, 3 kV/10 s and 3 kV/5 s. To examine the effect of these different CE settings on LT DNA profiles, 20, 25 and 30 pg DNA007 were each amplified in 30 replicates.

A dilution series of 63, 31, 16 and 8 pg DNA007 was amplified in 6 replicates to compare our standard PCR and detection technique (29 PCR cycles and CE at

3 kV/15 s) with two LT techniques. For the first LT technique (29 + 5 cycles), after the standard 29 PCR cycles 10 µL PCR product was transferred to a new PCR tube, and after addition of 0.5 µL AmpliTaq™ Gold Polymerase (AB) 5 additional PCR cycles were performed. This method was followed by standard CE, and STR profile analysis was performed with stutter ratio thresholds that were multiplied by 1.5 (see the Results and discussion section on Low template DNA analysis). The second LT technique is based on a standard PCR followed by CE with a raised injection voltage of 9 kV/10 s. For samples that were injected at 9 kV, a Performa™ DTR gel filtration step (Edge Bio, Gaithersburg, MD, USA) preceded the CE to prevent dye blobs in the DNA profiles as described in Westen et al. [14]. For these samples, 2 µL DTR-filtered sample or 1:20 diluted allelic ladder was combined with 7.0 µL Hi-Di™ formamide and 1.0 µL 1:100 diluted GeneScan™ 500 LIZ™ Internal Size Standard (AB).

Results and discussion

Stochastic threshold

In order to determine the stochastic threshold for NGM™ STR profiles, 511 single alleles from heterozygous loci were compared with 138 homozygous peaks occurring in the same low template data set. Fig. 1A shows the empirical cumulative distribution of the homozygous and heterozygous single allele peak heights. The solid horizontal line at 0.99 crosses the data points between 387 and 435 rfu. Hence, we have set the stochastic threshold at 400 rfu (vertical line). The stochastic threshold intersects the homozygous data points at 0.75 (dotted horizontal line). Thus, for a stochastic threshold at 400 rfu, 75 % of the single peaks at homozygous loci are marked as {a,F} and 25 % are correctly designated as {a,a}, while for the single alleles at heterozygous loci 99 % are correctly marked as {a,F} and 1 % is misdesignated as {a,a}. For the latter category, the peak heights ranged between 435 and 613 rfu (compared to a maximum peak height of 994 rfu for homozygous loci). A consequence of determining the stochastic threshold as such is that allele drop-outs may still occur in profiles that have peak heights above the stochastic threshold, albeit uncommon.

For the analysis of SGM Plus™ DNA profiles we used a stochastic threshold of 175 rfu. These profiles were generated after 28 amplification cycles and a CE injection at 3 kV/15 s. For NGM™, the same CE settings, but an additional 29th PCR cycle was performed. We infer that the higher stochastic threshold for NGM™ relates to this extra PCR cycle.

After the in-house validation of NGM™ (that included determination of the stutter ratio thresholds and assessment of low template DNA techniques, as described in more detail below), NGM™ has been implemented in routine casework. However, using the

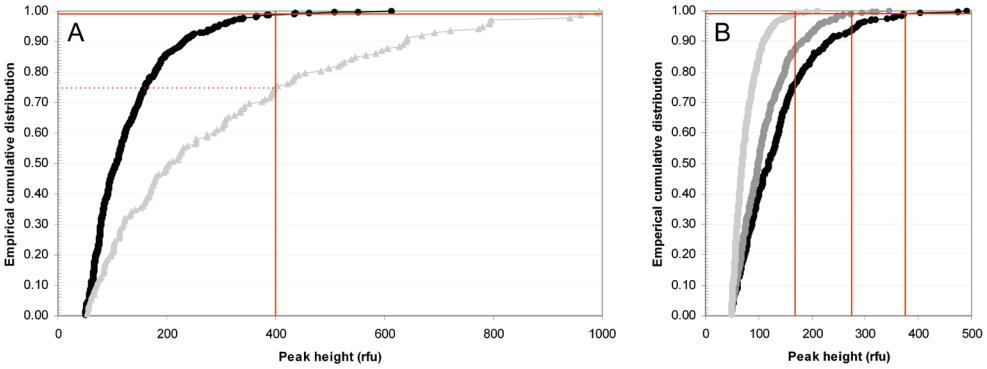

Fig. 1 (A) Empirical cumulative distribution of homozygous and heterozygous single allele peak heights for standard CE settings (3 kV/15 s). Homozygous peaks are plotted as grey triangles (n = 138), and heterozygous alleles (with drop-out of the sister allele) are represented by black dots (n = 511). The solid horizontal line at 0.99 intersects the single heterozygous alleles around 400 rfu (99 % of the alleles has a lower peak height), which is set as stochastic threshold (vertical line). The stochastic threshold crosses the homozygous data at 0.75 (75 % of the alleles has a lower peak height; dotted horizontal line). (B) Empirical cumulative distribution of heterozygous single allele peak heights for CE settings at 3 kV/15 s (black dots, n = 331), 3 kV/10 s (dark grey dots, n = 468) and 3 kV/5 s (light grey dots, n = 583). The solid horizontal line at 0.99 intersects the single heterozygous alleles around 372, 273 and 168 rfu (99 % of the alleles has a lower peak height for that CE setting), as indicated by the vertical lines, for 3 kV/15 s, 3 kV/10 s and 3 kV/5 s, respectively.

standard CE injection settings (3 kV/15 s) in routine casework for some time, profiles showing pull-up peaks (especially for homozygous peaks) were encountered repeatedly. To lower the number of pull-up peaks, we tested reduced injection times of 3 kV/10 s and 3 kV/5 s. These settings lowered the average peak height for DNA profiles with a PCR input of 500 pg DNA007 (n = 2) from 3552 rfu, to 2298 rfu and 1188 rfu for 3 kV/15 s, 3 kV/10 s and 3 kV/5 s, respectively. To assess how these CE injection times affect the stochastic threshold, the percentage detected alleles, the average peak height and the peak height ratio, a set of 90 LT DNA samples was examined (Table 1). When lowering the injection settings from 3 kV/15 s to 3 kV/5 s, the percentage detected alleles drops from 84 to 49 %, while the number of single alleles on heterozygous loci increases (Table 1). The average peak height goes down from 170 to 80 rfu, resulting in a decrease of the empirically determined stochastic thresholds (Table 1). Fig. 1B shows the empirical cumulative distribution of the heterozygous single allele peak heights for 3 kV/15 s, 3 kV/10 s and 3 kV/5 s. The stochastic thresholds for these three settings have been determined empirically in the same way as described above, and decrease from around 400 to 175 rfu (rounded values, Table 1 and Fig. 1B). The peak

Table 1
DNA profile characteristics for different CE settings.

Setting	# single heterozygous alleles[a]	Empirically determined stochastic threshold[b]		Average percentage detected alleles	Average peak height	Peak height ratio (*n*)
		Precise	Round-off			
3 kV/15 s	331	372 rfu	400 rfu	84%	170 rfu	0.62 (1048)
3 kV/10 s	468	273 rfu	300 rfu	74%	125 rfu	0.66 (825)
3 kV/5 s	583	168 rfu	175 rfu	49%	80 rfu	0.73 (419)

[a] Based on 30 amplifications with an input of 20, 25 or 30 pg pristine DNA007 each (*n* = 90).
[b] 99% of single heterozygous alleles were below this value.

height ratio becomes better with shorter injection times, although far fewer loci (419 instead of 1048) remain to calculate this ratio. This is probably caused by the fact that the peak heights are generally lower for shorter injection times, which reduces the efficacy of detecting both alleles at a heterozygous locus. Thereby, especially heterozygous loci with good peak height balance will remain for peak height ratio calculation. Overall, the 3 kV/15 s CE settings show the most complete DNA profiles. Nevertheless, due to the repeatedly encountered pull-up peaks in profiles for routine casework and the additional time needed for re-running and re-analysing these samples, we have chosen to use the 3 kV/5 s CE injection settings for routine DNA analysis, with the possibility to rerun the samples at 3 kV/15 s (or to use a LT technique) when needed.

Stutter ratios

Stutter ratios were determined based on the DNA profiles from the reference set of 2085 Dutch blood donors. For all 15 STR markers -1 repeat unit stutter ratio thresholds were determined and the +1 stutter ratio threshold for D22S1045, as well. For the various loci, between 1279 and 3119 data points were obtained.

Locus-specific -1 repeat stutter

In order to determine whether the locus-specific -1 stutter ratios were normally distributed, the data were categorised in intervals of one percent and plotted against the number of observations per category. A normal distribution was plotted in the same graph, as is shown in Fig. 2 for the -1 stutter ratios of three loci (D10S1248, VWA and D16S539). Visual inspection suggests that the data are not normally distributed, which is confirmed by a Kolmogorov–Smirnov (K–S), a Lilliefors and a Shapiro–Wilk W test (Fig. 2). Similar results were obtained for the other loci (results not shown). Consequently, the stutter ratio thresholds could not be calculated using the average plus 2 or 3 standard deviations from the mean (to comprise 95.45 or 99.73 % of the data points, respectively) without

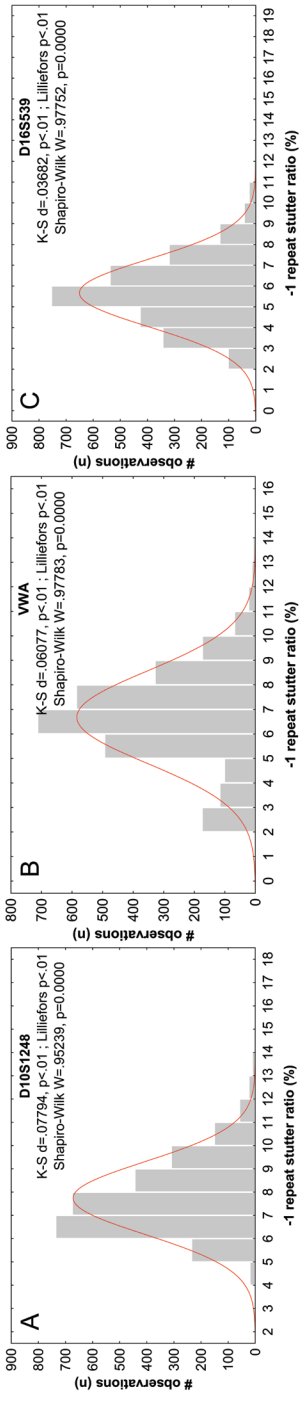

Fig. 2 Minus 1 repeat stutter ratio (in categories of 1 % intervals) plotted against the number of observations (grey bars) and compared to the normal distribution (curve) for locus D10S1248 (A), VWA (B) and D16S539 (C). Normal distribution test results by a Kolmogorov–Smirnov (K–S), a Lilliefors and a Shapiro–Wilk W test are shown in the upper right-hand corner.

normalisation, and we preferred to determine these thresholds empirically.

To determine the -1 stutter ratio threshold empirically, for each locus the -1 stutter ratio data points were sorted in increasing order. After exclusion of the 1 % highest data points, the maximum value was chosen as the empirical locus-specific stutter ratio threshold. Table 2 shows a comparison between the empirically determined stutter ratio thresholds (Supplementary Table S1) and the thresholds provided by Applied Biosystems. For thirteen of the NGM™ STRs, the stutter ratio thresholds could be lowered by 0.46 % to 1.79 %; a slight elevation by 0.04 % and 0.06 % was needed for two loci (D19S433 and TH01, respectively). The number of stutters on which the stutter ratio thresholds are based differs per locus (Table 2), which is due to aspects such as different rates of homozygous or heterozygous donors for the loci, or more or less alleles with stutter peaks below the detection threshold of 25 rfu. The finding that most of the -1 stutter ratio thresholds could be lowered is especially interesting to assist minor contributor allele detection in unequal mixtures with low template DNA components. Although lowering the stutter ratio filters may result in slightly more stutters to be designated as alleles, maintaining stutter filters that are set relatively high may prevent the minor component(s) of unequal mixtures from being called. In our experience with unequal mock casework mixtures (for which the donors are known), the gain of additional alleles from the minor component(s) is more valuable than the drawback of a sporadically called stutter peak [16] (data not shown).

A noteworthy observation on locus TH01 was the presence of different stutter products

Table 2
Locus-specific −1 repeat stutter ratio thresholds.

Locus	D10S1248	VWA	D16S539	D2S1338	D8S1179	D21S11	D18S51	D22S1045	D19S433	TH01[c]	FGA	D2S441	D3S1358	D1S1656	D12S391
n[a]	2629	2756	2658	3119	2891	2865	2903	2641	2760	1279	2966	2681	2748	3119	2953
emp[b] (%)	12.12	11.08	10.11	12.55	10.13	10.91	12.66	16.30	11.10	5.32	11.82	7.68	12.12	13.21	14.82
AB (%)	12.89	11.82	10.57	13.55	10.82	11.40	13.89	17.99	11.06	5.26	12.61	9.47	13.77	14.16	15.84
emp – AB (%)	−0.77	−0.74	−0.46	−1.00	−0.69	−0.49	−1.23	−1.69	+0.04	+0.06	−0.79	−1.79	−1.65	−0.95	−1.02

[a] Number of −1 stutters >25 rfu.
[b] Empirically determined −1 stutter ratio threshold comprising 99% of the stutters.
[c] Allele 9.3 on locus TH01 showed stutter products on position 8.3 (*n* = 191), but also on position 9 (*n* = 52) or on both (*n* = 30); only the 8.3 stutter products were used to determine the stutter ratio threshold.

for allele 9.3. The most frequently occurring stutter peak appeared on position 8.3 (n = 191), but also on position 9 (n = 52) or on both positions (n = 30) stutter products occurred (Fig. 3A–C). The general repeat sequence for locus TH01 is $[AATG]_n$. For allele 9.3 a deletion of one adenosine in the seventh repeat is reported: $[AATG]_6ATG[AATG]_3$ [17,18]. According to the slipped strand displacement model, stutter peaks are formed when the DNA polymerase dissociates from the template during extension and the template strand loops out followed by (out-of-register) re-annealing and further extension of the fragment [8]. A possible explanation for the different stutter products preceding allele 9.3 is that looping out occurs not only for full tetra-nucleotide repeats, but also for the tri-nucleotide repeat number 7.

When following this hypothesis, the occurrence of the two -1 stutter products is independent of the amplification kit employed. For the determination of the -1 repeat length stutter ratios, only the stutters on position 8.3 were taken into account. When analysing a DNA profile with allele 9.3 on TH01, however, one has to realise that a stutter peak on position 9 may be present.

All the analyses described in this paper have been performed using the early version of the NGM™ kit (AB). In the meantime, AB has replaced this version with a new version of NGM™ containing primer adjustments for amelogenin, D2S441 and D22S1045 to avoid a number of known null alleles. AB has not adjusted the stutter filters for this updated version of NGM™. In order to analyse whether the new primers for D2S441 and D22S1045 influence the stutter ratios in our hands, we evaluated the genotyping results from 776 reference samples that were typed with the new version of NGM™ for the Dutch national DNA database. The results did not require changes to be made in the stutter ratio thresholds for the new version of NGM™. Thus, we continue to use the -1 and +1 repeat stutter ratios as determined in this study for use in both database and casework DNA analysis.

Locus-specific +1 repeat stutter

Tri-nucleotide repeat loci, like D22S1045, are known to show higher -1 and +1 stutters than tetra-nucleotide repeat loci [9,11]. This is illustrated in Fig. 3D, where allele 15 is followed by a stutter product of 4.3 %, while the +1 stutter ratios for other loci did not

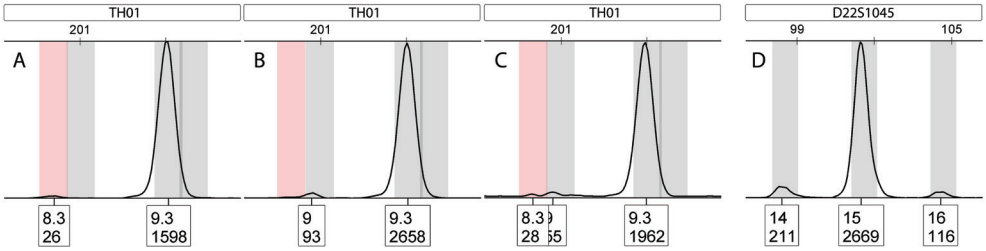

Fig. 3 Examples of stutter products for locus TH01 allele 9.3 on position 8.3 (A), 9 (B) or both (C), and a random example of -1 and +1 stutter on locus D22S1045 (D). In the boxes below the peaks, the upper value represents the allele call and the lower value the peak height in rfu.

exceed 2.50 % in over 99 % of the cases. We determined the empirical +1 stutter ratio threshold for D22S1045 on 2153 data points in the reference DNA profiles. Based on these results, a +1 stutter ratio threshold of 7.27 % is appropriate to comprise 99 % of the +1 stutter products.

Allele-specific -1 repeat stutter

Next to locus-specific stutter ratio thresholds, we evaluated the possibility to use allele-specific -1 stutter ratio thresholds. The empirically determined -1 stutter ratios are plotted per allele for each locus, and Fig. 4 shows an example for three loci (D10S1248, D21S11 and D2S441). For most loci, a rise in stutter ratio is seen with increasing numbers of "full" repeats (Fig. 4A and B). For x.1, x.2 or x.3 microvariants, the stutter ratios are lower than expected based on their length alone, but also they increase with increasing length (Fig. 4B). For these microvariants, the series of repeats is interrupted by a modified repeat. The length of uninterrupted stretches of repeats affects the occurrence of stutters and stutter ratio; the longer the stretches are, the higher the ratio of stutter products [10,11]. This explains the lower stutter ratios for the microvariants. The full alleles at locus D2S441 do not follow the general trend of increasing stutter ratio with higher allele number. Actually, a double pattern of increasing allele-specific stutter ratio seems to be present: one from alleles 10 to 13, and the other from alleles 12 to 16, with two clusters of stutter ratios for alleles 12 and 13. We hypothesise that two different repeat sequences underlie these differences in stutter ratio. This could be tested by sequencing (a number of) these samples, but that is beyond the scope of this study. Interestingly, Phillips et al. [3] did sequence a number of DNA samples for D2S441 and found single nucleotide polymorphisms (SNPs; predominantly in repeat number 6) for various alleles and populations. Such SNPs do interrupt the repeat sequence and shorten the number of uninterrupted repeats significantly, and their results thus support our hypothesis.

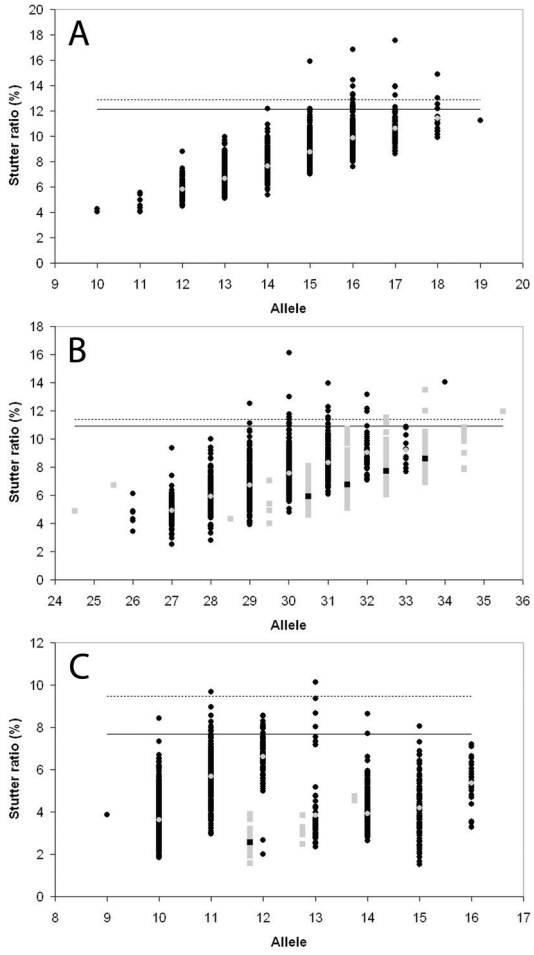

Fig. 4 Empirically determined allele-specific stutter ratios plotted per allele for locus D10S1248 (A), D21S11 (B) and D2S441 (C). Black dots represent allele-specific stutter ratios for "full" alleles, with grey dots as their average when at least 10 data points were present. Grey squares correspond to allele-specific stutter ratios for "x.2" (B) or "x.3" (C) alleles, with black squares as their average. The horizontal lines represent the locus-specific stutter ratio thresholds provided by AB (dotted) or empirically determined (solid).

The use of allele-specific -1 repeat stutter ratio thresholds could aid the analysis of unequal mixtures when peaks at stutter position have a peak height around the locus-specific stutter ratio threshold. To determine allele-specific stutter ratio thresholds that comprise at least 99 % of the stutters (our empirical approach), per allele 100 data points are needed as a minimum. In our data set this is achieved only for the most frequent alleles. Since the stutter data are not normally distributed, extrapolation of the data to values for less frequent alleles is not appropriate. Another disadvantage of the allele-specific stutter ratio thresholds is that they cannot be entered into GeneMapper® ID-X (standard in many laboratories) and can therefore not be applied automatically during DNA profile analysis.

Low template DNA analysis

To determine which LT DNA technique is most fit for use with NGM™, we increased the number of PCR cycles from 29 to 29 + 5 or the CE injection settings from 3 kV/15 s to 9 kV/10 s. A dilution series with an input range from 63 to 8 pg DNA was used. For the method with 29 + 5 PCR cycles, the STR profiles with

Table 3
NGM performance for LT DNA analyses; dilution series of pristine DNA007 ($n=6$).

Input	Average percentage detected alleles			Average number of drop-ins/profile								
	3 kV/15 s	9 kV/10 s	29+5 cycles	3 kV/15 s			9 kV/10 s			29+5 cycles		
				−1	+1	[a]	−1	+1	[a]	−1	+1	[a]
63 pg	99%	100%	n.a.[b]	0.0	0.0	0.0	1.0	0.8	0.0	n.a.	n.a.	n.a.
31 pg	90%	92%	n.a.	0.5	0.0	0.0	0.3	0.7	0.0	n.a.	n.a.	n.a.
16 pg	66%	78%	79%	0.0	0.0	0.0	0.3	0.0	0.0	0.2	2.0	1.0
8 pg	41%	57%	57%	0.0	0.0	0.0	0.7	0.0	0.2	0.2	1.0	0.0

[a] Drop-ins at other positions than −1 and +1 repeat stutter distance.
[b] Not analysed due to over-loaded profiles.

an input of 63 or 31 pg DNA were highly overloaded and could not be analysed (Table 3); the profiles with a PCR input of 16 or 8 pg DNA for this method were analysed with 1.5 times the stutter ratio thresholds (see details below). The average number of drop-ins, resulting from contaminating alleles, elevated stutters, or other artefacts [12], was calculated per profile. The position on which the drop-ins occurred was divided into three categories: -1 stutter position, +1 stutter position, or other position. Our standard method shows the lowest number, while the method with 29 + 5 PCR cycles relatively shows the highest number of drop-ins per profile. The method with CE at 9 kV/10 s shows a number of drop-in alleles in-between the other two methods. For all methods, drop-ins occur predominantly at -1 or +1 stutter position, thereby most likely representing elevated stutter artefacts (also termed as stutter drop-ins [6]).

Stutter ratios tend to increase when performing additional PCR cycles, such as 28 + 6 cycles for SGM Plus™ [13] or 29 + 5 cycles for NGM™. In order to determine the magnitude of this increase, a comparison was made between the (previously mentioned) DNA007 profiles with a PCR input of 8 and 16 pg that were each replicated 6 times with 29 + 5 cycles for NGM™, and 29 cycles NGM™ amplifications with an input of 250 or 500 pg DNA007 in 6 replicates each, as these two sets showed comparable peak heights. Stutters were determined in the same way as described in the Materials and methods section on Stutter ratios. In Supplementary Table S2 is shown that, in total, 198 stutters were obtained for DNA profiles after 29 + 5 cycles and 375 stutters after 29 cycles. For each allele in the DNA007 profiles for which it was possible to determine a stutter ratio, we calculated the average stutter ratio over the 12 DNA profiles that were amplified with either 29 + 5 or 29 PCR cycles. Next, the ratio between them was determined by dividing the average stutter ratio for example for allele 12 on D10S1248 after 29 + 5 cycles by the average stutter

ratio for the same allele after 29 PCR cycles. Such a ratio could be determined for 24 alleles in the DNA007 profiles and the ratios ranged between 1.0 and 1.5. When analysing SGM Plus™ DNA profiles after 28 + 6 PCR cycles, we used to multiply the stutter ratio thresholds by 1.5, and, based on the results, it is appropriate to use this factor of 1.5 for the analysis of 29 + 5 cycles NGM™ DNA profiles, as well.

We do not use a stochastic threshold for any of the LT DNA techniques as we always consider that allele drop-out may have occurred. For these LT methods, it is needed to perform multiple PCR amplifications of the same DNA extract in combination with a consensus method [6]. In our laboratory, the *n*/2 method is used, for which an allele is included in the consensus when it is designated in at least half of the replicates (with $n = 3$ or $n = 4$ as optimal replicate number) [6].

Conclusion

Our conclusions and decisions for practical use are summed up below.

- The stochastic threshold is dependent on the injection settings used; for 3 kV/5 s 175 rfu, for 3 kV/10 s 300 rfu, and for 3 kV/15 s 400 rfu is appropriate.
- Since the stochastic threshold includes 99 % of the single alleles on heterozygous loci, some may remain un-flagged. The maximum observed peak height in this data set is 613 rfu.
- Stutters are not normally distributed and stutter ratio thresholds are best determined empirically.
- Thirteen -1 stutter ratio thresholds are lowered by up to 1.79 % compared to the ones provided by AB; two are elevated slightly (with a maximum of 0.06 %). This will assist allele calling of the minor contributor in unequal mixtures.
- Especially for the tri-nucleotide repeat locus D22S1045 a +1 stutter ratio threshold is needed, which is set at 7.27 %.
- Allele-specific -1 stutter ratio thresholds can only be determined for the most frequent alleles and cannot be entered into GeneMapper® ID-X. Consequently, they will not (yet) be applied.
- Low template DNA analysis can be performed with 9 kV/10 s CE injection settings; only for the very low ranges (<31 pg) the use of 29 + 5 PCR cycles is recommended.

In conclusion, when introducing a new STR kit for routine use, we recommend in-house validation of several aspects, such as the stochastic threshold, -1 and +1 repeat stutter ratio thresholds and low template DNA analysis methods. These parameters will optimise the analyses of complex mixtures and low template DNA samples.

Acknowledgements

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Supplementary data

Supplementary Table S1
Insight into the composition of the -1 stutter dataset based on the 6-FAM-channel of NGM DNA profiles from 2085 Dutch reference samples. Data are presented based on the parent allele peak height and on the stutter peak height. When less than 100 data points are available, the values are shown in grey, and the highest stutter ratio becomes the stutter ratio filter value.

		parent peak height (rfu)				stutter peak height (rfu)		
		>400[a]	<400[b]	400-1000	>1000	>25[a]	>50	25-50
D10S1248	*n*	2629	21	211	2418	2629	2562	67
D10S1248	emp.thres.	12.12	18.13	13.95	12.00	12.12	12.16	11.38
D10S1248	avg	7.74	10.32	8.11	7.71	7.74	7.77	6.72
D10S1248	stdev	1.56	2.80	1.99	1.51	1.56	1.55	1.49
vWA	*n*	2756	14	376	2380	2756	2487	269
vWA	emp.thres.	11.08	14.37	12.88	10.91	11.08	11.10	9.82
vWA	avg	6.68	10.79	7.12	6.61	6.68	6.91	4.62
vWA	stdev	1.88	2.55	1.90	1.87	1.88	1.73	1.98
D16S539	*n*	2658	6	336	2322	2658	2297	361
D16S539	emp.thres.	10.11	14.53	10.82	9.85	10.11	10.23	9.07
D16S539	avg	5.67	10.18	6.20	5.59	5.67	5.87	4.37
D16S539	stdev	1.63	2.52	1.80	1.59	1.63	1.54	1.58
D2S1338	*n*	3119	51	888	2231	3119	2833	286
D2S1338	emp.thres.	12.55	19.00	13.50	12.15	12.55	12.63	10.42
D2S1338	avg	7.86	10.15	8.17	7.73	7.86	7.97	6.77
D2S1338	stdev	1.78	2.62	1.94	1.70	1.78	1.78	1.44

[a] The empirically determined -1 stutter thresholds that are presented in the Results and discussion section on Locus-specific -1 repeat stutter, are based on parent peaks >400 rfu and stutter peaks >25 rfu.
[b] Parent peaks <400 rfu (representing LT DNA) show relatively high stutter thresholds based on small numbers of stutter peaks and were left out of the overall stutter threshold determination; for LT DNA multiple independent amplifications should be performed in combination with a consensus method to accommodate stochastic amplification effects, such as elevated stutters.

Supplementary Table S2
Ratio between the average stutter ratio per allele for DNA007 after 29+5 (8 and 16 pg DNA input, 6 replicates each) or 29 (250 and 500 pg DNA input, 6 replicates each) NGM PCR cycles.

		29+5 cycles		29 cycles		29+5/29
marker	allele	average stutter ratio	*n*	Average stutter ratio	*n*	ratio
D10	12	6.66	8	5.80	16	1.15
	15	10.58	8	8.58	16	1.23
D12S391	18	7.17	8	6.82	16	1.05
	19	9.88	2			
D16S539	9	3.85	9	3.65	16	1.06
	10	4.65	3			
D18S51	12	5.39	6	4.98	16	1.08
	15	8.92	6	7.32	16	1.22
D19S433	14	7.91	10	6.35	16	1.25
	15	7.56	2			
D1S1656	13	7.61	5	6.89	16	1.10
	16	12.45	10	9.98	16	1.25
D21S11	28	7.37	10	5.87	16	1.26
	31	10.48	8	8.01	16	1.31
D22	11	4.36	9	2.91	16	1.50
	16	11.01	10	9.53	16	1.15
D2S1338	20	9.24	9	8.80	16	1.05
	23	8.51	7	8.28	16	1.03
D2S441	14	5.60	7	3.75	16	1.49
	15	4.17	1			
D3S1358	15	8.94	9	7.57	16	1.18
	16	8.42	1			
D8S1179	12	8.47	10	6.67	16	1.27
	13	8.96	2			
FGA	24	8.79	7	7.74	16	1.14
	26	9.64	7	9.54	16	1.01
TH01	7	3.38	8	2.23	15	1.52
	9.3	2.17	1	1.47	8	1.48
VWA	14	3.74	8	3.11	16	1.20
	16	8.05	7	6.07	16	1.33
n total			198		375	24

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Chapter 6

Combining results of forensic STR kits: HDplex validation including allelic association and linkage testing with NGM and Identifiler loci

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Abstract

The autosomal short tandem repeat (STR) kits that are currently used in forensic science have a high discrimination power. However, this discrimination power is sometimes not sufficient for complex kinship analyses or decreases when alleles are missing due to degradation of the DNA. The Investigator™ HDplex™ kit contains nine STRs that are additional to the commonly used forensic markers, and we validated this kit to assist human identification. With the increasing number of markers it becomes inevitable that forensic and kinship analyses include two or more STRs present on the same chromosome. To examine whether such markers can be regarded as independent, we evaluated the 30 STRs present in NGM™, Identifiler™ and HDplex™. Among these 30 markers, 17 syntenic STR pairs can be formed. Allelic association between these pairs was examined using 335 Dutch reference samples and no linkage disequilibrium was detected, which makes it possible to use the product rule for profile probability calculations in unrelated individuals. Linkage between syntenic STRs was studied by determining the recombination fraction between them in five three-generation CEPH families. The recombination fractions were compared to the physical and genetic distances between the markers. For most types of pedigrees, the kinship analyses can be performed using the product rule, and for those cases that require an alternative calculation method (Gill et al., Forensic Sci Int Genet 6:477–486, 2011), the recombination fractions as determined in this study can be used. Finally, we calculated the (combined) match probabilities, for the supplementary genotyping results of HDplex™, NGM™ and Identifiler™.

Introduction

The expansion of the European standard set (ESS) of autosomal short tandem repeat (STR) markers [2, 3] has resulted in the development of new forensic STR kits such as the AmpF*l*STR® NGM™ (SElect) PCR Amplification Kit (Applied Biosystems (AB), Foster City, CA, USA), the PowerPlex® ESX™ and ESI™ Systems (Promega Corporation (Promega), Madison, WI, USA) and the Investigator® ESSplex Plus™ Kit (Qiagen Benelux B.V. (Qiagen), Venlo, the Netherlands). These kits combine the gender-determining Amelogenin marker with the ten commonly used AmpF*l*STR® SGM Plus™ (AB) STRs and the five new ESS markers, with or without the addition of SE33 (ACTBP2) as a 16th STR. The five new ESS markers show a higher discrimination power than the five STRs in the AmpF*l*STR® Identifiler™ Kit (AB) that are additional to SGM Plus™ [4]. Nevertheless, in complex kinship analyses or in (missing person) cases in which the DNA has been severely degraded, the power of discrimination of the DNA profile may not be high enough to identify a person. In these cases, it is opportune to analyse additional highly discriminative STR markers.

In 2010, the Investigator® HDplex™ Kit (Qiagen; formerly known as Mentype® Chimera™ PCR Amplification Kit, Biotype Diagnostic GmbH, Dresden, Germany) became available in the European forensic market. This kit contains nine highly discriminative STRs (D2S1360, D3S1744, D4S23 66 , D5S25 00 , D6 S474 , D7S15 17 , D8S1132 , D10S2325 and D21S2055), next to the Amelogenin gender marker and three STRs that are included in the abovementioned commercial forensic kits: D12S391, D18S51 and SE33. We validated the HDplex™ for human identification and generated allele frequencies based on 335 Dutch reference samples.

With the increasing number of forensically available STRs, it becomes inevitable that two or more markers are present in the same chromosome (a.k.a. syntenic markers). To ensure independent inheritance, syntenic markers are ideally situated on different arms of the chromosome or at least 50 centiMorgans (cM) apart. The latter means that there is at least a 50 % chance of recombination between the two syntenic markers, which are therefore regarded as unlinked [5]. The vWA and D12S391 markers are only 6.36 Mb apart, and several studies have addressed the possible linkage or allelic association (a.k.a. linkage disequilibrium) between them [1, 4, 6–8]. From their results, the authors expect no interpretation problems at the population level, but they do express their concerns for the interpretation of data from closely related individuals. In our study, we evaluated the 30 STRs present in NGM™ (AB), Identifiler™ (AB) and HDplex™ (Qiagen), from which 17 pairs of syntenic loci can be formed. For these pairs, we determined the recombination fraction in five three-generation CEPH families and compared our results with those of Phillips et al. [7], who studied the recombination landscape around a broad spectrum of forensically relevant STRs based on HapMap

data. In addition, we evaluated whether linkage disequilibrium was detectable at the population level. Finally, we determined the random match probability when combining two of the three (NGM™, Identifiler™ or HDplex™) or all three kits.

Material and methods

DNA samples

Validation tests for the HDplex™ were performed using the pristine DNA samples DNA XY5 (positive control HDplex™ (Qiagen)), DNA007 (positive control NGM™ (AB)) and hDNA (Quantifiler™ Human DNA standard (AB)). 2085 DNA samples, representative for the Dutch population, were used with informed consent of the donors. Five three-generation CEPH families (Coriell Institute, Camden, NJ, USA) were analysed: one French family with pedigree number 0066 and four Utah families with pedigree numbers 1362, 1423, 1454 and 1463. Each family consisted of four grandparents, two parents and seven to 11 children. To prepare artificially degraded DNA, hDNA (200 ng/µL) was treated with UV–light for 0, 10, 30 and 60 min, following the protocol described by Westen et al. [9].

PCR amplification, capillary electrophoresis and DNA profile analysis

All 2,085 population samples were amplified with the NGM™ (AB) and Identifiler™ (AB) PCR amplification kits (de Knijff and Sijen, in preparation). 335 of the 2,085 Dutch population samples were amplified with the HDplex™ kit (Qiagen) using a 750-pg PCR input. The CEPH family samples were amplified with all three kits using PCR inputs of 500 pg for NGM™, 1 ng for Identifiler™ and 750 pg for HDplex™. During the HDplex™ validation, study various amounts of template DNA were used: a series from 8 to 750 pg during sensitivity assays, 750 pg for mixture studies, 1 ng when assessing resistance to PCR inhibitors and 1 ng artificially degraded hDNA (1 µL of 200-fold diluted UV-treated hDNA). PCR products were detected by capillary electrophoresis (CE) on an ABI Prism 3130xl Genetic Analyzer (AB). PCR amplifications and CE detection were performed according to the manufacturer's instructions. DNA profiles were analysed using GeneMapper® ID-X v. 1.1.1 (AB). When analysing HDplex™ profiles, we found that all D10S2325 peaks were detected on the left-hand side within or adjacent to the bin for the amplified samples, but not for the allelic ladder. We solved this issue by diluting the allelic ladder 1,000-fold and re-amplifying it for 15 additional PCR cycles [10]. We suspect that the D10S2325 primers used to amplify the allelic ladder and those provided in the kit originate from different synthesis batches, resulting in a shift of the amplified alleles of approximately 0.5 nt compared to the allelic ladder.

Statistical analyses

For the 2,085 population samples, allele frequencies were calculated using the Excel Microsatellite Toolkit [11]. Departure from the Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) testing was performed using Arlequin v. 3.5 [12]. HWE exact tests were performed with 100,000 dememorisation steps and 1,000,000 steps in the Markov chain. LD between pairs of loci was calculated with three initial conditions for the Expectation–Maximisation algorithm and 10,000 permutations. Power of discrimination, power of exclusion, polymorphic information content and match probabilities were calculated using the Genetic Identity PowerStats v. 12 Excel spreadsheet (Promega) [13]. We estimated the genotypic linkage disequilibrium correlation coefficients (r^2) using the LINKDIS program [14] implemented in GENETIX v. 4.05.2 [15]. Allele frequencies and descriptive statistics for NGM™ and Identifiler™ will be published elsewhere (de Knijff et al., in preparation). In order to determine the recombination fraction between pairs of syntenic loci, the three-generation CEPH family data were analysed with LINKAGE [16, 17] (see Supplementary Note 1 for additional information).

Results and discussion

Validation of the Investigator HDplex™ kit

In order to test the sensitivity of the HDplex™, a dilution series between 750 and 8 pg DNA XY5 was genotyped in threefold. Full profiles were detected down to 63 pg DNA (for one of the three replicates, Table 1). In our laboratory, the optimal input was found to be 750 pg template DNA, which resulted in heterozygous peak balances between 0.79 and 0.93 (calculated by dividing the height of the lower peak by that of the higher peak) compared to 0.64 to 0.92 for 500 pg template DNA, and the average peak heights for heterozygous alleles were around 2,300 rfu (relative fluorescence unit) for 750 pg and around 1,376 rfu for 500 pg template DNA.

For the analysis of low template DNA, a comparison was made between the standard protocol (30 PCR cycles with 3 kV/10 s CE injection settings), two additional PCR cycles (32 cycles with CE at 3 kV/10 s, as recommended by the manufacturer for DNA inputs <100 pg) and increasing the CE injection voltage to 9 kV (30 PCR cycles with CE at 9 kV/10 s) [18]. A dilution series of 63, 31, 16 and 8 pg DNA007 was amplified in threefold. Supplementary Fig. 1A shows that both increased cycling and increased CE injection voltage are functional to obtain a higher percentage of detected alleles, with 9 kV injection voltage giving a slightly higher percentage of detected alleles for most profiles. This method is easily performed, without the use of additional DNA extract, to increase the sensitivity of STR typing [18]. In our

Table 1 HDplex sensitivity characteristics based on a dilution series of pristine XY5 DNA (n=3)

	750 pg	500 pg	250 pg	125 pg	63 pg	31 pg	16 pg	8 pg
Average % detected alleles	100±0.00	100±0.00	100±0.00	100±0.00	94±0.06	72±0.21	51±0.24	35±0.10
Average peak height (rfu)	2,293±309	1,376±309	1,009±233	731±94	245±118	185±63	108±25	83±10
Minimum peak height (rfu)	1,182	1,022	682	632	193	130	58	30
Maximum peak height (rfu)	2,658	1,493	967	1,460	489	547	190	146

laboratory (NFI), for low template DNA samples, multiple independent amplifications are performed, and the results are interpreted in combination with the consensus method as described by Benschop et al. [19], irrespective of the sensitising technique used.

Artificially degraded samples were genotyped in duplicate for both HDplex™ and NGM™. Supplementary Fig. 1B shows that NGM profiling is less sensitive to DNA degradation than HDplex™ analysis, as full NGM™ profiles are found up to 30 min of UV irradiation, while the average percentage of detected alleles for HDplex™ starts to decrease with 10 min of irradiation. Both for HDplex™ and NGM™, allele drop-out is most prominent in larger sized markers, which is in agreement with earlier findings [20–22]. HDplex™ carries relatively more large-sized amplicons, ranging from 70 to 475 bp, while NGM™ spans 76 to 352 bp. This is probably due to the fact that HDplex™ makes use of a four-dye chemistry, instead of a five-dye chemistry as used with NGM™ and Identifiler™, thereby providing less room for markers with small amplicon sizes.

Further characteristics, for which the performance of the HDplex™ was tested, were resistance to PCR inhibitors and DNA mixture analysis. Also for these aspects, the HDplex™ performed within the boundaries we had set (results not shown); HDplex™ tolerated 50 μM hematin and correctly analysed two- and three-person mixtures that were within the sensitivity range of the kit (Table 1).

DNA profile characteristics

Several aspects of the HDplex™, such as the inter-locus balance, the intra-locus peak height ratio and the stutter ratio thresholds, were evaluated based on the genotyping results for 335 Dutch reference DNA samples. The inter-locus balance is calculated by dividing the average peak height on a locus by the average peak height of the complete profile. As apparent from Supplementary Fig. 2A, the shorter loci have an inter-locus balance that is above one (one is a perfect balance), while the longer loci have a balance below one. Overall, a general decreasing trend is visible with increasing amplicon length, although

Table 2 Locus-specific −1 repeat stutter ratio filters for HDplex

Locus	D2S1360	D3S1744	D4S2366	D5S2500	SE33	D6S474	D7S1517	D8S1132	D10S2325	D12S391	D18S51	D21S2055
Number[a]	448	594	139	505	504	356	587	639	519	611	546	394
emp[b,c] (%)	14.61	15.96	9.76	8.91	16.11	10.28	13.38	17.28	10.67	18.13	13.25	20.58
Qia[d] (%)	9.00	11.00	6.00	6.00	10.00	8.00	10.00	13.00	5.00	14.00	11.00	15.00
emp-Qia (%)	+5.61	+4.96	+3.76	+2.91	+6.11	+2.28	+3.38	+4.28	+5.67	+4.13	+2.25	+5.58

[a] Number of −1 stutters >25 rfu; this number differs between loci from variation in the numbers of stutters above detection threshold, homozygous alleles (which give only one stutter at a locus) and overlapping alleles at heterozygous loci (e.g., for an 11/12 genotype, the −1 stutter of allele 12 overlaps with allele 11)

[b] Empirically determined −1 stutter ratio threshold comprising 99 % of the stutters

[c] Presented with two decimals to fit GeneMapper ID-X entry

[d] Stutter ratio filter as provided by Qiagen

D8S1132 shows slightly higher values than the other short loci and SE33 shows slightly lower values than the other markers.

The intra-locus peak height ratio (PHR, also known as heterozygous peak balance) should, for standard PCR conditions, be between 0.6/0.7 and 1 (perfect balance) [23, 24] to enable correct interpretation of the DNA profile. Supplementary Fig. 2B illustrates that the shorter loci all have median values >0.80 and, thus, better PHRs (i.e. closer to one) than the longer loci that show median values down to 0.74. Nevertheless, all loci show PHRs that meet the requirements for correct interpretation of the DNA profile.

The HDplex™ consists of 11 tetra- and one pentanucleotide (D10S2325) repeat markers and does not contain trinucleotide STRs (such as D22S1045 in NGM™) that are prone to increased stuttering at both -1 and +1 position (one repeat unit shorter or longer than the parent allele, respectively) [25, 26]. Accordingly, the (pristine) DNA profiles did not invoke inference of locus-specific +1 stutter ratio filters. However, several -1 repeat stutters were called, indicating that the locus-specific -1 stutter ratio filters provided by the manufacturer do not suffice. Therefore, we determined the -1 stutter ratio thresholds empirically using the same method as in Westen et al. [26]. These stutter ratio thresholds comprise 99 % of the -1 stutters and are based on 139 to 639 observations per locus (Table 2). Table 2 shows the empirically determined -1 stutter ratio thresholds, which are compared to the ones provided by Qiagen. For all 12 loci, the empirically determined -1 stutter ratio thresholds were higher than the thresholds provided by Qiagen, and we elevated the stutter ratio filters with 2.25 % to 6.11 % in the profile analysis software. The large differences between our thresholds and those suggested by Qiagen may have several reasons. It could result from our relatively small number of observations (when compared to our NGM™ validation [26]), the

method by which stutter thresholds are calculated (empirically by us, not known for Qiagen), whether stutters at -1 and +1 position (heterozygous pair with two repeat lengths size difference) are included (included by us, not known for Qiagen) or how alleles not showing detectable stutters are regarded (excluded by us, not known for Qiagen). Nevertheless, we feel that the stutter ratios as provided by Qiagen are too low, as we observed several called stutter peaks when using their stutter ratio filters, already when analysing pristine DNA and optimal inputs (increased stutters are well-known for low template samples). Therefore, we recommend determining the -1 stutter ratio thresholds in-house when working with the HDplex™.

Statistical analysis

Hardy–Weinberg equilibrium, linkage disequilibrium and linkage

The genotyping results of the 335 Dutch reference samples were used to determine the allele frequencies and summary statistics for the HDplex™ (Supplementary Table 1). One important aspect of the summary statistics is the Hardy– Weinberg equilibrium (which refers to the independent association of alleles within one locus [27]). The data should not deviate significantly from HWE to enable assessment of gametic disequilibrium or linkage between syntenic STR pairs. Gametic disequilibrium is also known as linkage disequilibrium (LD) and refers to the non-random association of alleles at different loci into gametes [28]. Since we aim to assess these aspects for all syntenic STR pairs residing in HDplex™, NGM™ and Identifiler™, the p value for HWE testing was also determined for the syntenic markers in NGM™ and Identifiler™ (based on the complete DNA reference set of 2,085 samples; results not shown). For the syntenic markers in our population data, no significant deviation from HWE was detected after Bonferroni correction (Supplementary note 2).

An overview of all 30 markers that are present in HDplex™, NGM™ and Identifiler™ and their chromosome location is presented in Table 3. The 17 syntenic STR pairs that can be formed out of these 30 markers are shown in Table 4. For these pairs, we tested for departure from linkage equilibrium using the Arlequin software. The results are presented in Table 4, both for the subset of 335 Dutch reference samples (meaning 335 HDplex™, 335 NGM™ and 335 Identifiler™ DNA profiles) and for the full set extending to 2,085 samples (which means 2,085 NGM™, 2,085 Identifiler™ and 335 HDplex™ DNA profiles). No significant departure from linkage equilibrium was detected after Bonferroni correction (Supplementary Note 2). Using the GENETIX software, we found genotypic correlation coefficients between 0.014 and 0.051. As a comparison, r^2 values as high as 0.35 [29] or 0.45 [30, 31] have been found for Dutch or European populations, albeit for much smaller physical distances. Thus, the correlation coefficients that we found seem to indicate low genotypic LD for all 17 STR pairs tested in our population samples.

Table 3 Overview of the STR markers present in HDplex (HD), NGM and Identifiler (Id)

chr	STR	kit[a]	Location	Location (Mb)
01	D1S1656	NGM	1q42.2	230.91
02	TPOX	Id	2p25.3	1.49
02	D2S1360	HD	2p24-p22	17.49
02	D2S441	NGM	2p14	68.24
02	D2S1338	NGM, Id	2q35	218.88
03	D3S1358	NGM, Id	3p21.3	45.58
03	D3S1744	HD	3q24	147.09
04	D4S2366	HD	4p16-p15.2	6.48
04	FGA	NGM, Id	4q28	155.51
05	D5S2500	HD	5q11.2	58.70
05	D5S818	Id	5q23.2	123.11
05	CSF1PO	Id	5q33.1	149.46
06	SE33	HD	6q14	88.99
06	D6S474	HD	6q21-22	112.88
07	D7S820	Id	7q21.1	83.79
07	D7S1517	HD	7q31.3	123.50
08	D8S1132	HD	8q23.1	107.33
08	D8S1179	NGM, Id	8q24.1	125.91
10	D10S2325	HD	10p12	12.71
10	D10S1248	NGM	10q26.3	131.09
11	TH01	NGM, Id	11p15.5	2.19
12	vWA	NGM, Id	12p13.3	6.09
12	D12S391	NGM, HD	12p13.2	12.45
13	D13S317	Id	13q31.1	82.72
16	D16S539	NGM, Id	16q24.1	86.39
18	D18S51	NGM, Id, HD	18q21.3	60.95
19	D19S433	NGM, Id	19q12	30.42
21	D21S11	NGM, Id	21q11.2-q21	20.55
21	D21S2055	HD	21q22	41.19
22	D22S1045	NGM	22q12.3	37.54

HD HDplex, *Id* Identifiler

[a] 2,085 genotypes were assessed for all loci present in NGM, Identifiler and locus SE33; 335 genotypes were determined for the nine (remaining) loci in HDplex

To determine the recombination fraction between the 17 above-mentioned syntenic STR pairs, 5 three-generation CEPH pedigrees were profiled for HDplex™, NGM™ and Identifiler™. The genotypes of the 78 individuals are provided in Supplementary Table 2. Figure 1A visualises four aspects of the 17 syntenic STR pairs (the corresponding numeric values are shown in Supplementary Table 3): (1) the physical distance between the markers as derived from the NCBI UniSTS database [32], (2) the genetic distance between the nearest HapMap SNP proxies as determined by Phillips et al. [7] together with (3) the Kosambi-derived recombination fractions (Rc) that were converted from the genetic distance using the Kosambi mapping function [7] and (4) the recombination fractions based on the CEPH pedigree data that were determined using the LINKAGE program (for which estimates greater than 0.5 are interpreted as being equal to 0.5 (Prof. J. Ott, personal communication)). As apparent from Fig. 1A and Supplementary Table 3, the physical distance can present both an overestimation and an underestimation of the genetic distance. The largest underestimations are made for the STR pairs vWA-D12S391 and TPOXD2S1360, which might be due to the fact that both these pairs are situated near the telomeres, where crossover rates are generally higher [7, 33]. The recombination fractions that we found based on the CEPH pedigree data are comparable to the Kosambi-derived Rc values (that were generated based on the cM interval of the HAPMAP SNP proxies [7]), as shown in Fig. 1A and Table 4. Another representation of these data is given in Fig. 1B, from which it is clear that the data points reside around the diagonal line, indicating similar results for both methods.

Table 4 Overview of pairwise linkage disequilibrium test results for syntenic STR loci in HDplex, NGM, and Identifiler

chr	STR pair	position[a]	Arlequin LD p-value 335NL	Arlequin LD p-value 2085NL	GENETIX corr.coef. 335NL	GENETIX p-value 335NL	GENETIX corr.coef. 2085NL	GENETIX p-value 2085NL
02	TPOX-D2S1360		0.038	0.076	0.045	0.040	idem[b]	idem
02	TPOX-D2S441		0.221	0.314	0.046	0.340	0.016	0.482
02	TPOX-D2S1338	*	0.953	0.449	0.035	0.994	0.016	0.894
02	D2S1360-D2S441		0.796	0.915	0.041	0.456	idem	idem
02	D2S1360-D2S1338	*	0.702	0.498	0.040	0.114	idem	idem
02	D2S441-D2S1338	*	0.418	0.507	0.042	0.462	0.015	0.968
03	D3S1358-D3S1744	*	0.402	0.580	0.042	0.562	idem	idem
04	D4S2366-FGA	*	0.191	0.135	0.050	0.000	idem	idem
05	D5S2500-D5S818		0.771	0.913	0.040	0.878	idem	idem
05	D5S2500-CSF1PO		0.037	0.138	0.051	0.023	idem	idem
05	D5S818-CSF1PO		0.334	0.822	0.043	0.658	0.014	0.956
06	SE33-D6S474		0.188	0.265	0.040	0.661	idem	idem
07	D7S820-D7S1517		0.946	0.957	0.041	0.921	idem	idem
08	D8S1132-D8S1179		0.493	0.211	0.043	0.020	idem	idem
10	D10S2325-D10S1248	*	0.845	0.889	0.039	0.860	idem	idem
12	vWA-D12S391		0.821	0.591	0.038	0.659	0.015	0.963
21	D21S11-D21S2055		0.731	0.332	0.034	0.999	idem	idem

For the Arlequin and GENETIX results, two datasets were used: "335NL" referring to a set of 335 samples profiled with NGM, Identifiler and HDplex, and "2085NL" which refers to 2,085 samples profiled with NGM and Identifiler, complemented with the genotyping data for HDplex for a subset of 335 of the samples

[a] Syntenic STR pairs on different chromosomal arms are marked by an asterisk in this column and shown in grey

[b] Equal to the value for 335NL

The pair with the smallest physical distance and the second smallest recombination fraction is vWA and D12S391 (Supplementary Table 3). Since these STRs are both present in the current generation STR kits, several studies have assessed their possible allelic association [1, 4, 6–8]. As confirmed by our results, none of these studies have found indications for linkage disequilibrium between these markers at the population level, and it is inferred that it is legitimate to use the product rule for DNA profile probability calculations involving unrelated individuals. Regarding the assessment of linkage, a different approach is needed. In our and one of the other studies [6], three-generation CEPH families are used to determine the recombination fraction between vWA and D12S391. We find a recombination fraction of 0.17; Budowle et al. [6] estimate a value of 0.11. The Kosambi-derived Rc for vWA-D12S391 is 0.12 [1, 7], which is in the same range. Taken together, all values indicate the presence of (loose) physical linkage, which may influence the interpretation of genotyping data from (closely) related individuals.

According to Buckleton and Triggs [34], recombination fractions of 0.197 and 0.316 (derived using the Haldane mapping function for the STR pairs CSF1PO–D5S818 (25 cM) and Penta D–D21S11 (50 cM), respectively) are sufficiently small to affect match probability calculations for relatives and some pedigree analyses. An influence of physical linkage was also found by Nothnagel et al. [35], who simulated pairwise kinship analyses with or without taking linkage between STR markers into account. The overall results for both strategies were very similar, although

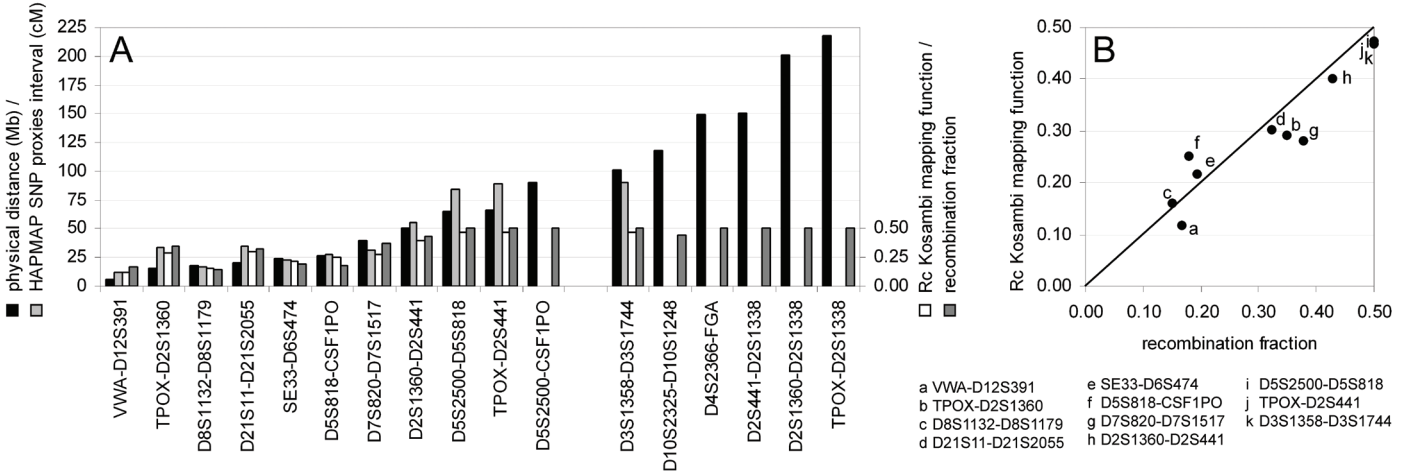

Fig. 1 (A) Four aspects of the 17 syntenic STR pairs: (1) the physical distance in Mb (black bars), (2) the cM interval of the closest HAPMAP SNP proxies [7] (light gray bars) together with (3) the associated recombination fraction (Rc) as derived from the Kosambi mapping function by Phillips et al. [7] (white bars) and (4) the recombination fraction as determined from CEPH families (dark grey bars) for which the values >0.5 are presented as 0.5. Syntenic STR pairs on the same chromosome arm are shown left and those on different chromosome arms are shown right from the dividing space. STR pairs are ordered by physical distance. The physical and genetic distances are shown on the left Y-axis, while the Kosambi-derived Rc and the CEPH-based recombination fraction are shown on the right Y-axis. (B) The Rc derived from the Kosambi mapping function (for syntenic STR pairs for which the Kosambi-derived Rc was presented by Phillips et al. [7]) is plotted against the CEPH-based recombination fraction. The diagonal line represents the theoretical situation in which the Kosambi-derived Rc is equal to the estimated recombination fraction. Exact values for the plotted data can be found in Supplementary Table 3.

the assessment of certain kinships (such as full siblings versus half siblings) could be affected by ignoring linkage [35]. The three kits that are assessed in our study each contain one syntenic STR pair residing on one chromosomal arm. When using genotyping data from one kit, the influence of one loosely linked pair among the set of 12 or 15 markers might not be substantial. However, when combining kits, the number of paired loci on the same chromosomal arm having recombination fractions <0.50 increases substantially as NGM™ with Identifiler™ presents two, NGM™ with HDplex™ five, Identifiler™ with HDplex™ seven and all three kits together eight of these pairs. Such numbers of loosely linked syntenic pairs may affect kinship analyses. Gill et al. [1] elaborated on this kind of kinship analyses using the vWA-D12S391 pair as an example (and they say their methods can be extended to evaluate linkage effects between any pair of loci with known recombination rate). Under the assumption of linkage equilibrium at the population level, they state that linkage has no effect and should not be considered in a pedigree unless at least one individual is involved in at least two transmissions of genetic material, as a parent and/or a child, and that individual is a double heterozygote at the loci involved. When the pedigree is informative of phase and the recombination rate between the markers is known, linkage can be accounted for statistically with the equations given in their paper [1]. Otherwise (under the assumption of linkage equilibrium at the population level), both loci can be used for kinship analysis employing the product rule. An overview and more details are given in Supplementary Textbox 1.

Match probabilities for combined kits

In the previous section, it is shown that for unrelated individuals and for kinship analyses in which linkage has no effect, all 30 loci residing in NGM™, Identifiler™ and HDplex™ can be employed in profile probability calculations using the product rule. Therefore, we evaluate the combined power of the three STR kits. Table 5 shows

Table 5 Match probability (when assuming independence) and number of available loci for NGM, Identifiler, HDplex and combinations thereof, based on genotyping data of 335 Dutch reference samples

	Complete profile		<200 bp[a]		<150 bp[b]	
	MP	# STRs	MP	# STRs	MP	# STRs
NGM	1.8E-19	15	4.9E-07	6	1.3E-03	3
Identifiler	5.2E-18	15	4.7E-06	5	6.5E-03	2
HDplex	6.1E-18	12	2.6E-06	4	3.3E-02	1
NGM+Id	3.3E-24	20	6.2E-09	8	8.4E-06	5
NGM+HD	1.6E-33	25	1.3E-12	10	4.3E-05	4
Id+HD	9.9E-34	27	1.2E-11	9	2.1E-04	3
NGM+Id+HD	2.9E-38	30	1.6E-14	12	2.8E-07	6

MP match probability, *Id* Identifiler, *HD* HDplex

[a] Loci <200 bp: the loci described for "b" (loci <150 bp) and the following loci: NGM: D3S1358, D8S1179, D19S433; Id: D5S818, D8S1179, TH01; HD: D3S1744, D8S1132, D10S2325

[b] Loci <150 bp: NGM: D2S441, D10S1248, D22S1045; Id: D3S1358, D19S433; HD: D7S1517

the match probabilities for NGM™, Identifiler™, HDplex™ and combinations thereof. NGM™ has the best match probability of these three kits; Identifiler™ and HDplex™ have a comparable match probability even though Identifiler™ contains three STRs more. Thus, the match probability per locus is more favourable for HDplex™ than for Identifiler™. Combining the results of two kits gives the most informative match probability for Identifiler™ together with HDplex™ (9.9×10^{-34}), which is based on genotyping data from 27 unique STRs. The combination of the 25 different STRs of NGM™ and HDplex™ gives a very informative match probability (1.6×10^{-33}) as well. Table 5 also shows the match probabilities and number of available loci for amplicon sizes <200 bp and <150 bp that apply to DNA of different degradation levels. The most informative combination for fragments <200 bp is NGM™ with HDplex™ (1.3×10^{-12}), and for fragments <150 bp NGM™ with Identifiler™ (8.4×10^{-6}). Combining all three kits further improves the match probability up to 2.8×10^{-7} for degraded DNA (<150 bp) and up to 2.9×10^{-38} for non-degraded DNA.

Conclusion

The Investigator™ HDplex™ is suitable for forensic DNA analysis (when used with a re-amplified ladder to prevent binning problems for D10S2325). No linkage disequilibrium was detected between the syntenic STRs of HDplex™ and those of NGM™ or Identifiler™, and we infer that the product rule can be applied for profile probability calculations in unrelated individuals. In kinship analyses, the product rule cannot always be applied (depending on the pedigree), and readers are referred to the paper by Gill et al. [1] for a description of these cases and for methods to implement the recombination rate between markers (like determined in this study) into the calculations. HDplex™ has many non-overlapping markers with NGM™ and Identifiler™, and the power of discrimination per marker is, on average, higher than for the other kits. In conclusion, the HDplex™ is a good complementary STR kit that can be used for complex kinship analyses and may aid the analysis of degraded DNA.

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Supplementary material

Supplementary Note 1

In order to determine the recombination fraction between pairs of syntenic loci, the three-generation CEPH family data were analysed with the DOS version of LINKAGE [16]. Pedigree files were prepared with MAKEPED1.EXE, while data files were prepared using web-preplink [17]. CFACTOR.EXE was used to prepare these files before using CILINK.EXE to determine the recombination fractions per chromosome. A number of mutations were found between parents and children (Supplementary Table 2, indicated in light grey), and the records of these children were removed for the analysis of that chromosome (maximum of 2 records per STR pair), since LINKAGE cannot handle these mutations. There were two cases where a mutation was found between a grandparent and a parent (Supplementary Table 2, indicated in middle grey); in these cases, we denoted this locus as 00 (= unknown) in the grandparent to be able to run the program.

Supplementary Note 2

A *p* value (e.g. for HWE or LD) represents the probability of finding values at least as extreme as the observed value, assuming that the null hypothesis (i.e. no difference) is true. Rejecting the null hypothesis while it is true, results in a Type I error. The probability of making a Type I error is called α. This α is a pre-defined value, which is often set at 0.05 (i.e. there is a 5 % chance of making a Type I error). When performing multiple tests the chance of making a Type I error becomes bigger. One way to set an α-value of 0.05 for the whole family of (for example) 17 tests, would be to compare each individual test against a level of $\alpha/n = 0.05/17 = 0.003$. This type of correction for the number of tests is called a Bonferroni correction.

Supplementary table 1. HDplex allele frequencies and summary statistics based on 335 Dutch samples.

	D2S1360	D3S1744	D4S2366	D5S2500	SE33	D6S474	D7S1517	D8S1132	D10S2325	D12S391	D18S51	D21S2055
7									14.63			
8									5.37			
9			34.93	0.30					8.81		0.15	
10			18.06	9.25					10.15		0.45	
11			5.97	26.12	0.15				16.42		1.49	
12			15.22	18.36					19.55		14.33	
12.2					0.15							
13		0.30	15.67	7.01	0.75	26.87			14.63		13.43	
13.2					0.15							
13.3					0.15							
14		10.15	9.10	8.21	2.54	19.40		0.15	8.06		18.36	
15		6.27	1.04	21.94	3.88	14.63			2.24	4.93	14.63	
15.2					0.15							
16		12.84		6.42	4.18	28.21	0.30	1.19		2.69	12.39	
16.1												5.67
16.2					0.15							
17		31.94		1.94	5.82	10.45	0.45	13.13	0.15	10.75	9.85	
17.1												0.90
17.3										1.94		
18	0.30	16.12		0.45	8.06	0.30	4.18	20.00		17.01	8.21	
18.1												1.04
18.3										2.69		
19	1.34	15.22			8.36	0.15	12.54	13.13		11.79	4.03	
19.1												25.82
19.2					0.30							
19.3										0.90		
20	10.75	5.82			5.07		12.69	13.88		10.30	1.19	
20.1												5.22
20.2					1.19							
21	9.25	1.34			3.13		10.30	12.99		12.24	0.75	0.15
21.1												0.60
21.2					2.24							
22	29.85				0.30		11.64	11.49		9.85	0.30	0.15
22.1												0.15
22.2					2.24							
23	13.58				0.15		8.06	10.45		7.31	0.15	1.19
23.2					2.99							
24	6.12						14.48	3.28		4.63	0.30	0.90
24.2					3.13							
25	8.51						21.04	0.30		1.79		13.13
25.2					3.58							
26	9.85						3.28			0.90		14.78
26.2					4.78							
27	4.78						0.90			0.15		1.94
27.2					9.25							
28	2.84						0.15			0.15		1.34
28.2					7.16							
29	1.64											1.64
29.2					7.31							
30	0.90											2.09
30.2					6.57							
31	0.15											2.69
31.2					2.54							
32	0.15				0.15							2.99
32.2					0.90							
33					0.60							5.82
33.2					1.19							
34					0.30							6.57
35					0.30							4.03
35.2					0.15							
36												0.75
37												0.45
	D2S1360	D3S1744	D4S2366	D5S2500	SE33	D6S474	D7S1517	D8S1132	D10S2325	D12S391	D18S51	D21S2055
Obs. het.[a]	0.842	0.827	0.782	0.854	0.958	0.776	0.872	0.854	0.875	0.884	0.884	0.869
Exp. het.[b]	0.849	0.816	0.787	0.826	0.946	0.779	0.871	0.865	0.865	0.899	0.874	0.877
HWE[c]	0.530	0.547	0.629	0.830	0.568	0.563	0.577	0.047	0.338	0.087	0.810	0.466
PD[d]	0.961	0.940	0.924	0.944	0.991	0.914	0.967	0.964	0.964	0.978	0.968	0.973
PE[e]	0.679	0.650	0.566	0.702	0.915	0.555	0.738	0.702	0.744	0.762	0.762	0.732
PIC[f]	0.834	0.792	0.757	0.803	0.942	0.743	0.856	0.849	0.849	0.889	0.859	0.865
MP[g]	0.039	0.060	0.076	0.056	0.009	0.086	0.033	0.036	0.036	0.022	0.032	0.027

[a]Observed heterozygosity. [b]Expected heterozygosity. [c]p-value for Hardy-Weinberg equilibrium. [d]Power of discrimination. [e]Power of exclusion. [f]Polymorphic information content. [g]Match probability.

Supplementary table 2. Genotypes of five three-generation CEPH pedigrees (78 individuals) for HDplex, NGM and Identifiler.

	D1S1656	TPOX	D2S1360	D2S441	D2S1338	D3S1358	D3S1744	D4S2366	FGA
1423_01_PGF_NA11917	12 16	8 11	25 25	10 14	18 24	15 16	14 14	9 11	20 21
1423_02_PGM_NA11918	11 17,3	8 10	21 23	15 15	19 23	16 18	16 19	10 13	20 21
1423_03_MGF_NA11919	13 13	11 11	22 24	10 10	17 18	16 17	17 18	10 13	24 24
1423_04_MGM_NA11920	12 15,3	8 8	22 22	11 14	17 20	14 16	14 17	13 15	20 20,2
1423_05_F_NA10842	16 17,3	8 10	23 25	14 14	23 24	16 16	14 19	11 13	20 20
1423_06_M_NA10843	13 15,3	8 11	22 22	10 11	17 17	14 17	14 18	10 13	20,2 24
1423_07_S1_NA11909	13 17,3	8 8	22 25	11 14	17 24	14 16	14 18	11 13	20 20,2
1423_08_D1_NA11910	15,3 16	8 10	22 23	10 14	17 24	16 17	14 14	10 11	20 20,2
1423_09_D2_NA11911	15,3 16	8 11	22 25	10 14	17 23	16 17	18 18	13 13	20 24
1423_10_S2_NA11912	15,3 16	10 11	22 23	10 14	17 23	16 17	14 18	10 11	20 20,2
1423_11_S3_NA11913	15,3 16	10 11	22 23	11 14	17 23	14 16	14 19	11 13	20 20,2
1423_12_D4_NA11914	15,3 16	8 8	22 25	10 14	17 23	14 16	14 18	13 13	20 24
1423_13_D5_NA11915	15,3 16	10 11	22 23	10 14	17 23	14 16	14 18	11 13	20 24
1423_14_S4_NA11916	13 17,3	8 10	22 23	11 14	17 24	14 16	18 19	10 13	20 20,2
1423_15_D6_NA11921	13 16	10 11	22 23	10 14	17 24	16 17	14 14	13 13	20 24
1362_01_PGF_NA11992	14 15,3	9 11	22 23	11 13	17 18	15 18	14 17	9 12	21 22
1362_02_PGM_NA11993	14 17,3	11 11	22 25	10 14	18 20	15 16	17 18	10 13	22 22
1362_03_MGF_NA11994	11 12	10 11	22 24	11 14	16 19	16 17	17 18	13 14	19 21
1362_04_MGM_NA11995	11 18,3	8 8	25 28	11 11	17 19	15 16	17 17	9 13	25 25
1362_05_F_NA10860	15,3 17,3	9 11	22 22	10 13	18 20	16 18	17 18	9 10	22 22
1362_06_M_NA10861	11 18,3	8 10	22 28	11 11	19 19	16 17	17 17	9 13	21 25
1362_07_D1_NA11982	17,3 18,3	9 10	22 28	10 11	18 19	16 18	17 17	10 13	22 25
1362_08_D2_NA11983	15,3 18,3	8 9	22 28	11 13	18 19	16 18	17 18	9 9	22 25
1362_09_S1_NA11984	11 15,3	10 11	22 22	11 13	18 19	17 18	17 17	9 9	22 25
1362_10_D3_NA11985	11 17,3	9 10	22 22	11 13	18 19	16 17	17 17	9 13	22 25
1362_11_D4_NA11986	11 17,3	9 10	22 28	11 13	19 20	16 17	17 18	9 10	21 22
1362_12_S2_NA11987	11 17,3	8 9	22 28	11 13	19 20	17 18	17 18	9 13	22 25
1362_13_D5_NA11988	15,3 18,3	8 9	22 22	11 13	19 20	16 17	17 17	9 9	21 22
1362_14_D6_NA11989	17,3 18,3	10 11	22 22	11 13	18 19	16 18	17 18	9 10	22 25
1362_15_S3_NA11990	11 15,3	8 9	22 22	11 13	18 19	16 18	17 18	9 9	21 22
1362_16_D7_NA11991	15,3 18,3	8 9	22 28	10 11	19 20	16 16	17 17	9 10	22 25
1362_17_S4_NA11996	11 15,3	8 11	22 22	10 11	19 20	16 18	17 18	9 10	22 25
0066_01_PGF_NA12556	15 17,3	11 11	22 22	10 13	16 24	14 16	17 18	9 10	20 23
0066_02_PGM_NA12557	12 15	8 11	22 23	11 14	16 18	14 17	18 19	9 9	18 21
0066_03_MGF_NA12558	17,3 17,3	8 11	22 26	10 14	17 24	14 14	17 17	10 14	20 22
0066_04_MGM_NA12559	16 16,3	6 11	20 22	14 15	18 18	15 16	14 18	9 11	25 25
0066_05_F_NA12547	12 15	11 11	22 23	11 13	18 24	16 17	18 18	9 9	18 23
0066_06_M_NA12548	16 17,3	6 8	22 22	10 15	18 24	14 15	17 18	9 14	22 25
0066_07_S1_NA12549	15 17,3	8 11	22 23	10 13	18 24	15 16	18 18	9 9	23 25
0066_08_D1_NA12550	12 17,3	6 11	22 22	10 13	18 18	14 16	17 18	9 14	23 25
0066_09_D2_NA12551	12 17,3	6 11	22 22	13 15	18 24	14 17	18 18	9 9	22 23
0066_10_S2_NA12552	12 16	6 11	22 22	10 13	18 24	14 16	17 18	9 14	18 22
0066_11_D3_NA12553	15 16	6 11	22 23	10 11	24 24	15 17	17 18	9 14	22 23
0066_12_D4_NA12554	12 17,3	6 11	22 22	10 11	18 24	15 16	17 18	9 14	18 22
0066_13_S3_NA12555	15 16	8 11	22 23	11 15	24 24	14 17	18 18	9 14	22 23
1454_01_PGF_NA12812	13 16,3	9 11	22 25	11 11	18 26	16 18	15 18	9 14	19 20
1454_02_PGM_NA12813	11 17	8 8	20 27	11 12	19 23	16 16	17 20	9 14	20 24
1454_03_MGF_NA12814	16,3 18,3	8 11	22 30	11 11	17 17	16 18	15 18	9 13	22 23
1454_04_MGM_NA12815	15 16,3	8 9	20 23	10 14	16 18	14 16	17 18	10 13	20 23
1454_05_F_NA12801	11 16,3	8 9	20 25	11 11	18 23	16 16	18 20	14 14	20 20
1454_06_M_NA12802	16,3 16,3	8 9	22 23	10 11	17 18	14 16	15 17	9 13	20 22
1454_07_D1_NA12803	16,3 16,3	8 9	23 25	11 11	18 23	14 16	15 18	13 14	20 20
1454_08_D2_NA12804	16,3 16,3	8 8	20 22	10 11	18 18	14 16	15 20	9 14	20 22
1454_09_S1_NA12805	11 16,3	8 8	20 22	11 11	18 23	16 16	15 20	9 14	20 22
1454_10_S2_NA12806	11 16,3	9 9	22 25	11 11	18 18	14 16	15 18	13 14	20 20
1454_11_D3_NA12807	16,3 16,3	8 9	20 22	10 11	18 23	16 16	15 20	9 14	20 22
1454_12_D4_NA12808	11 16,3	8 9	20 22	10 11	17 23	14 16	15 18	13 14	20 22
1454_13_D5_NA12809	16,3 16,3	9 9	20 22	11 11	18 18	16 16	17 20	13 14	20 22
1454_14_S3_NA12810	11 16,3	9 9	20 22	11 11	17 18	16 16	17 20	9 14	20 22
1454_15_S4_NA12811	16,3 16,3	9 9	20 23	10 11	17 18	14 16	15 18	13 14	20 20
1454_16_S5_NA12816	11 16,3	8 8	20 22	11 11	18 18	16 16	17 18	13 14	20 22
1463_01_PGF_NA12889	15 16,3	8 9	20 26	10 10	20 24	16 16	14 18	12 13	20 24
1463_02_PGM_NA12890	16 18,3	8 11	20 26	14 14	23 24	14 16	17 18	9 13	20 25
1463_03_MGF_NA12891	14 14	8 8	24 25	14 14	20 24	16 16	16 17	9 14	22 23
1463_04_MGM_NA12892	15,3 16,3	8 9	22 22	10 11	17 25	17 17	14 19	10 12	18 24
1463_05_F_NA12877	16 16,3	9 11	20 20	10 14	24 24	16 16	17 18	9 12	24 25
1463_06_M_NA12878	14 15,3	8 8	22 25	11 14	17 20	16 17	14 16	9 10	22 24
1463_07_D1_NA12879	14 16	8 11	20 25	11 14	17 24	16 17	14 18	9 12	24 25
1463_08_D2_NA12880	15,3 16	8 11	20 22	10 14	17 24	16 16	14 18	9 9	22 24
1463_09_D3_NA12881	15,3 16,3	8 11	20 25	10 11	20 24	16 17	16 18	9 9	22 25
1463_10_S1_NA12882	15,3 16	8 11	20 22	10 11	20 24	16 16	16 17	10 12	22 24
1463_11_S2_NA12883	14 16	8 11	20 25	10 11	20 24	16 17	16 18	9 12	24 25
1463_12_S3_NA12884	15,3 16,3	8 9	20 25	10 11	20 24	16 17	14 17	9 9	22 24
1463_13_D4_NA12885	15,3 16,3	8 9	20 25	10 14	17 24	16 16	16 17	9 12	24 24
1463_14_S4_NA12886	15,3 16,3	8 9	20 22	11 14	17 24	16 16	14 18	9 9	22 24
1463_15_D5_NA12887	15,3 16,3	8 9	20 25	14 14	20 24	16 16	16 18	9 10	24 24
1463_16_S5_NA12888	14 16	8 11	20 22	10 14	20 24	16 16	16 17	10 12	24 25
1463_17_S6_NA12893	14 16,3	8 11	20 22	10 11	17 24	16 17	14 17	10 12	24 25

PedigreeNumber_MemberNumber_Relation_RepositoryNumber; Relation: PGF/MGF: paternal/maternal grandfather, PGM/MGM: paternal/maternal grandmother, F: father, M: mother, D: daughter, S: son

Mutation.

Due to mutation in parent, genotype grandparent changed to 0/0 for recombination fraction calculations.

No data available due to unresolvable tri-allelic pattern in genotyping data.

D5S2500	D5S818	CSF1PO	SE33	D6S474	D7S820	D7S1517	D8S1132	D8S1179	D10S2325	D10S1248
10 11	11 13	12 12	19 29,2	15 17	7 11	22 25	17 18	12 13	7 11	13 14
10 12	11 11	10 10	22,2 26,2	13 16	10 11	18 25	20 24	10 12	13 15	11 13
12 14	11 12	10 11	17 19	15 16	8 9	20 24	18 20	12 14	7 11	15 16
14 15	11 12	10 12	24,2 27,2	14 15	7 8	19 24	18 21	8 14	7 9	13 15
10 10	11 13	10 12	19 26,2	13 15	11 11	18 22	17 24	12 13	11 13	11 14
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10 13	11 12	10 10	17 24,2	13 13	10 13	24 26	20 22	12 13	10 12	13 16

TH01	vWA	D12S391	D13S317	D16S539	D18S51	D19S433	D21S11	D21S2055	D22S1045	AMEL
6 8	15 17	18 18	12 14	9 11	12 16	11 14	30,2 31	35 36	15 15	X Y
7 7	16 18	15 20	8 11	12 13	12 15	14 15	28 31,2	19,1 25	17 18	X X
6 9,3	14 15	18 24	11 12	12 12	14 16	13 15	26 29	29 33	15 16	X Y
6 9,3	16 17	17,3 23	10 12	9 11	12 14	14 14	30 32	19,1 26	11 15	X X
6 7	15 18	15 18	11 14	11 12	12 12	14 15	28 30,2	19,1 36	15 17	X Y
6 9,3	14 17	17,3 18	10 11	11 12	14 14	13 14	26 30	26 29	11 15	X X
6 9,3	14 15	18 18	10 14	11 12	12 14	13 15	26 30,2	29 36	15 15	X Y
6 6	14 18	15 18	11 14	11 12	12 14	14 14	26 30,2	26 36	15 15	X X
7 9,3	17 19	18 18	11 14	12 12	12 14	14 15	26 30,2	26 36	11 15	X X
6 7	15 17	17,3 18	11 11	11 12	12 14	14 14	30 30,2	29 36	15 15	X Y
6 9,3	14 15	18 18	11 11	12 12	12 14	13 14	26 28	29 36	15 17	X Y
7 9,3	14 18	18 18	11 11	11 11	12 14	14 14	26 28	19,1 26	11 15	X X
6 6	17 19	15 17,3	11 14	11 12	12 14	14 14	26 28	19,1 29	15 17	X X
7 9,3	14 18	18 18	10 14	12 12	12 14	13 14	30 30,2	29 36	15 15	X Y
6 6	14 15	18 18	11 11	11 12	12 14	13 14	26 28	19,1 26	15 15	X X
8 9,3	16 16	20 22	12 12	11 12	14 17	13 13	30 31,2	25 26	15 15	X Y
7 9,3	16 17	18 18	8 10	11 12	13 16	12 13	29 33,2	16,1 21,1	16 16	X X
6 9	16 18	16 19,3	10 11	10 14	12 15	13 13	28 30	17,1 19,1	15 16	X Y
6 8	16 18	22 22	8 11	13 13	14 15	13 17,2	29 30	19,1 19,1	15 15	X X
9,3 9,3	16 17	18 20	8 12	12 12	13 14	13 13	30 33,2	16,1 25	15 16	X Y
6 9	16 18	19,3 22	8 10	13 14	14 15	13 17,2	28 29	17,1 19,1	15 16	X X
6 9,3	17 18	18 19,3	8 12	12 13	14 14	13 17,2	29 33,2	16,1 19,1	16 16	X X
9 9,3	17 18	18 19,3	8 12	12 13	14 15	13 17,2	28 33,2	16,1 17,1	15 16	X X
9 9,3	16 18	20 22	8 8	12 14	14 15	13 17,2	28 30	17,1 25	15 16	X Y
6 9,3	16 17	18 22	8 8	12 14	13 15	13 17,2	29 30	16,1 17,1	15 15	X X
6 9,3	16 18	19,3 20	8 8	12 13	13 15	13 17,2	28 30	17,1 25	15 15	X X
9 9,3	17 18	18 19,3	8 8	12 13	13 14	13 17,2	29 30	19,1 25	15 15	X Y
6 9,3	17 18	18 19,3	10 12	12 13	13 15	13 17,2	28 33,2	19,1 25	15 16	X X
9 9,3	17 18	18 19,3	8 8	12 13	13 14	13 17,2	29 33,2	16,1 19,1	15 16	X X
6 9,3	16 16	19,3 20	10 12	12 14	14 14	13 13	29 33,2	16,1 19,1	15 16	X Y
9 9,3	17 18	18 19,3	10 12	12 14	13 15	13 13	29 33,2	16,1 17,1	16 16	X X
6 9,3	16 17	18 22	8 8	12 14	13 15	13 17,2	28 33,2	17,1 25	16 16	X Y
7 9,3	15 18	17 22	12 13	12 13	13 18	14 15	29 29	16,1 19,1	15 15	X Y
8 9,3	17 18	19 22	8 11	13 15	13 17	12 13	28 31	21,1 25	15 17	X X
7 9	17 19	18 23	8 11	12 12	14 17	14 15,2	29 30	19,1 35	15 16	X Y
6 9,3	14 17	17 20,3	11 12	11 13	11 13	14 16	28 29	18,1 34	16 16	X X
7 9,3	17 18	19 22	11 13	12 15	17 18	12 15	28 29	16,1 21,1	15 17	X Y
6 7	14 19	18 20,3	8 11	12 13	13 17	14 15,2	28 29	19,1 34	15 16	X X
6 7	14 17	18 19	11 11	12 12	17 18	14 15	28 28	19,1 21,1	15 16	X Y
6 9,3	18 19	20,3 22	8 11	13 15	13 17	15 15,2	29 29	16,1 34	15 16	X X
7 9,3	18 19	18 22	11 11	13 15	17 18	12 14	28 29	16,1 34	15 17	X X
7 7	18 19	18 22	11 13	13 15	17 18	14 15	28 28	21,1 34	16 17	X Y
6 7	14 18	18 22	8 11	13 15	13 18	14 15	28 28	16,1 34	15 15	X X
7 7	14 17	19 20,3	8 11	12 13	13 18	14 15	29 29	16,1 19,1	15 15	X X
7 9,3	18 19	18 22	11 13	12 15	13 18	12 15,2	28 29	19,1 21,1	15 16	X Y
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8 9	16 18	19 22	10 12	11 12	12 15	14 16,2	28 29	26 29	11 11	X X
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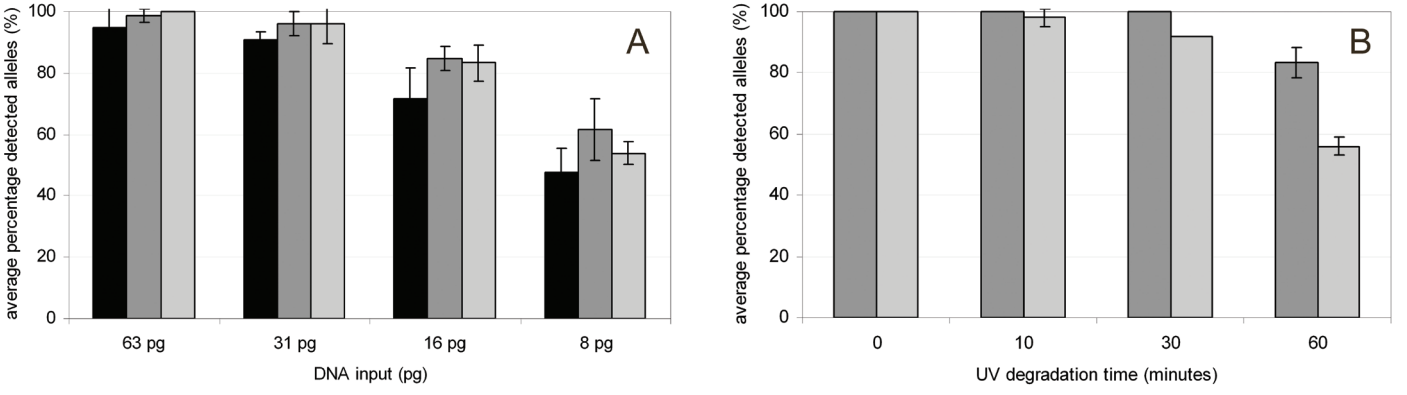
7 7	16 17	22 23	8 12	11 13	15 16	14 14	29 29	26 31	11 16	X X
7 8	14 16	18 19	10 12	12 12	12 15	13 16,2	29 31	26 26	11 16	X Y
6 7	16 16	22 23	8 10	12 13	15 16	14 14	29 30	25 26	11 15	X X
7 8	16 16	18 23	8 12	12 12	12 16	13 14	29 29	26 26	15 16	X X
6 7	16 16	18 23	8 12	12 12	12 15	14 16,2	30 31	26 26	15 16	X X
6 8	14 16	18 23	8 12	12 13	12 16	14 16,2	30 31	26 26	11 15	X Y
7 7	16 16	19 23	8 10	12 13	12 15	14 16,2	29 29	26 26	11 16	X Y
7 8	16 16	19 23	10 12	12 13	15 15	14 16,2	29 30	25 26	11 16	X X
6 7	16 16	19 23	10 10	12 13	15 15	13 14	29 29	26 26	11 15	X X
6 7	14 16	18 23	8 12	12 13	12 16	14 16,2	30 31	25 26	11 11	X X
7 7	16 16	19 23	8 12	12 13	12 16	14 16,2	29 29	25 26	15 16	X Y
6 8	16 16	19 22	8 12	12 12	15 15	13 14	30 31	25 26	11 16	X Y
6 8	14 16	18 23	8 10	12 13	15 15	13 14	29 31	25 26	11 11	X Y
7 10	18 19	18 20	8 13	11 12	15 17	13 16,2	28 29	19,1 25	15 15	X Y
6 7	14 16	19 19	11 13	11 13	12 15	14 14	29 32,2	25 34	15 17	X X
6 9,3	17 17	17 18	8 11	10 13	14 17	12 14	25,2 30	16,1 31	13 16	X Y
7 9,3	15 18	16 20	9 12	11 12	14 16	14 14	30 32,2	19,1 19,1	15 16	X X
6 10	16 19	18 19	13 13	11 11	12 15	14 16,2	28 29	25 25	15 15	X Y
7 9,3	15 17	16 17	11 12	10 11	16 17	12 14	30 30	19,1 31	13 15	X X
6 7	15 16	17 19	11 13	11 11	12 17	14 16,2	29 30	19,1 25	15 15	X X
7 10	16 17	17 19	11 13	11 11	12 17	14 16,2	28 30	25 31	13 15	X X
6 7	17 19	16 18	12 13	10 11	12 17	14 16,2	29 30	25 31	13 15	X X
9,3 10	17 19	17 18	11 13	10 11	12 16	14 14	28 30	25 31	13 15	X Y
7 10	17 19	17 18	11 13	10 11	12 17	14 14	29 30	25 31	15 15	X Y
7 10	16 17	17 19	12 13	10 11	12 17	14 14	29 30	19,1 25	13 15	X Y
7 10	15 16	16 18	12 13	11 11	12 17	12 16,2	28 30	25 31	13 15	X X
7 10	17 19	17 18	12 13	10 11	12 17	14 14	28 30	19,1 25	15 15	X Y
6 9,3	15 19	16 18	0 0	10 11	15 17	12 16,2	28 30	19,1 25	13 15	X X
7 10	15 19	17 18	11 13	10 11	12 17	14 14	28 30	25 31	13 15	X Y
6 7	15 19	17 18	11 13	10 11	12 16	14 14	28 30	25 32	15 15	X Y

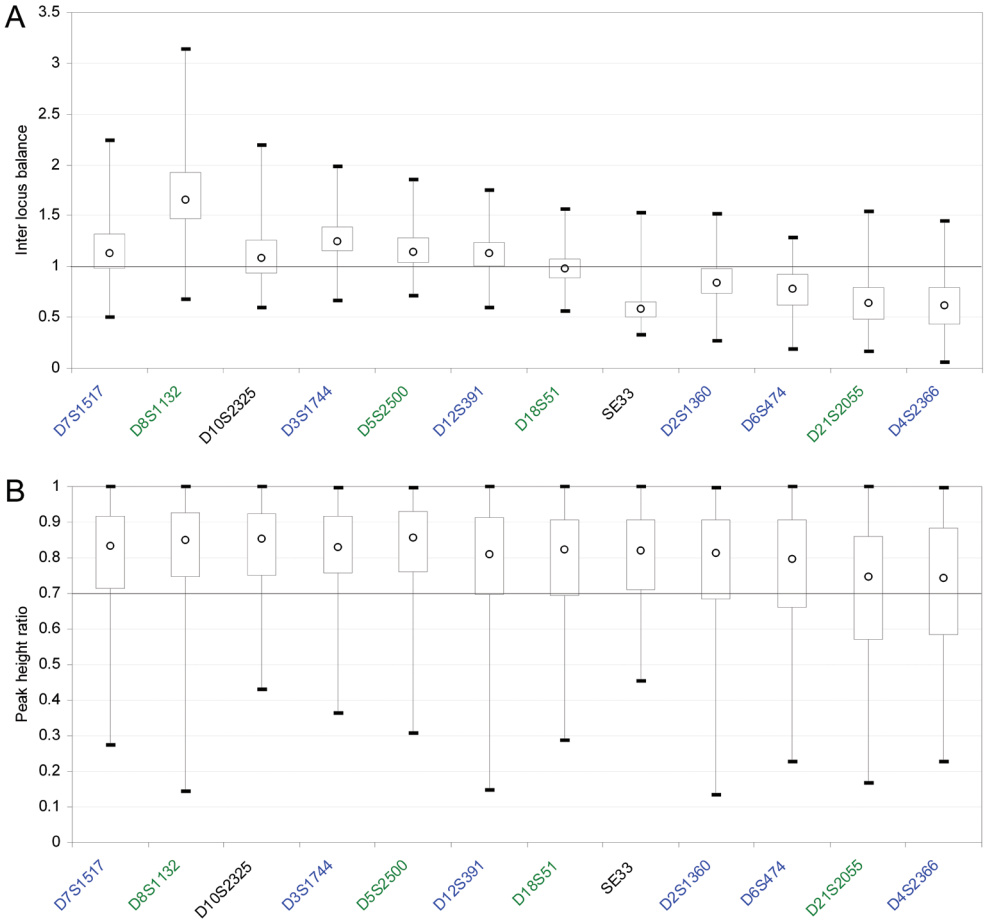
Supplementary table 3. Overview of physical and genetic distance and recombination fraction between pairs of syntenic STR loci in HDplex, NGM and Identifiler.

chr	STR pair	position[a]	physical distance (Mb)	cM interval HapMap SNP proxies[b]	Rc from Kosambi mapping function[b]	recombination fraction[c,d]
02	TPOX-D2S1360		16.00	33.39	0.29	0.35
02	TPOX-D2S441		66.75	88.81	0.47	0.53
02	TPOX-D2S1338	*	217.39	-	-	0.51
02	D2S1360-D2S441		50.75	55.42	0.40	0.43
02	D2S1360-D2S1338	*	201.39	-	-	0.58
02	D2S441-D2S1338	*	150.64	-	-	0.58
03	D3S1358-D3S1744	*	101.51	90.06	0.47	0.64
04	D4S2366-FGA	*	149.03	-	-	0.51
05	D5S2500-D5S818		64.41	84.11	0.47	0.57
05	D5S2500-CSF1PO		90.76	-	-	0.66
05	D5S818-CSF1PO		26.35	27.76	0.25	0.18
06	SE33-D6S474		23.89	23.21	0.22	0.19
07	D7S820-D7S1517		39.71	31.87	0.28	0.38
08	D8S1132-D8S1179		18.58	16.48	0.16	0.15
10	D10S2325-D10S1248	*	118.38	-	-	0.45
12	vWA-D12S391		6.36	11.94	0.12	0.17
21	D21S11-D21S2055		20.64	34.82	0.30	0.32

[a] Syntenic STR pairs on different chromosomal arms are marked by an * in this column and shown in grey.
[b] As determined by Phillips et al. [7].
[c] Recombination fraction based on LINKAGE analyses of data from five three-generation CEPH families.
[d] LINKAGE recombination fraction estimates > 0.5 can be interpreted as being equal to 0.5 (Prof. J. Ott, personal communication).

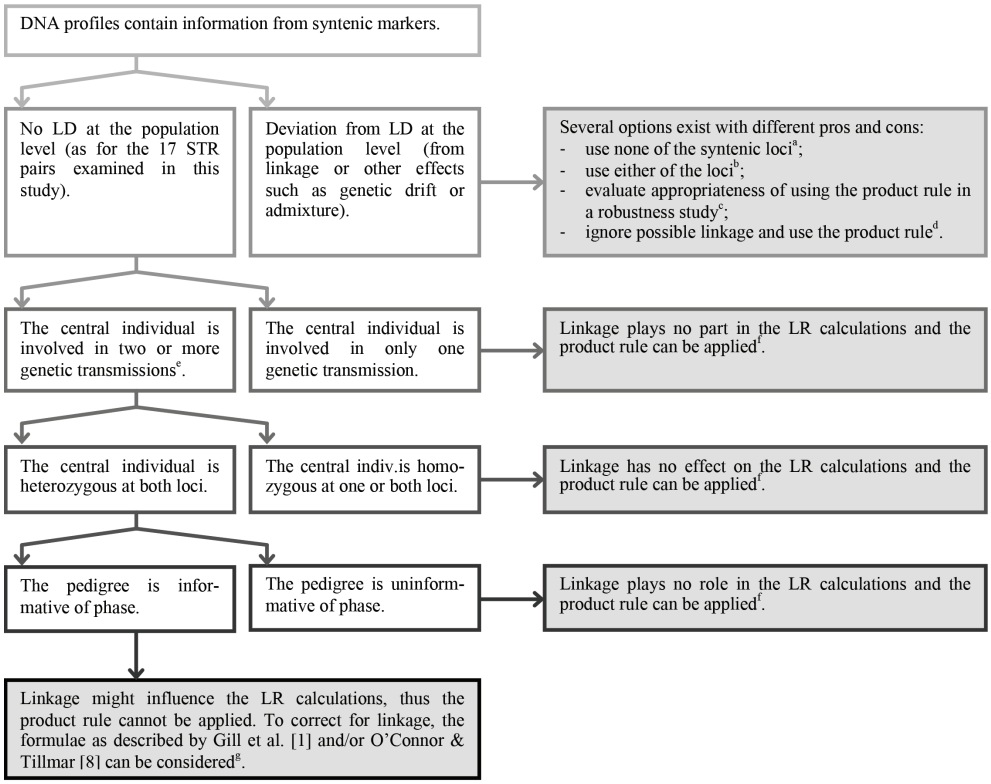


Supplementary Figure 1. HDplex™ performance. (A) Comparison between two methods to sensitise low template DNA profiling of a dilution series of pristine DNA007 in three-fold. Black bars show the average percentage of detected alleles for the standard setting with 30 PCR cycles and 3 kV CE. Dark grey bars represent 30 PCR cycles with 9 kV CE and light grey bars 32 PCR cycles with 3 kV CE. Error bars show the standard deviation. (B) Comparison between NGM™ (dark grey bars) and HDplex™ (light grey bars) for time-wise UV-degraded hDNA. The experiment is performed in duplicate and the average percentage of detected alleles is plotted against the UV degradation time. Error bars represent the standard deviation (when present).



Supplementary Figure 2. Inter-locus balance (A) and intra-locus peak height ratio (B). Loci are ordered from shorter (left) to longer (right) amplicon sizes. Circles represent the median of the data points, boxes indicate the first to the third quartile and whiskers show the minimum and maximum values. The colour in which the markers are presented indicates the dye-channel (blue = 6-FAM, green = BTG and black = BTY). The horizontal line (at 1) in (A) represents perfect balance between loci, and the line in (B) indicates the preferred value (of 0.7) above which the data points should reside.

Textbox 1: Flowchart of possibilities for likelihood ratio (LR) calculations when linked loci are considered in kinship analysis.



[a] By using none of the syntenic loci, information from two loci is disregarded.
[b] Choosing between loci [6] holds a risk, especially when one locus is in favour of the prosecution hypothesis (H_p) and the other is in favour of the defence hypothesis (H_d); which one to choose?
[c] The robustness of the results when applying the product rule can be evaluated by simulating different genotypes for the central individual for different recombination fractions.
[d] Although all available genotyping information is used, the possible effect of linkage [8,35] is disregarded.
[e]Examples in which the central individual is involved in two or more genetic transmissions include a parent with at least two children, or an incestuous pedigree [1], but also a parent with a grandparent and a child (P. Gill, personal communication).
[f]The product rule is preferably combined with a theta-correction to correct for possible low level LD due to population substructure effects [1].
[g] These formulae make use of recombination fractions [1,8], like determined in our study.

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Chapter 7

Improved analysis of long STR amplicons from degraded single source and mixed DNA

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Abstract

DNA profiles from degraded samples often suffer from information loss at the longer short tandem repeat (STR) loci. Sensitising the reactions, either by performing additional PCR cycles or increasing the capillary electrophoresis injection settings, carries the risk of over-amplifying or overloading the shorter fragments. We explored whether profiling of degraded DNA can be improved by preferential capturing of the longer amplified fragments. To this aim, a post-PCR purification protocol was developed that is based on AMPure® XP beads that have size-selective properties. A comparison was made with an unselective post-PCR purification system (DTR gel filtration) and no purification of the PCR products. Besides a set of differently and serially degraded single source samples, unequal mixtures of degraded DNAs were analysed, in order to extract more genotyping information for the minor contributor without overloading the major component at the shorter amplicons. Purification by the AMPure® protocol resulted in higher peak heights especially for the longer amplicons, while DTR gel filtration gave higher peaks for all amplicon sizes. Both purification methods presented more detected alleles, with the AMPure® protocol performing slightly better, on average. In conclusion, the in-house developed AMPure® protocol can be employed to improve STR profile analysis of degraded single source and (unequally) mixed DNA samples.

Introduction

When DNA is degraded, the longer STR (i.e., short tandem repeat) amplicons of a DNA profile tend to have lower peak heights than the shorter amplicons, or they may not be detected at all [1, 2]. When sufficient DNA extract is remaining, it is often possible to retrieve this missing STR information by using another amplification kit with different primer designs, resulting in shorter amplicons (a.k.a. mini-STRs) for these loci [2–7]. When this approach fails, for instance because no additional DNA extract is available, there are still a few options to sensitise the reactions, such as performing additional PCR cycles [8], using increased capillary electrophoresis (CE) settings [9, 10], or applying post-PCR purification [9–12]. These strategies exploit the remaining PCR products (at the NFI, generally 24 µL is left, as only 1 µL of the 25-µL PCR product is used for CE analysis) that otherwise would be discarded. However, with degraded DNA samples, these approaches may provoke the shorter STR loci to become over-amplified during PCR or overloaded during CE. This may lead, for instance, to bleed through signals in other dye channels [12, 13], collapsed and/or shifted peaks and minus A shoulder peaks. Similar problems may be encountered when unequal mixtures are analysed; sensitising the reactions to obtain more information for the minor contributor could lead to over-amplification or overloading of the major component [14, 15]. Again, loss of genotyping information is often particularly a problem at the longer loci, as compromised casework samples tend to have some sloping in the DNA profiles. To our knowledge, no forensic method exists to specifically recover the information residing at the longer STR loci in PCR products.

Post-PCR purification removes salts (that were needed during PCR) and, depending on the method, also primers that compete with amplified DNA fragments for injection into the capillary during electrophoresis [9–12], thereby increased peak heights are obtained. From the research area of next generation sequencing, a size-selective post-PCR purification method (i.e. Agencourt™ AMPure® XP beads; Beckman Coulter, Woerden, The Netherlands) came to our attention. Fragments of different lengths can be captured by using different ratios of PCR product and AMPure® XP magnetic beads [16, 17]. In theory, by combining size selection and post-PCR purification, signals of especially the longer amplicons would increase. In this study, we assess whether the size-selective properties of the AMPure® XP beads can be employed to improve the recovery of the genotyping information at the longer STR amplicons from single source and unequally mixed degraded DNA samples.

Material and methods

A description of the degraded DNA samples [18, 19] and the mixtures used in this study, together with the methods for quantification (ALU-assay [20]), PCR (NGM™), capillary electrophoresis and DNA profile analysis, is given in the electronic supplementary material (Supplementary Text 1).

Post-PCR purification

Both post-PCR purification approaches used in this study make use of Performa® DTR (Dye Terminator Removal) Gel Filtration Cartridges (EdgeBio, Gaithersburg, MD, USA). The first method uses DTR cartridges only; the second method is preceded by a size-selective purification approach as described below. DTR cartridges were prepared for sample loading by spinning them in the accompanying tube for 3 min at 13,200 rpm (17,532 rcf) in an Eppendorf centrifuge 5430, turning them 180° in the centrifuge and spinning them for an additional 2 min at 13,200 rpm. Then, the cartridge was placed on a new (labelled) tube, the PCR products were pipetted on the gel, the lid was closed and the cartridge was spun for 2 min at 9,600 rpm (9,273 rcf). When PCR products were purified by DTR only, 4 µL of the PCR product was used.

To achieve size selection of PCR products, Agencourt™ AMPure® XP beads (and the corresponding Agencourt™ SPRI-Plate 96 Ring Super Magnet Plate (Beckman Coulter, Woerden, The Netherlands)) were used. The protocol was optimised to comply with forensic genotyping kits and reads as follows: "Transfer 10 µL PCR product to a new PCR plate (or strip). Shake the bottle with AMPure® XP beads gently to resuspend the magnetic particles. Add 12 µL AMPure® XP beads to the PCR product, mix 10 times by pipetting and incubate at room temperature for 5 min to bind the PCR products to the beads. Place the PCR plate onto the Super Magnet Plate and wait for 3 min to separate the beads from the solution. While the plate is on the Super Magnet Plate, aspirate the cleared solution from the wells without touching the beads and discard this solution. Take the PCR plate from the Super Magnet Plate; resuspend the beads in 10 µL ddH2O and pipette 10 times up and down to elute the DNA. Transfer the complete bead suspension to a prepared DTR cartridge and spin for 2 min at 9,600 rpm."

Results and discussion

The development of a protocol for size-selective post-PCR purification is described in the electronic supplementary material (Supplementary Text 2 and Supplementary Fig. 2).

Improved analysis of degraded DNA

Thirty-nine DNA samples are subjected to several degradation methods and degrees, using UV-light, freeze/thaw cycles, sonication, microbial overgrowth and long preservation time, to mimic the various causes of degradation that may affect forensic samples. After standard DNA analysis of these samples, the resulting DNA profiles are categorised by degradation state: nine samples show little or no signs of degradation, 20 exhibit moderate degradation and ten present severe degradation. NGM™ PCR amplifications of these 39 samples are compared after exposure to three methods: (1) no post-PCR purification, (2) purification via DTR gel filtration and (3) purification by the AMPure® protocol (which includes DTR gel filtration as well).

To determine the effect of the post-PCR purification methods on the alleles called at various loci, we compared the peak heights of alleles in profiles from purified amplification products to those of the corresponding alleles in the profiles derived from non-purified PCR products and determined the average fold increase in peak height for each locus. When signals approach saturation, alleles are excluded from analyses (as these are quantitative assessments [21]). First, we examined the samples categorised as moderately degraded, and Fig. 1A shows the average fold increase per locus for these samples. After DTR gel filtration, the fold increase is relatively constant for all amplicon lengths and ranges between 2.0 and 2.3 times. For the size-selective AMPure® purification protocol, the fold increase varies with amplicon length: alleles at the shorter amplicons do not change peak height (fold increase of 1.0) and those at longer amplicons increase up to 3.7 times. The preferential recapturing of the longer amplicons by the AMPure® XP beads is well illustrated in Fig. 1B that shows the average ratio between the fold increase after AMPure® purification and DTR gel filtration at each locus. Notwithstanding relatively high standard deviations, these values are in general below one for the shorter and above one for the longer amplicons. Actually, the NGM™ loci can be divided into three groups: (1) less selected by AMPure® purification (D2S441, D22S1045, AMEL and D10S1248; size range 70–125 bp); (2) equally selected by the AMPure® and DTR protocols (D19S433, D3S1358 and D8S1179; size range 125–170 bp); and (3) preferably selected by the AMPure® procedure (vWA, THO1, D1S1656, D21S11, D16S539, D12S391, FGA, D18S51 and D2S1338; size range 170–370 bp). When examining the data for the samples in the categories with little or no degradation or severe degradation, the various loci appear to respond similarly (Supplementary Fig. 3A and B). Since for the longest four amplicons in the severely degraded samples no alleles are detected for the non-purified PCR products, the fold increase cannot be determined.

Next, we analysed the effect of post-PCR purification on the number of detected donor alleles (Supplementary Fig. 4). For the group of samples with little or no degradation, the DNA profiles are already complete without purification, so no

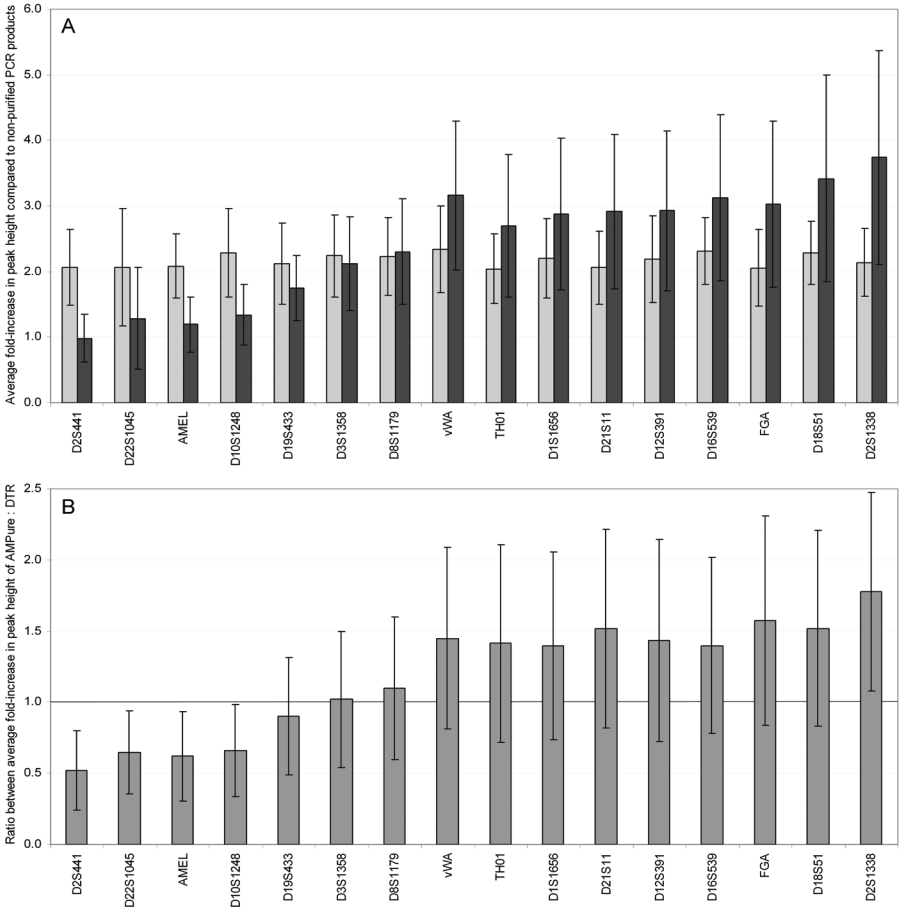

Fig. 1 (A) Average fold increase in peak heights when PCR products from moderately degraded samples are purified by either DTR gel filtration (light grey bars) or the in-house developed AMPure® protocol (black bars). This graph is based on a total of 522 alleles that were detected (i.e. above 50 rfu), but not saturated (i.e. below 6,000 rfu) in profiles of both non-purified and purified amplifications. The number of alleles per locus ranged between 10 (for D2S1338) and 39 (for D1S1656). (B) Average ratio between the fold increase in peak height of AMPure® purification and DTR gel filtration. In total, 510 ratios could be calculated (for samples for which the average fold increase in peak height could be determined for both purification methods), varying between 10 (for D2S1338) and 39 (for D1S1656) ratios per locus. The horizontal line at ratio 1 represents an equal fold increase in peak height after DTR gel filtration and AMPure® purification. For both (A) and (B), the loci are ordered by fragment length (from shorter ones on the left-hand to longer fragments on the right-hand side). The error bars represent the standard deviation.

Table 1 Number of detected alleles and saturated peaks after post-PCR purification compared to non-purified PCR products. DNA samples are categorised by degradation status

	Little degradation		Moderate degradation		Severe degradation	
	Total # (gained) alleles	# saturated peaks	Total # (gained) alleles	# saturated peaks	Total # (gained) alleles	# saturated peaks
No purification	270	n.a.	528	n.a.	162	n.a.
DTR gel filtration	270 (+0)	26	563[a] (+36)	16	185 (+23)	4
AMPure protocol	270 (+0)	31	567 (+39)	6	184 (+22)	2

n.a. not applicable.

[a]One allele was not detected after DTR purification

additional alleles can be detected (Table 1). In fact, for these samples, post-PCR purification has a negative effect, as saturated or overloaded (with peak heights >6,000 rfu) peaks occur (after DTR gel filtration on the shorter loci, and after AMPure® purification on the mid-sized and longer loci). In forensic analyses, saturated peaks are regarded undesirable [21]: (1) because they can be accompanied by apparently increased stutters, due to an underestimated peak height of the parent peak; (2) because minus A or shoulder peaks can occur; and (3) because they induce pull-up signals in other dye channels. To account for this effect, we marked these peaks as saturated when determining the number of detected alleles. Much less saturated peaks are seen for the moderately and severely degraded samples (Table 1 and Supplementary Fig. 4), especially with the AMPure® protocol. Alleles at the longer loci that remained undetected for the non-purified amplification products were retrieved after purification by both the AMPure® and the DTR procedure (Table 1), and for the moderately degraded samples, the AMPure® method performs slightly better than the DTR procedure (Table 1). Both for DTR gel filtration and for AMPure® purification, some low artefact signals of aberrant peak morphology were seen together with few stutter peaks above the stutter ratio threshold. The frequency of these detected stutter peaks is around 1 % of the peaks and is thus in line with a filter value set at 99 % [22]. Non-template controls did not show drop-in alleles for either method. An important advantage of post-PCR purification is that there is no need to use additional DNA extract, as it is performed on PCR products that otherwise would have been discarded (after

some time). We recommend analysing DTR-purified PCR products soon after purification, as they gradually destabilise in desalted conditions and the fluorescent dye tags start to detach from the primers after a few days, resulting in an increase of so-called dye blobs and a decrease of allele peak heights in the DNA profiles.

The efficiency of post-PCR purification may vary per sample (as is for instance demonstrated by the relatively large standard deviations in Fig. 1), resulting in a variable amount of salts within the purified PCR products. Consequently, the fold increase in peak height varies per DNA profile. Due to this variable increase, the stochastic threshold (below which peaks are prone to drop out due to stochastic amplification effects [22]) cannot be used reliably. Therefore, we regard the use of post-PCR purification as a low level technique and recommend the use of low template strategies such as replicate PCR analyses and derivation of a consensus profile [23].

The above-described data illustrate that post-PCR purification methods can efficiently increase the number of detected alleles at the longer loci in DNA profiles of degraded samples. The methods

Table 2 Average percentage of detected alleles and average peak height at locus D10S1248 or D2S1338 for mixtures having ratios of 1:5, 1:10 and 1:15

	Purification	1:5		1:10		1:15	
		Major	Minor	Major	Minor	Major	Minor
Average percentage of detected alleles	No	93 %	58 %	89 %	43 %	85 %	33 %
	DTR	95 %	74 %	90 %	57 %	95 %	45 %
	AMPure	99 %	79 %	96 %	57 %	95 %	42 %
Average peak height of D10S1248 (number of non-saturated alleles)	No	2,145 (5)	602 (5)	1,860 (5)	370 (5)	1,747 (5)	254 (5)
	DTR	3,547 (3)	1,328 (5)	3,822 (4)	827 (5)	4,168 (5)	611 (5)
	AMPure	2,578 (5)	658 (5)	2,093 (5)	382 (5)	1,895 (5)	253 (5)
Average peak height of D2S1338 (number of non-saturated alleles)	No	83 (6)	n.a.	81 (7)	n.a.	68 (3)	n.a.
	DTR	126 (8)	77 (3)	132 (8)	n.a.	123 (7)	n.a.
	AMPure	249 (9)	73 (2)	257 (8)	n.a.	161 (7)	n.a.

n.a. not applicable, due to lack of genotyping information

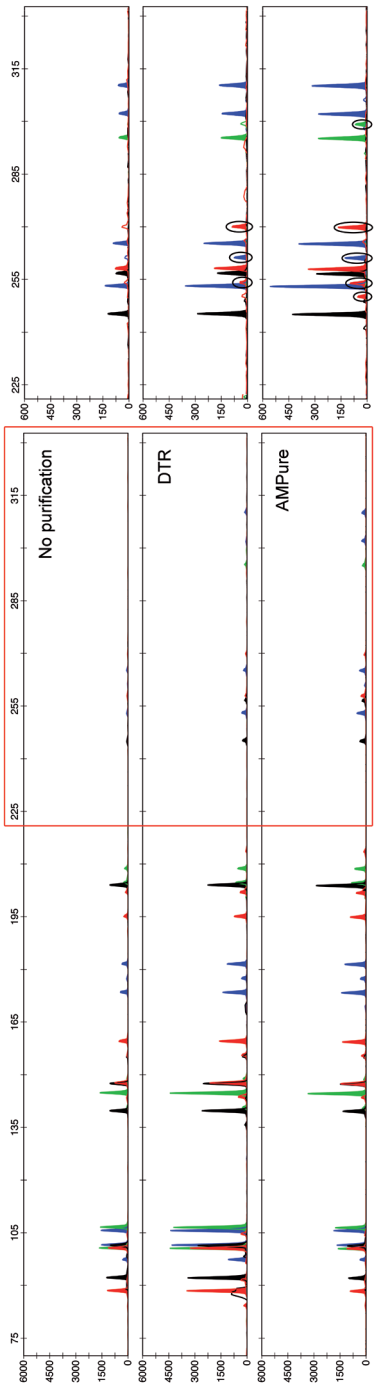

Fig. 2 Overlay electropherograms for a mixture with a minor/major ratio of 1:10 without purification (upper panes), after DTR gel filtration (middle panes) and after AMPure® purification (lower panes). The panes on the left show the full electropherograms, with the Y-axes scaled to 6,000 rfu; after DTR gel filtration, the peak heights on all loci are increased, while after AMPure® purification, the peak heights increase for all but the short amplicons. The panes on the right show a ten times zoom-in of the boxed area, and additionally detected alleles are circled.

hold the most potential for (moderately) degraded samples that show signs of peaks below the detection threshold. For those samples, the AMPure® purification procedure (with its size-selective properties) may be especially useful as it increases peak heights at the longer, but not at the shorter loci, thereby bringing more balance to the DNA profiles.

Improved analysis of unequal DNA mixtures

The analysis of degraded samples is further complicated when the samples represent mixtures with an unequal ratio between the contributors. The high amount of DNA from the major component at the shorter loci leads to over-amplification or overloading when the analysis is sensitised in order to retrieve genotyping information from the minor contributor at the longer loci. Thus, we prepared 15 mixtures of degraded DNA samples in various ratios (i.e. 1:5, 1:10 and 1:15) and assessed the performance of the AMPure® procedure in comparison to no and DTR purification. The corresponding unmixed samples (1:0 and 0:1) were analysed as well. For the non-purified samples, the average percentage of detected alleles for the major component (which was kept at a

fixed DNA input) is around 90 %; for the minor component, this percentage drops to 58, 43 and 33 % for the ratios 1:5, 1:10 and 1:15, respectively (Table 2). Both components show the allele drop-outs specifically at the longer loci. The effects observed for the single source degraded samples occur in the mixed samples as well. Firstly, peak heights show an overall increase after DTR gel filtration and a size-selective increase at the longer loci after AMPure® purification. Illustrative electropherograms are provided in Fig. 2, and the average peak height of the detected alleles for both the major and minor components at a short (D10S1248) and a long (D2S1338) locus is shown in Table 2. Secondly, saturated peaks (heights above 6,000 rfu) occur somewhat more after DTR gel filtration ($n = 14$) than after AMPure® purification ($n = 3$). Thirdly, more alleles are detected for both mixture components after both purification strategies (Table 2), with the AMPure® protocol retrieving slightly more genotyping information.

The specifics of the difference in the number of detected alleles between purified (either by DTR gel filtration or AMPure® purification) and non-purified PCR products are presented in Fig. 3. This graph differentiates between short, middle range and long loci and between unshared major and minor component alleles and shared alleles. At the short loci (red shades), saturated peaks occur after DTR gel filtration.

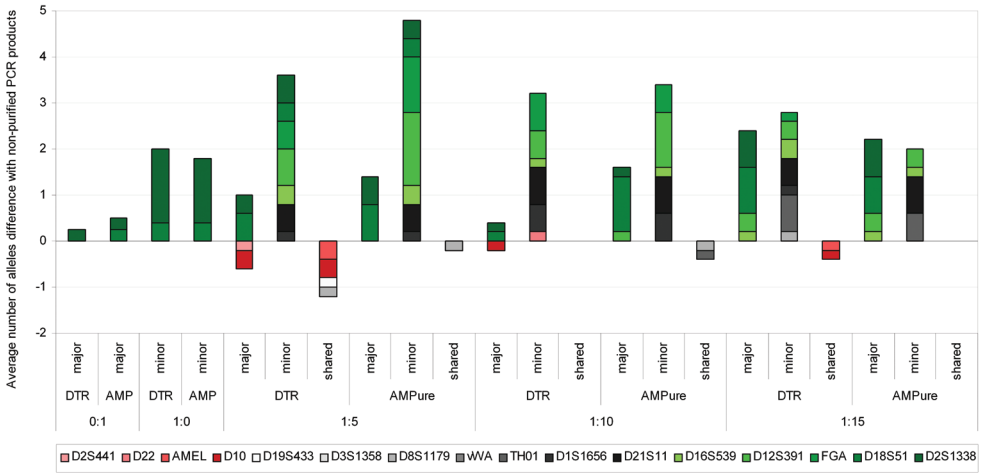

Fig. 3 Average number of allele difference between purified and non-purified PCR products. A comparison was made between DTR gel filtration and AMPure® post-PCR purification after differentiation between major, minor and shared alleles. Gained alleles are shown as a positive number, saturated alleles as a negative value. The markers are shaded in red tones for the shorter loci, in grey for the mid-range and in green tones for the longer fragments. Mixture ratios 1:5, 1:10 and 1:15 were performed in five replicates, and the corresponding unmixed samples were tested in fivefold for 1:0 and in fourfold for 0:1. Standard deviations are not shown for clarity.

Alleles for which the major contributor is homozygous and alleles shared between both contributors appear especially prone to saturation. For the mid-range loci (grey shades), a gain of alleles is seen especially for the minor component in the mixtures having 1:10 and 1:15 ratios (the minor component shows only few drop-out alleles at these loci in the non-purified 1:5 mixtures). The effects of DTR and AMPure® purification are similar at these loci, although few shared alleles become saturated after applying the AMPure® procedure. Most of the effect of the post-PCR purification methods is seen at the long loci (green shades), as many alleles of both contributors are gained. The largest gain in detected alleles occurs for the minor contributor in 1:5 mixtures, and the AMPure® protocol retrieves on average slightly more alleles than the DTR procedure.

These results show that post-PCR purification can improve genotyping of both single source and (unequally) mixed degraded DNA samples. The in-house developed AMPure® protocol (including DTR gel filtration) has the potency to bring more balance to profiles showing a degraded pattern.

Concluding remarks

To improve STR profile analysis of (unequally mixed) degraded DNA samples, we developed a post-PCR purification method that takes advantage of the size-selective properties of AMPure® XP beads. This method was compared to DTR gel filtration and no purification of the PCR products. While DTR purification results in an approximately twofold increase of peak heights at all loci, AMPure® purification increases the signals at the longer loci three- to fourfold, while those at short loci are not increased. Both methods can provoke the occurrence of saturated peaks that are undesirable and should be excluded from quantitative assessments [21]. DTR purification produces more saturated peaks that reside predominantly at the shorter amplicons, while the AMPure® procedure gives less saturated peaks that are present mainly at the mid-range amplicons. We do not anticipate that adaptations in the AMPure® protocol could reduce the occurrence of saturated peaks; when the PCR product to beads ratio would be lowered, the recovery of fragments at the longer loci would be reduced as well. Overall, slightly more alleles are detected, especially for the longer amplicons, with the AMPure® protocol than with DTR purification alone. In general, alleles are only retrieved when signals appear present below the detection threshold in the profiles of non-purified PCR products, and the success of the approach is influenced by mixture ratio and degradation state. In conclusion, the in-house developed AMPure® protocol can be an efficient new forensic tool to increase the number of detected alleles in STR profiles of single source and (unequally) mixed degraded DNA samples.

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Supplementary material

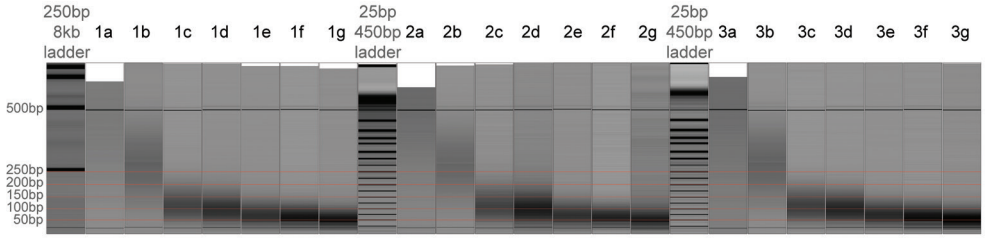
Supplementary Text 1

DNA samples

For assay optimisation pristine control DNA007 (Applied Biosystems (AB), Foster City, CA, USA) was used. To test the performance of the assay on samples with various levels of DNA degradation, 39 samples were subjected to several degradation methods as described below. Six samples were extracted from 450-550-year-old bone samples excavated in Delft (the Netherlands) [18]. One sample originated from a vaginal swab that was overgrown with microbes. Four samples were prepared with pristine DNA from the Quantifiler™ Human DNA standard (200 ng/µL) and irradiated with UV-light for 0, 10, 30 and 60 minutes, following the protocol as described by Westen et al. [19]. Seven samples, extracted from blood of different donors, were diluted to ± 0.5 ng/µL and exposed to several freeze (-20 °C) and thaw (room temperature) cycles. Three other samples derived from blood donations were subjected to seven different sonication settings each (Supplementary Table 1) that created fragments of different lengths (QIAxcel images for all 21 DNA samples are shown in Supplementary Figure 1). Sonication was performed on a Covaris™ S2 instrument (Covaris, Woburn, MA, USA) in microTUBEs with 110 µL 1*TE-buffer and 10 µL sample (± 50 ng/µL). Except for the bone samples, DNA profiles were known and volunteers had provided informed consent.

Supplementary Table 1 Covaris settings for degradation of DNA by sonication.

	Duty cycle	Intensity	Cycles/burst	Time (s)	# Cycles
a	-	-	-	-	-
b	5%	3	200	20	1
c	10%	4	200	120	2
d	10%	5	200	180	3
e	10%	5	200	360	6
f	10%	5	200	540	9
g	10%	5	200	900	15



Supplementary Fig. 1 DNA integrity of Covaris-degraded samples. Three DNA samples were subjected to seven Covaris settings (Supplementary Table 1) to fragment the DNA. DNA quality is visualised on a QIAxcel system (Qiagen, Venlo, the Netherlands) using DNA size markers of 250 bp – 8 kb (four fragments can be seen: 250 bp, 500 bp, 750 bp and 1 kb) and 25 bp – 450 bp (fragments at 25 bp intervals).

These 39 DNA samples, which included four non-degraded samples (0 minutes UV-irradiation and setting "a" for the three Covaris-degraded samples), were genotyped using standard DNA analysis as described in the next paragraph. Based on the profiling results, they were divided into three categories: 1) little or no signs of degradation (all alleles detected); 2) moderate degradation (peak heights at the longer loci in the region of the detection threshold and ranging from just above to well below) and 3) severe degradation (no peaks for the longer loci, not even below the detection threshold). To link these categories and the apparent DNA integrity on gel (Supplementary Figure 1): DNA samples subjected to Covaris settings "a" and "b" become categorised as no or little degradation (DNA fragments predominantly above 200 bp), those exposed to settings "c" and "d" illustrate the category moderate degradation (predominant size range 200-50 bp) and DNAs affected by settings "e", "f" and "g" become regarded as severely degraded (DNA fragments mostly 125-25 bp in length).

DNA mixtures were prepared from the Covaris-degraded samples 1c and 2c (Supplementary Table 1). Besides using both samples unmixed (0:1 with $n = 4$ and 1:0 with $n = 5$), they were mixed in the ratios 1:5, 1:10 and 1:15 (all with $n = 5$). In these mixtures, the major component was fixed at 1 ng DNA per reaction (quantified after sonication) and the minor components contained 200, 100 and 67 pg DNA, respectively.

DNA quantification and PCR

After the degradation procedures were performed, all DNA samples were quantified using an ALU-assay, based on the publication by Nicklas and Buel [20]. The amplicon size in this assay is 127 bp (for total DNA quantification), which makes the assay predictive of STR amplification success with degraded samples [20]. Indeed, the serially degraded samples showed a decrease in measured DNA quantity with

stronger treatment, while all samples within one series were prepared from one stock solution. DNA amplification was performed with the AmpF*l*STR® NGM™ PCR Amplification Kit (AB), according to the manufacturer's protocol using 0.5 ng DNA as input if available (except for the mixtures, see previous paragraph).

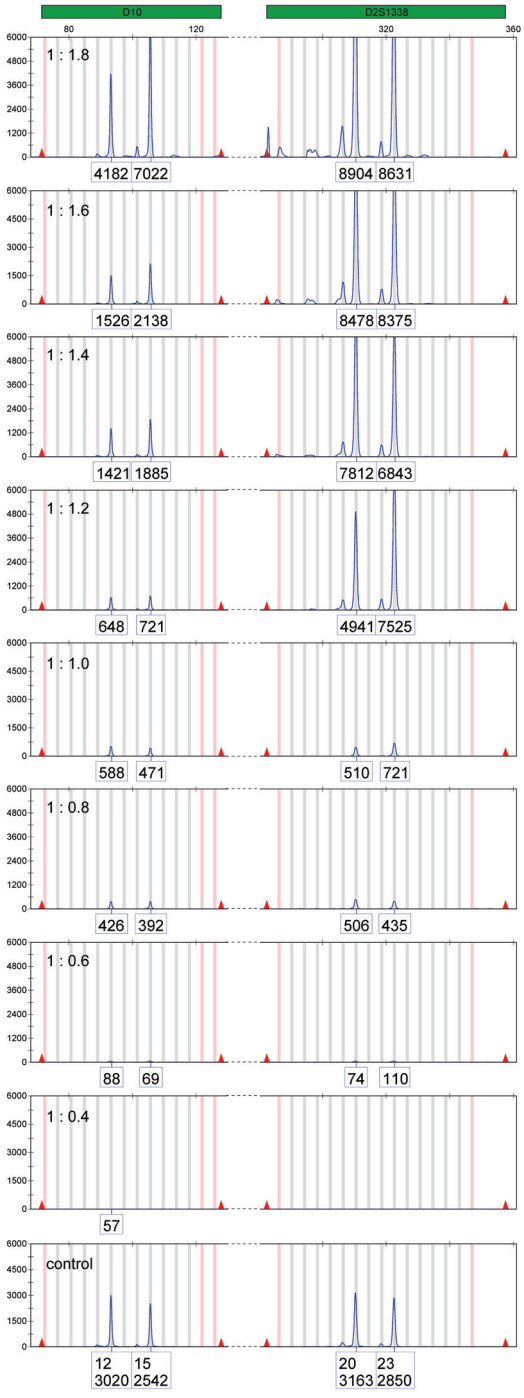
Capillary electrophoresis and DNA profile analysis

PCR products were detected by capillary electrophoresis on an ABI Prism 3130xl Genetic Analyser (AB). Non-purified samples were analysed in a blend of 8.7 µL Hi-Di™ formamide (AB), 0.3 µL GeneScan™-500LIZ™ size standard (AB) and 1.0 µL PCR product or allelic ladder (AB). Purified samples (after DTR gel filtration or the AMPure® protocol) were injected in a blend of 6.5 µL Hi-Di™ formamide, 1.5 µL 1:100 diluted GeneScan™-500LIZ™ and 2.0 µL PCR product. After 5 min of denaturation at 95 °C and 5 min on ice, the PCR products were analysed with injection settings of 3 kV for 10 s for the experiments assessing single source samples and 3 kV for 5 s for the mixture experiments. These reduced settings were used to prevent too high overloading for the major component upon sensitised injection. Also, in the mixture experiment only 1.0 µL purified PCR product was taken instead of 2.0 µL. DNA profiles were analysed using GeneMapper® ID-X v. 1.1.1 (AB). The detection threshold was set at 50 relative fluorescent units (rfu) and alleles with peak heights above 6000 rfu (that may have signal saturation) were excluded from calculations that involved peak heights and marked as a saturated peak when determining the number of detected alleles. In all profiles, each peak was counted as one allele irrespective of zygosity state as the genotypes were unknown for some of the samples (the bones).

Supplementary Text 2

Developing a protocol for size-selective post-PCR purification

The size-selective properties of AMPure® XP magnetic beads depend on the ratio between PCR product and AMPure® XP beads [16,17], and therefore we first explored how various ratios function when applied to forensic STR genotyping products (Supplementary Figure 2). When using the recommended ratio of 1 volume of PCR product to 1.8 volumes of AMPure® XP beads, the NGM™ DNA profiles show increased peak heights for all loci (i.e. both the shorter (70 to 125 bp) and the longer (225 to 370 bp) amplicons) compared to the non-purified amplification products. When the relative amount of magnetic beads is lowered to 1:1.6, 1:1.4 or 1:1.2, the increase in peak height for the shorter amplicons reduces, while the peak heights for the longer amplicons remain augmented. When the ratio is lowered further to 1:1.0 or below, all peak heights decrease significantly, and for the ratio of 1:0.4

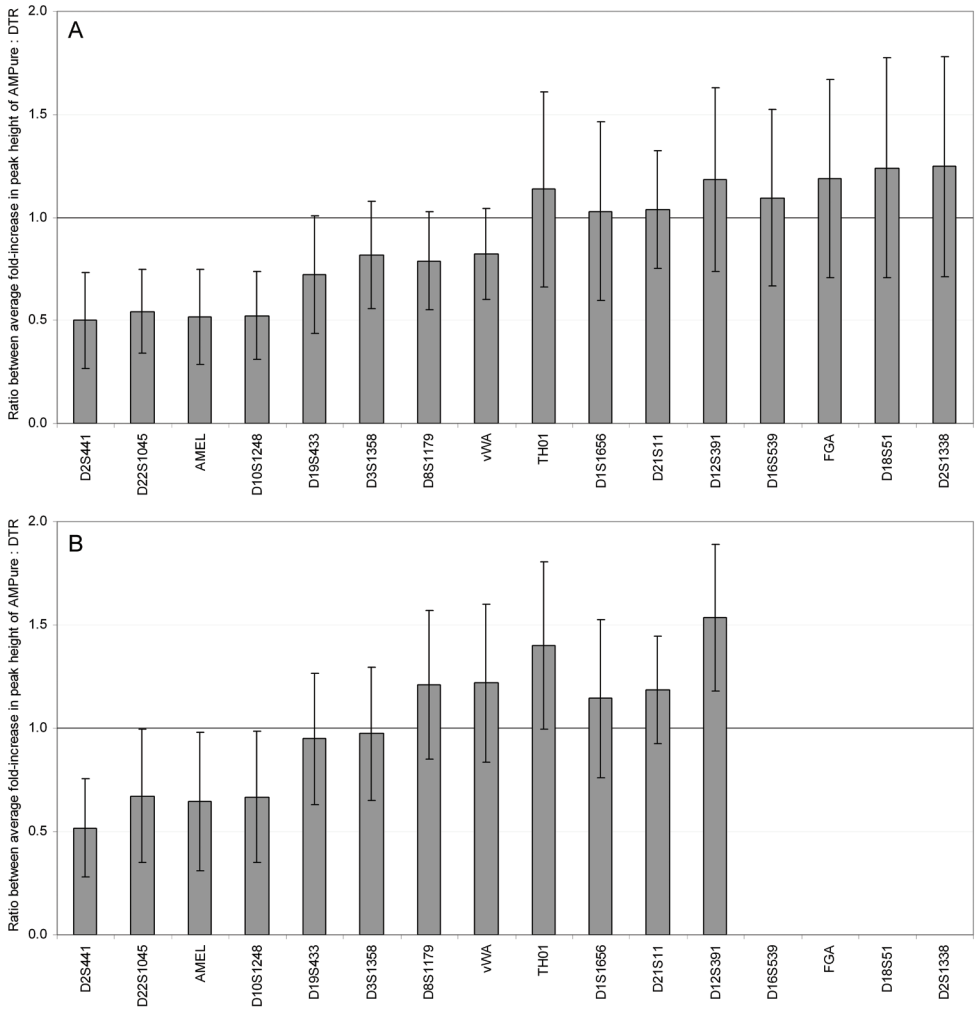


allele drop-outs start to occur. Size-selectivity of the AMPure® XP beads was also reported by David Edwards [16], although he observed slightly different recovery effects, as fragments below 150 bp and 200 bp were not retrieved with ratios of 1:0.8 and 1:0.6 respectively. The finding that in our study all fragments in the DNA profiles (that range from 70 to 370 bp) are recaptured for these ratios, is probably due to the higher sensitivity of our detection method (agarose gels for [15] versus fluorescently-labelled fragments on CE here). We regard the ratio of 1:1.2 as most potent to improve forensic DNA profiles of degraded samples,

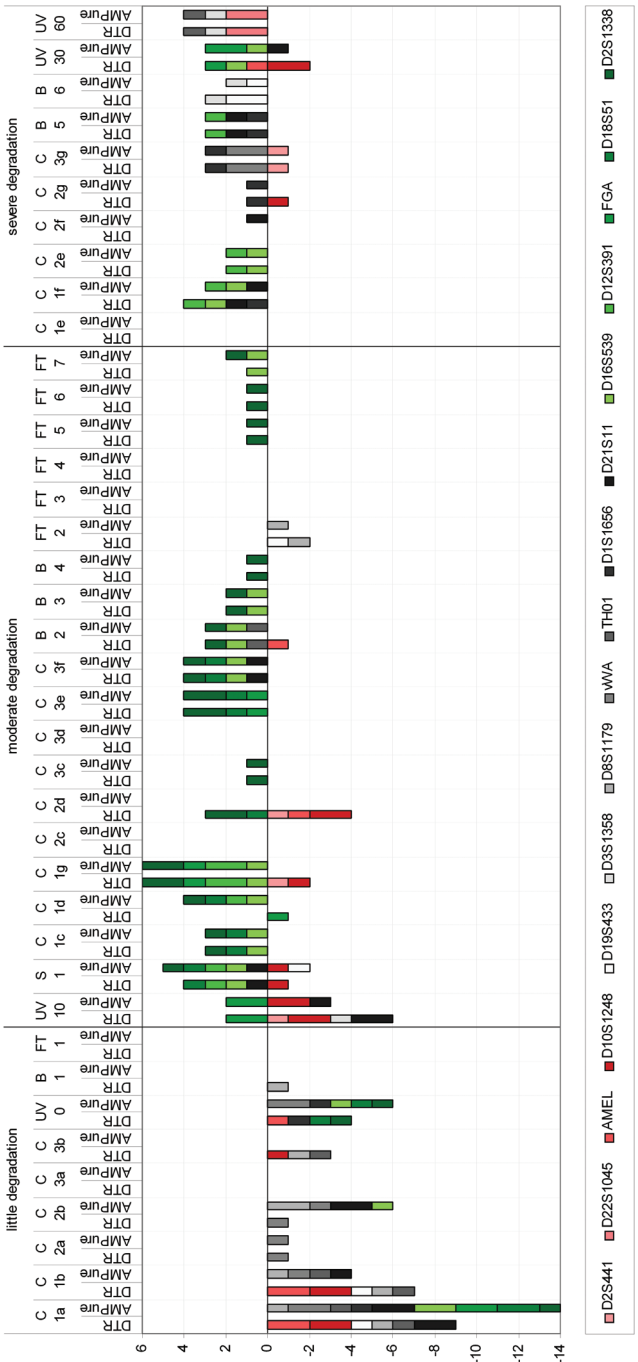
Supplementary Fig. 2 Post-PCR purification by AMPure® XP magnetic beads (according to the manufacturer's protocol) in various volume ratios of PCR product and beads (1:1.8 down to 1:0.4). These experiments are all based on 500 pg pristine DNA007 NGM™ PCR amplifications of which 24 µL were used during the AMPure® purification and 1 µL purified PCR product was analysed by CE. A non-purified control sample is shown in the bottom row. On each row, the first (D10S1248; 70-125 bp) and the last locus (D2S1338; 280-360 bp) of the blue (6-FAM) channel are shown. The peaks of the control sample are labelled both with the allele call and the peak height; the other samples are labelled with the peak height only. The Y-axis is scaled to 6000 rfu in all panels.

as the peak heights for the longer loci become substantially increased, while those at the shorter amplicons are somewhat reduced; it is anticipated that this will not lead to loss of genotyping information as degraded DNA profiles typically have high peaks for the short fragments. Thus, based on this ratio, we optimised the protocol for forensic applications.

Aspects for protocol optimisation included: 1) the input volume of PCR product (10 or 24 µL; 10 µL would save some PCR product for other analyses and fits with lower PCR volumes), 2) the number of ethanol washing steps (0 or 2; less washes are preferred as washing generally induces sample loss), 3) the elution volume (10, 20, 30 or 40 µL; the amplified fragments are higher concentrated in a lower elution volume, which enables more fragments to be injected during CE), 4) elution in ddH2O or Hi-Di™ formamide (Hi-Di™ formamide would allow to use a larger portion of the eluted liquid for CE analysis), 5) elution by diffusion (according to manufacturer's protocol) or active resuspension by pipetting beads and elution liquid 10 times up and down (to increase fragment elution) and 6) recovery of eluate by careful pipetting (beads collected at the magnet) or using DTR gel filtration (beads remain on top of filtration gel). The optimised protocol is described in the Material and methods section. Next, we assessed the performance of this protocol on NGM™ amplifications of single source and unequally mixed degraded DNA samples.



Supplementary Fig. 3 Average ratio of the fold-increase in peak heights of the AMPure® protocol and DTR gel filtration for (A) no or little degraded samples and (B) severely degraded samples. The loci are ordered by fragment length (from shorter ones on the left-hand to longer fragments on the right-hand side). The error bars represent the standard deviation. The horizontal line at ratio 1 represents an equal fold-increase in peak height after DTR gel filtration and AMPure® purification. (A) For the no or little degraded samples 224 ratios could be calculated, varying between 9 (for D8S1179 and vWA) and 18 (for D22S1045 and D12S391) ratios per locus. (B) For the severely degraded samples 157 ratios could be calculated, varying between 3 (for D12S391) and 18 (for D2S441 and D3S1358) ratios per locus. No ratios could be determined for the longer loci, due to the lack of genotyping information in the profiles of non-purified PCR products.



Supplementary Fig. 4 The effect of post-PCR purification by DTR gel filtration or AMPure® purification on the number of donor alleles detected for non-purified PCR products. Gained alleles are shown as a positive number, saturated alleles as a negative value. Sample degradation methods are abbreviated as: C = Covaris, UV = UV-light, B = bone sample, FT = freeze/thaw cycles and S = swab overgrown with microbes. Alleles on short loci are shown in red, on mid-range loci in grey and on long loci in green.

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Chapter 8

General discussion

Human identification is employed in diverse situations. When a clandestine grave is discovered in a forest, the circumstances are completely unlike those at an airplane crash site where bodies and body parts are scattered around. A tsunami area presents again an entirely different scenario as not only many casualties may occur, but also the complete infrastructure has flooded away. Nevertheless, in all such cases one of the main questions to answer is: "To whom belongs this body (part)?" Especially when bodies are beyond recognition, DNA-based methods can be very useful for identification. DNA, however, is subject to decay particularly when it has been exposed to harsh conditions, and this may hamper obtaining an interpretable DNA profiling result. The research described in Chapters 1 to 7 of this thesis (published as [1,2,3,4,5,6,7]) has been aimed at developing additional or alternative methods to extract information from a person's DNA when standard DNA methodology is not sufficient for human identification. This chapter reviews various aspects of human identification and the technical and ethical choices at hand. Focus lies on (disaster) victim identification, although human identification also applies to relationship testing and forensic analysis.

Sample collection, DNA extraction and quantification

In case of a (mass) disaster, it is helpful when disaster victim identification (DVI) team members can rely on predefined guidelines, such as provided in "Mass fatality incidents: A guide for human forensic identification" by the National Institute of Justice [8] or the Interpol DVI guide [9]. These guides broadly outline the steps that need to be taken during DVI, but do not give specifics on how to perform certain procedures. In Chapter 1 (published as [1]), we have described a guideline for bone and tooth sample collection and contamination prevention for these samples to aid DNA-based victim identification. This guideline, which has been spread under the participants of "The use of DNA in disaster victim identification meeting" in Berlin in 2009, is based on common sense and practical experience. Additional experiences may provide further improvements. The two most important requirements for DNA-based identification are 1) collection of representative, high quality tissue samples from a victim and 2) the availability of reference samples, either from the alleged victim or from family members, with which the profiling results of the tissue samples can be compared [10,11,12,13]. Since the quality of the DNA is affected by time since death and environmental conditions (Box 1), samples need to be collected as soon as possible and stored under optimal conditions after sample collection to prevent further deterioration of the DNA [1,14,15]. To determine which samples are the most promising for DNA analysis, Milos et al. [16] analysed genotyping success of various skeletal elements (using skeletal samples from war victims in the former Yugoslavia). They verified empirically that the densest compact bones and teeth are the optimal samples for DNA recovery,

Box 1. DNA degradation

After death, cells and organelles disintegrate, thereby releasing lysosomal endonucleases that mediate DNA degradation. Further degradation can be caused by, for instance, exogenous nucleases from micro-organisms (that may grow on released nutrient-rich fluids after cell death) and environmental invertebrates. These enzymatic reactions can be followed by non-enzymatic or spontaneous DNA degradation processes that are generally much slower. These include hydrolytic reactions (at the glycosidic base-sugar bond, which can result in abasic sites, DNA cleavage or deamination for DNA bases with secondary amino groups), oxidative reactions (induced by O_2 or H_2O_2 and resulting in modifications such as formation of hydantoins that may block PCR), DNA cross-linking (within or between DNA strands or between DNA and proteins, which also blocks PCR) and radiation (that may induce a variety of lesions) [146,147]. The extent and manner of DNA degradation is influenced by environmental factors and exposure time to these factors. These environmental factors include (amongst others) river- or seawater, warm humid air, fire, nuclear radiation, UV-light, highly acidic soil and chemical agents that are used in deliberate attempts to degrade the DNA of war victims [1,148,149,150,151,101].

as is often assumed. Mundorff et al. [17] performed similar analyses (based on samples taken after the World Trade Center terrorist attacks) and suggest to sample patellae, metatarsals or foot phalanges since these provided similar genotyping results as femora and tibiae, but are much easier accessible; these skeletal elements can be excised with a disposable scalpel, thereby reducing contamination risk from reuse of instruments. Steinlechner et al. [18] advise to take (in addition to bone and tooth samples) two swabs from the internal organ or muscle surfaces that appear most intact (based on samples from the Sri Lanka site after the 2004 tsunami) and analyse these swabs first. The main advantage is that swabs are much easier to process than bone samples, but since DNA in soft tissues typically degrades faster than in bones and teeth, the chance of success is lower [18]. Evidently, any swab used for DNA-identification needs to be sterile and free of contaminating human DNA [19,20]. The actual sample collection procedure that is followed will depend on the condition of the remains, which to a large extent will rely on the type of disaster that has occurred as every disaster has its own specifics [17,21].

After (DVI) samples are taken, the DNA analyses are preferably performed in specialised and/or accredited (forensic) laboratories. When DNA is to be extracted from bone fragments or teeth, pulverisation of the samples is needed beforehand, which is usually done by cryogenic grinding (Box 2.1). According to Loreille et al., the DNA

yield can be increased by total demineralisation of the bone powder after grinding [22] although some bone samples cannot be demineralised completely (personal communication, T. Parsons), which results in a smaller increase in yield. Next, the DNA has to be extracted from the sample and purified from possible PCR-inhibiting substances, such as humic acid (that can be present in soil) or soot (after fire). Many DNA extraction methods exist that mostly rely on phenol/chloroform extraction or silica (column or bead)-based purification (e.g. [23,24,25,26]). Comparative studies on human bones showed either that these methodologies perform equally well [27,28], or that the silica-based strategy (e.g. QIAgen kits, Venlo, the Netherlands) outperforms phenol/chloroform extraction [29,30] (L. Schoenmakers & A.A. Westen, unpublished results). An interesting finding by Guo et al. [28] is that the DNA extraction method has an influence on the ratio between the extracted nuclear DNA and mitochondrial DNA (mtDNA). They conclude that when a high yield of mtDNA is essential for your research, organic extraction is preferred over column-based extraction methods, while for nuclear DNA phenol/chloroform and QIAgen extraction methods performed equally well [28]. In general, silica-based methods are very good (and better than the organic extraction procedures) in removing non-DNA compounds that may negatively affect the PCR [29,31,32]. In specific cases, a pre-PCR DNA clean-up kit can be used such as NucleoSpin gDNA Clean-up (Macherey-Nagel) [33,34] or Powerclean DNA Clean-Up [35], to obtain DNA as pure as possible.

A completely different approach to obtain DNA from (bone) cells is based on pressure cycling technology (PCT). PCT makes use of hydrostatic pressure that alternates between ultrahigh and ambient levels to physically disrupt the cellular material. This method is reported to extract proteins, lipids and nucleic acids in parallel from the same sample [36]. The DNA recovery is dependent on the cell type and the efficiency (compared to DNeasy (Qiagen) methodology) ranges between 100 % and 30 % [36]. An additional interesting aspect of PCT is the ability to change the conformation of compounds under high pressure, which may positively affect the PCR when inhibiting substances are present [37].

After isolation, the DNA is quantified, preferably by a human-specific methodology, to determine the PCR input in order to obtain balanced results in the generally multi-target DNA profiling assays. Different quantification methods amplify DNA targets of different fragment lengths. Since the DNA in DVI samples is often degraded, it is important to realise that quantification methods relying on short DNA amplicons (such as Quantifiler™ Human (Applied Biosystems (AB)), having a 62 bp target [38]) may provide higher quantifications than methods with longer targets (such as Plexor HY™ (Promega) with a 99 bp [39], Alu-assay with a 127 bp [40], Quantifiler™ Duo (AB) with a 140 bp [38] or Quantiplex HYres™ (Qiagen) with a 146 bp target). Given that most short tandem repeat (STR) multiplex systems currently comprise amplicons between 70 and 400 bp, the quantification methods with the longer DNA targets will give more accurate predictions of genotyping success than those with shorter targets.

STR profiling of compromised samples

Usually, the next step towards human identification is an STR PCR to amplify and fluorescently label DNA markers before detection by capillary electrophoresis (CE). The PCR may be inhibited by several substances that were co-isolated with the DNA, resulting in no profiles or profiles with lower peak heights than expected from the amount of template DNA. PCR inhibitors can be classified into three groups by their source: 1) intrinsic inhibitors, such as heme in blood or melanin in hair, 2) inhibitors from the substrate, such as humic acid in soil or indigo dye in denim and 3) other inhibitors, such as substances used during the extraction process (e.g. SDS or chelex resin) or EDTA, which is used to buffer the DNA extracts [41]. Using less of the DNA extract may improve the results, but may also lead to partial DNA profiles when the quantity of the DNA is low, as is often the case in human identification samples. The PCR process may benefit from modified DNA polymerases that are more tolerant to PCR inhibition. Some of these modified enzymes are even able to directly amplify whole blood such as Hemo KlenTaq (a *TaqI* polymerase that lacks the first 280 amino acids and has several additional mutations) [42] and Phusion™ Flash Polymerase (a *Pyrococcus*-like polymerase to which a DNA-binding domain is fused) [43]. As Phusion™ Flash Polymerase is in addition accurate and fast, this enzyme was incorporated in an in-house developed "DNA-6-hours" service that assists police investigations by rapidly deriving DNA information from trace evidence [44,45]. Besides, different DNA polymerases vary for the capacity to deal with difficult templates such as GC-rich or looped sequences. STRs are complex templates in the sense that they comprise repeated sequences, which invokes slipped strand displacement during amplification due to which back and forward stutter products are formed [5]. As stutter peaks resemble real alleles, they can hamper the interpretation of unequal mixtures. DeepVent$_R$ DNA polymerase [46] is a polymerase adapted for use with difficult templates, but it did not produce less stutter products than the standard AmpliTaq™ Gold polymerase (Box 2.2). Other options that have been explored to improve PCR performance relate to adjustment of the PCR parameters, such as adding twice the recommended amount of Taq polymerase [47,48], elongation of the annealing time to reduce allele drop-out [48], reducing PCR volume, or (and this strategy is the most widely used) increasing the number of PCR cycles for the analysis of low template DNA [49,47,50,51]. Some of these aspects have been accounted for during the development of the current generation STR kits (e.g. AmpFlSTR® NGM™ (AB), the PowerPlex® ESX™ and ESI™ systems (Promega) and Investigator® ESSplex™ (Qiagen)); these kits show an improved resistance to PCR inhibitors due to optimised buffer systems, have elongated annealing times to reduce allele drop-out rates and make use of one or two additional PCR cycles (i.e. 29 or 30 cycles) to increase sensitivity [52]. The newest kits aim specifically for efficient analysis of unprocessed reference samples. These kits accommodate direct amplification of both

buccal and blood specimens and achieve amplification in impressively short times (e.g. below 50 minutes for the Investigator® IDplex Direct™ kit (beta testing for Qiagen)). Since commercial companies developed these kits, the compounds effectuating these improvements are unknown.

Low template DNA amplification (as is frequently encountered in human identification and forensic research) is typically affected by stochastic effects such as allele drop-out, near-threshold peaks, heterozygous peak imbalances, increased stutters and allele drop-in. To aid analyses, stochastic thresholds can be derived, and when allele peak heights are below this threshold it is anticipated that stochastic effects may have occurred. In Chapter 5 (published as [5]) we determined stochastic thresholds for the analysis of NGM™ DNA profiles. There are several options to improve low template DNA profiling results, such as the above-described methods to increase PCR performance (of which performing additional PCR cycles [50,51,47] is most widely and frequently used). In addition, adaptations at the post-PCR level can be made such as purification of amplified products (as described in Chapter 7 (published as [7])) or enhancing the CE injection settings (as described in Chapter 2 (published as [2])) [53,54,55,56,57]. Performing additional PCR cycles (e.g. 34 cycles as used at the NFI [51,5]) is only recommended for samples with very low DNA inputs (< 31 pg, using NGM™ [5]), as with higher inputs the profiles will become overloaded [51]. For low template samples with higher inputs, enhanced injection settings are advised preceded by post-PCR purification to reduce dye-blobs in the DNA profile (e.g. 9 kV for 15 seconds combined with DTR gel filtration, as suggested in Chapter 2 (published as [2])). This method retrieves almost as many alleles as 34 cycle PCR, but shows less drop-ins, and can be performed on the remaining PCR product after standard analysis (thus no additional PCR or use of extra DNA extract is needed) [51,5]; this method is embedded in the ISO17025 accreditation of the NFI and in use since November 2007. When some of the peaks in the standard DNA profile are relatively high, for instance on the shorter loci in profiles of degraded samples or for the major component in an unequal mixture, specific post-PCR purification strategies (such as a size-selective method based on AMPure® XP beads that especially enriches for longer amplicons) may be beneficial, as described in Chapter 7 (published as [7]). The above-described methods are applied when standard STR profiling resulted in peaks below the stochastic threshold and/or allele drop-outs. Consequently, these methods are to be regarded as low template techniques, and low template interpretation strategies are appropriate. The most applied interpretation strategy involves replicate PCR analyses from which a consensus profile is derived [51]. Benschop et al. have compared several methods to generate a consensus profile, and recommend the *n*/2 method in which alleles are included in the consensus when they are designated in at least half of the replicates (with $n = 3$ or $n = 4$ as optimal replicate number) [51]. An upcoming alternative is the use of statistical interpretation tools that harbour the occurrence

of drop-outs and drop-ins [58,59,60]. When peaks in the DNA profiles are low and sufficient DNA extract remains, a final possibility is to concentrate the DNA extract to a volume befitting the PCR set up (for instance by ethanol precipitation). Since this will consume most (if not all) DNA extract, this method should only be performed when it is anticipated that the resulting allele peak heights will be above the stochastic threshold. Otherwise, it might be sensible to store the DNA extract in expectation of future development of more sensitive methods.

Whole genome amplification (mostly used for single cells in pre-implantation genetic diagnostic research) has been proposed as an amplification method for forensic samples with extremely low amounts of template DNA [61,62]. Several techniques exist and have been compared [62], and increased amplification success was obtained especially with multiple displacement amplification [63,62]. However, WGA suffers from preferential amplification and this difficulty (that translates into locus and allele drop-out) has not yet been overcome (Box 2.3) [64]. Another concept that has been tested for relatively low template DNA samples, is employing the unused genomic DNA that still resides in PCR mixtures (of which only a small amount has been used for CE) after amplification as template for a second PCR based on a distinct marker system. Although products for the second marker system were generated, the primers from the first amplification strongly interfered in the second amplification and this approach was regarded unsuccessful (Box 2.4). Compromised samples may suffer not only from reduced quantity, but also from low quality, which means that the DNA is degraded. Therefore, attempts have been made to repair the degraded DNA before amplification by using DNA repair enzymes that *in vivo* correct genomic DNA damage. Several groups applied the commercially available PreCR™ Repair Mix (New England Biolabs, MA, USA), with variable success rates [4,65,66], which mainly relate to the amount of template DNA used. Full STR profiles are obtained when an input of 25 ng DNA (from an old bloodstain) is repaired [66], but thus far no success is reported for (mock) casework samples (Box 2.5) [4,65].

Box 2. Supplemental results generated during this thesis work

2.1 Optimisation of cryogenic grinding

In order to optimise the DNA yield from bone samples, these samples need to be grinded before DNA isolation. In a comparison of three cryogenic grinding systems, the Freezer/Mill® 6770 (SPEX SamplePrep, Metuchen, NJ, USA) produced bone powder with a finer structure (and, on average, more detected alleles per profile) than the Freezer/Mill® 6750 (SPEX SamplePrep), while the TissueLyser (Qiagen; with a liquid nitrogen cooled stainless steel grinding jar

set) was not capable of grinding the archaeological bone samples [152] at all (L. Schoenmakers & A.A. Westen, unpublished results). Thus, we regard the Freezer/Mill® 6770 as the optimal grinding system, and optimised the grinding process on this machine.

2.2 Reduction of stutter peak height

During amplification of STRs, slipped strand displacement may occur resulting in stutter products that are visible in the DNA profiles as peaks at one repeat length before (or after) the actual allele. These stutter peaks resemble real alleles and may hamper the analysis of unequal mixtures. DeepVent$_R$ DNA polymerase is a polymerase adapted for use with difficult templates, and we tested its effect on the formation of stutter products. The results were compared to AmpliTaq™ Gold (which is the standard DNA polymerase) in SGM Plus™ DNA profiles. Both DNA polymerases produced stutter peaks of similar heights, and the use of DeepVent$_R$ caused a decrease in the inter-locus balance. As no positive effect was seen from the use of DeepVent$_R$, it was decided not to proceed to tests involving unequal mixtures.

2.3 Multiple displacement amplification (MDA)

Whole genome amplification (WGA) can be used to generate manifolds of DNA from relatively low amounts of starting template. This could facilitate multiple analyses for samples otherwise allowing only one or few analyses. Many WGA methods exist which can be generally divided in two categories: those based on variations of PCR amplification (including techniques to attach random primers, degenerate primers and adaptors to genomic DNA fragments to create universal priming regions) and those with isothermal DNA amplification utilising multiple strand displacement amplification. Since the quality of the DNA in human identification and forensic analyses is usually low, fragmenting the DNA to create universal priming regions may lead to DNA that is too much fragmented to allow subsequent STR analysis. Multiple displacement amplification (MDA) methods generally require high quality DNA to be successful. An exception is the REPLI-g FFPE kit (Qiagen) that ligates all the short fragments before MDA takes place. Although the manufacturer recommends a minimum DNA input of several nanograms, we tested this kit with a DNA input of 500 pg to examine its performance for forensic use. Using ALU quantification [40], an increase in DNA concentration was measured. However, DNA profiles generated from this MDA-amplified DNA showed strong preferential amplification, which led to allele drop-out on the one hand and over-amplification of alleles on the other

hand within the same profile. Due to these extremely imbalanced DNA profiles we feel that this method is not fit for forensic practice, but we look forward to future developments that may comply with forensic use.

2.4 Reusing a DNA sample

At the NFI, the standard PCR volume is 25 µL of which 1 µL is analysed by CE. The remaining 24 µL are stored (for possible re-analysis of the PCR product) and discarded after a few months (as the instability of the fluorescent groups increases with time). This stored PCR product still contains the original genomic DNA and we tested whether this DNA could serve as template used for a second amplification with a distinct STR kit. When performing a second amplification, it is important to remove all primers used during the first PCR from the mixture (as these will induce amplification again, otherwise), together with the fluorescent labels from the PCR products (or the fluorescently labelled PCR products themselves). MiniFiler™ was chosen as first and Yfiler™ as second genotyping kit as these present very distinct profiling results. For primer removal ExoSAP-IT™ was tested that contains *Exonuclease I*, which can degrade single-stranded primers and DNA. Indeed it removed the primers from the PCR mixture, but at the same time also original DNA templates were degraded (possibly because these became single-stranded from the multiple denaturation steps). After purifying the PCR product using in-house prepared Sephadex G-50 or G-100 columns (with an exclusion limit of 20 or 25 bp, respectively) no loss of DNA was detected and (MiniFiler™) PCR primers seemed removed, as after the second (Yfiler™) PCR the Yfiler™ alleles were visible (which did not occur without applying the Sephadex columns). Nevertheless, the MiniFiler™ loci became further amplified, indicating that the MiniFiler™ primers were not removed completely. In addition, when using the G-50 and G-100 columns drop-ins occurred more frequently than in the control reactions (Yfiler™ reactions from untreated genomic DNA). As we did not find a good manner to fully dispose of the primers of the first PCR (in 2010), we decided not to continue this research line. However, with the latest experience with the size-selective AMPure® beads purification, as described in Chapter 7 (published as [7]), this line of research may be revisited.

2.5 Repair of DNA damage

DNA repair enzyme cocktails, such as Restorase™ and PreCR™, are developed to repair several kinds of DNA damage (e.g. abasic sites, nicks and thymine dimers) that may block processivity during PCR. Repairing damaged

DNA (as encountered in human identification research) may be advantageous and increase genotyping success. To simulate single strand breaks, a model system was developed using cassettes of synthetic oligos (A.A. Westen, B. Erkamp, C. Vervat, D. Mourik & T. Sijen, unpublished results). Each cassette was designed to represent double-stranded DNA of 110 bp with a single nucleotide gap in each strand. The region between the two gaps varied in length and was either 4, 8, 12, 16 or 20 nt. To achieve such a cassette, four oligos were annealed; two for the sense strand and two for the antisense strand. Both Restorase™ and PreCR™ showed promising results and repaired the templates having a region of 8, 12, 16 or 20 nt between the gaps; both kits could not repair the templates with a region between the gaps of 4 nt, which seems to act as a double strand break (for which is known that these kits cannot repair it). When these kits were used on UV-degraded human DNA and analysed using an SGM Plus™ PCR, variable results were obtained. Sometimes the repaired DNA showed additional alleles compared to unrepaired samples, but other times less alleles could be retrieved. These results are not robust enough to be confident that forensic samples will benefit from use of these repair cocktails. As the amount of sample is often limited, it seems not worthwhile to take the risk that genotyping may turn out less efficient. The results of this study were compared to other methods for the analysis of degraded DNA (standard SGM Plus™ profiling, MiniFiler™ and SNPs) and are described in Chapter 4 (published as [4]).

2.6 Follow-up on the tri-allelic SNP research

In August 2010, the Beijing Genomics Institute (BGI) kindly provided information on the potential tri- and tetra-allelic SNPs from the low-coverage (2-4X) study on 60 CEU, 60 CHB-JPT and 60 YRI samples from the 1000-genomes project. Almost 19.000 potential tri- and tetra-allelic SNPs were assessed, but within the provided dataset not one SNP appeared to be tri-allelic in all three population groups, although a few SNPs showed three alleles for two population groups. The discriminating power was assessed for a combined SNP set comprising the SNPs that were tri-allelic in two population groups and the 10 SNPs that were shown to be tri-allelic in our earlier study (as described in Chapter 3 (published as [3]), and this discriminating power did not reach a value powerful enough for human identification. Thus, we have not further tested the newly discovered tri-allelic SNPs. Since the low-coverage study has now expanded from 180 to 1.092 individuals [153] and will be extended to around 2500 samples in the near future, analysis of the new data may reveal additional SNPs that are tri-allelic in the major population groups worldwide.

2.7 Post-mortem interval determination based on molecular markers

In order to be able to determine whether a body could belong to a missing person, determination of the late post-mortem interval (PMI) could be of great value. Zimmermann et al. [132] analysed the nucleoside content of 5 to 40 year-old museum moth specimens by mass spectrometry. Promising results were obtained showing a time-dependent loss of deoxyguanosine (dG); the dA/dT ratio remained fairly constant, while the dG/dC ratio decreased gradually with time [132]. We performed a pilot study with a similar setup using human samples [154]. Femur and soft tissue samples were obtained from persons that were buried 2 to 40 years earlier. After sampling, the samples were cooled to 4 °C and upon arrival at the NFI directly stored in a -80 °C freezer. DNA was extracted, amplified and analysed according to standard NFI procedures. No signification correlation between DNA profiling success, lengths of amplified fragments and PMI was detected in these samples. After digestion of the DNA, separation of the nucleosides by high-pressure liquid chromatography (HPLC) and detection by tandem mass spectrometry (MS/MS), no decrease in the dG/dC ratio could be demonstrated. It is unknown what caused the differences in results of both studies. To our knowledge, no independent conformation of the findings by Zimmermann et al. [132] have been reported, which may indicate that either the detection method is technically challenging, or the findings do not apply generally. In addition, our sample set may have an unfavourable amount of heterogeneity because the samples were exposed to more variable conditions than the moth samples that were stored in a museum. Thus, we infer that this approach is not usable for late PMI determination in human remains.

Choice of marker type

Autosomal STRs have been the workhorses in human identification and forensic DNA analysis over the last two decades, and (national) DNA databases for missing persons and forensic casework mainly consist of autosomal STR profiles. These STR profiles can be used both for one-to-one comparisons with reference data and for pedigree analysis for which specialised software tools, such as Bonaparte [67,68], have been developed. When DNA is degraded to fragment lengths shorter than the STR amplicons, the resulting DNA profiles will have lower peak heights or allele drop out at the longer loci. To enable analysis of these loci in case of degraded DNA, mini-STRs were developed that have primers closer to the actual STR (to obtain amplicon sizes preferably below 200 bp). With the introduction of five new European standard set

(ESS) markers and the entry of a third commercial company that produces forensic STR kits (Qiagen, since 2010), various new STR kits became available, engineered to have most STRs in the mini- and mid-size range [69]. The diverse kits contain different markers as a mini-STR, and using another kit may retrieve information that was missing upon amplification with the first kit. At the NFI, the AmpFlSTR® NGM™ Kit (AB) [52], was selected for standard use and part of the in-house NGM™ validation is described in Chapter 5 (published as [5]), with focus on the determination of stochastic thresholds, back and forward stutter filters and low template DNA strategies. For specific cases, such as pairwise relationship testing in deficient pedigrees (e.g. grandparent-grandchild and avuncular relationships, or great grandparent-great grandchild and cousins), it can be helpful to type additional autosomal STR markers [70]. Nine of the 13 markers in the Investigator HDplex™ Kit (Qiagen) are uncommon to forensic analyses and traditionally used for bone marrow transplantation monitoring [6,71,72]; we determined Dutch allele frequencies for these loci as described in Chapter 6 (published as [6]). In addition, we calculated that, on average, the power of discrimination per marker is higher for HDplex™ than for NGM™ or Identifiler™, and no linkage disequilibrium was detected between the three kits (Chapter 6 (published as [6])). These additional markers, however, are not present in the current DNA databases and can therefore only be used in direct comparisons.

In case not enough STR information can be recovered from the DNA due to the high degree of degradation, it might still be possible to obtain genotyping information using single nucleotide polymorphisms (SNPs, Box 3) [73,74] and/or deletion-insertion polymorphisms (DIPs or InDels) [75,76,77]. Since only one or few nucleotides are analysed, SNP and DIP amplicons can be smaller than mini-STRs, although many more SNP or DIP markers need to be analysed due to the lower discrimination power per marker (the estimated number of markers required to reach a probability of identity comparable to 12-20 STRs lies between 45 and 65 [78,79,80,81,76]). Other advantages are that SNPs and DIPs are widely spread throughout the genome, possess low mutation rates, do not produce stutter artefacts, have high multiplexing capabilities and are amenable to automation. At the moment, no commercial kit is available to type the sequence information for SNPs (a commercial chip may be available soon [82], but this tool has not yet been tested by independent laboratories). Many different SNP typing techniques exist (Box 3), and although single base extension (SBE) is the most widely used method in forensic settings, there is no consensus on the method or the SNP set to be used between forensic laboratories. Recently, a commercial kit was introduced for the analysis of DIPs (i.e. Investigator® DIPplex™ Kit, Qiagen). Since DIPs are characterised by length-differences (and not sequence differences), they can be analysed in a similar manner as STRs, with a multiplex PCR followed by CE detection, which makes detection and (mixture) analysis easier than for SNPs [76,83]. When used for human identification, both SNPs and DIPs should ideally have similar

Box 3. Single nucleotide polymorphisms (SNPs)

A set of around 50 SNPs (based on the SNPforID assay by Sanchez et al. [73]) was broadly tested by the forensic community (e.g. [155,156,157,158,159,160]) and resulted in the development of an assay named GenPlex™ [161]. This assay was subjected to an inter-laboratory study [162,163], and although this study created support for GenPlex™ in many forensic laboratories, Applied Biosystems unfortunately decided to withdraw the chemistry on which it was based from the market. Several other assays have been developed, most of which are based on a multiplex PCR followed by different methods to detect the SNPs, such as single base extension (SBE) [73], Amplification Refractory Mutation System (ARMS) combined with Universal reporter primers (URP) [164], Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) [165], Invader assays [166], and micro-arrays [167]. Some of these techniques need high (above 25 ng) amounts of template DNA, while other techniques, such as SBE, also work well with DNA inputs of 1 ng or less, which makes them more suitable for human identification in DVI or forensic settings. One such SBE multiplex that is shown to be sensitive (with full profiles down to 78 pg/μL) is based on SNPs that are located inside nucleosomic regions, which were chosen as there is growing evidence that the histone-DNA complexes found in nucleosomes might offer protection from DNA degradation processes [168]. When regarding the average percentage of markers successfully detected, the nucleosome SNPs performed slightly better than the two SNPforID SBE multiplexes, and all these three SNP-based assays performed better than MiniFiler™ when degraded casework samples were tested [168]. Another study that showed a higher percentage of detected alleles for SNP-based assays compared to MiniFiler™, is described in Chapter 4 (published as [4]). The two SNP assays that outperformed MiniFiler™ were the GenPlex™ assay (based on bi-allelic SNPs) and an SBE assay based on the tri-allelic SNPs as described in Chapter 3 (published as [3]). Tri-allelic SNPs can assist in the recognition of mixed samples due to the third allele that can be present, while for bi-allelic SNPs only a change in peak height ratios can give an indication of a mixture (and as a result of the unequal peak heights for the different nucleotide-dyes in SBE analyses, ratio changes are more difficult to recognise than for STRs) [3]. Although it would be interesting to increase the number of tri-allelic SNPs available for human identification and forensic purposes [3,169,170], their discovery has proven difficult (box 2.6) as they often have a low frequency and are then regarded to be sequencing errors.

allele frequencies for different population groups. If (huge) variation between groups is present, they can be interesting as ancestry informative markers [3,84,85,86,87,88]. Due to their high mutational stability (when compared to STRs), SNPs and DIPs can also be informative (additional) markers in kinship analysis and (when several thousands SNPs are used) in distant pairwise relationship tests [70,83]. The use of thousands of SNPs (each with a low minor allele frequency) has also been proposed as a method to effectively identify the number of contributors in complex mixtures with multiple (up to 10) contributors [89].

Other proposed markers combine multiple polymorphisms. Haplotype blocks, for instance, contain multiple SNPs that are tightly linked, and when several haplotypes are observed at the population level, a haploblock has a higher discrimination power than the individual SNPs within the block [90]. SNP-STRs, which refers to sequence differences (SNPs) within STRs, can be determined by, for instance, (electrospray ionisation) mass spectrometry (ESI-)MS [91,92] or (next generation) sequencing (by for instance Illumina GAIIx short-read technology [93]). With ESI-MS, additional polymorphisms were identified in 11 of the 13 CODIS core STRs, thereby increasing the discrimination power significantly [92]. Next generation sequencing faced some difficulties in analysing the longer alleles as most reads had a maximum read length of 150 bp [93], but next generation sequencing methods hold great promise for mixture analysis. A big advantage of both techniques is that the obtained data are backwards compatible with existing STR information that is present in the national DNA databases, as the repeat lengths of the STRs can be inferred (next to the additional SNP sequence information). A disadvantage, however, is that they require specialised instruments that are not common in forensic laboratories. Another combined marker is the DIP-STR, which consists of a DIP near an STR (within 500 bp distance) [94]. This marker type has been developed for the analysis of minor components in DNA mixtures by using allele-specific amplifications of DIP-STR haplotypes [94]. An advantage of these markers is that they are sex-independent (unlike Y-STRs which are frequently used to examine male minor components when the major is female); a disadvantage is that relatively large sizes for the amplicons are needed, which makes them less suitable for degraded samples.

Next to autosomal DNA testing, analysis of Y-chromosomal, X-chromosomal and mitochondrial DNA (mtDNA) is of interest for human identity testing. Y-chromosomal markers are inherited from father to son, and therefore provide patrilineal information. Deep ancestral relationships can be analysed with Y-SNPs, as they have very low mutation rates. The Y-STRs currently used in forensic genetics, provide information on (more recent) relationships in the paternal lineage, while the recently described rapidly mutating Y-STRs seem to provide the possibility to distinguish between closely related males [95,96]. Both these types of Y-STRs can provide valuable information in paternity testing and in forensic cases involving a male component in mixtures with

an overabundance of female DNA. In contrast to the Y-chromosome that is inherited by sons, daughters inherit the X-chromosome of their fathers, which make X-STRs interesting markers for the analysis of, for instance, disputed father-daughter relationships [97,98]. Children always inherit their mitochondrial DNA from their mothers, and therefore mtDNA provides matrilineal information. Since several hundreds of copies of the mtDNA are present per cell (compared to two copies for the autosomes), mtDNA analysis is highly sensitive. Telogen hairs lack the hair roots that comprise nuclei and nuclear DNA, but still contain mtDNA up to at least 12 cm from the scalp (L. Clarisse & T. Sijen, unpublished results). Therefore, mtDNA is mostly applied on hairs, but it can be valuable for maternal lineage testing or analysis of any sample type having extremely degraded DNA. The analysis of degraded DNA (from hairs or other specimens) is even further improved when mini-amplicons are used for the mtDNA analysis [99,100].

All these different markers have their specific niche in which they can produce valuable results. Except for the core STRs, none of these markers are stored in the (national) DNA databases (yet), and thus for now their use is limited to one-to-one comparisons.

Other options for human identification beside comparative DNA analysis

Next to comparative DNA analysis, the main characteristics for human identification are fingerprints, dental status, facial recognition and body marks (such as scars, tattoos and medial implants) [101], but when reference data are absent, body parts are missing, or bodies are beyond recognition, these methods fail. When skeletal elements are present, physical anthropologists may be able to determine the sex and estimate the age at death and stature of the person, together with possible diseases he or she has gone through. For example, in an airplane crash in which several members of one family died, this information might help to discriminate between siblings of the same gender, but with different ages (who are not discernable based on their STR profiles without reference samples for direct comparison). Physical anthropology may also aid a facial reconstruction, which can be further supported by DNA-based information from the analysis of SNPs that are indicative of externally visible characteristics such as eye colour, hair colour and skin colour, and for which several multiplexes have been proposed in forensic literature [102,103,104,105,106,107,108]. Biogeographic ancestry markers cannot be used for this purpose, as they only pertain to geography and not to physical appearance [109]. Other externally visible characteristics that are being investigated include freckles, hair morphology, boldness, body height and facial

morphology [110], but further developments are needed before these can be used in human identification research. Especially body height (which is in addition strongly affected by non-genetic nurture effects) and facial morphology seem influenced by many genes [111,112] and can probably not be predicted by a few markers. Technologies that can detect many SNPs in one sample, such as micro-arrays and next generation sequencing, have the drawback that they generally need high amounts of input DNA (which is usually not present in human identification or forensic samples). Though, also in this area advances are being made as exemplified by the Identitas v1 Forensic Chip, which allows simultaneous analysis of appearance traits, biogeographic ancestry, relatedness and gender by analysing a total of 201,173 autosomal, X-chromosomal, Y-chromosomal and mtDNA SNPs, and for which positive results (genotype call rate >90 %) were reported for a sample with an input of 175 pg [82].

A conceptually difficult task is to differentiate between monozygotic twins, as their appearance is generally similar and their DNA sequence is virtually equal. Differences between fingerprints [113] and earprints [114] of monozygotic twins have been described and can be used when the decomposition status of the body allows collecting them and references for direct comparison are available (though earprints are probably more interesting for forensic burglary cases). Although the genotype of monozygotic twins is very similar, copy number variations between their genotypes were detected [115,116,117], which makes these markers interesting for further examination. Epigenetic differences in the genomic distribution of 5-methylcytosine DNA and histone modifications between monozygotic twins have been shown to accumulate with age (and to affect gene-expression) [118,119], and might be used to discriminate between them. Both for CNVs and epigenetic markers, additional research into the stability for these markers in different tissue types needs to be performed [120], as in forensics DNA from a buccal swab is compared to that derived from, for instance, a bloodstain. Another potential research topic focuses on personal variation for immunoglobulins produced during immune responses. Evidently, individual (disease) history determines to which antigens a person was exposed, but the most prominent source of variation derives from the biological process by which memory cells are generated from naïve B-lymphocytes. This involves the stochastic recombination of V, D, and J elements present on the immunoglobulin heavy (IgH) chain locus, which takes place in maturing B-cells. This recombination process results in extreme variation at a 45 bp region and this region can serve as a marker for human identification [121]. Interestingly, both disease history and VDJ recombination are believed to have inter-person variation, even between monozygotic twins. All these DNA-based markers clearly need further research and optimisation of techniques to enable analysis of low template amounts and/or degraded DNA, but they provide interesting starting points for research on monozygotic twin discrimination. Isotope ratios are non-DNA markers that could potentially be used to discriminate between monozygotic twins

that do not live in the same area. Drinking water and eating patterns have an influence on hydrogen ($\delta^{2}H$) and oxygen ($\delta^{18}O$) isotope ratios of organic matter, and the $\delta^{2}H$ and $\delta^{18}O$ values in precipitation and tap water vary along geographic gradients [122]. When, for example, hairs are being analysed, information can be obtained about the area in which someone has lived recently [122]. In contrast, bones are formed much slower and might present information about the living area roughly ten years before death, and teeth about the area in which someone has grown up (as dental crowns (except for the third molars) are formed by the age of 8 years) [123]. This information might assist human identification.

Another area of research focuses on age prediction of an unidentified person (deceased victim or perpetrator of a crime). Physical anthropology (combined with odontology, counting of tooth cementum annulations [124] and microscopic analysis of the bone structure [125]) can give a broad estimation of the age at death (depending on the number of markers that could be assessed). In addition, other methods have been explored in search for more accurate or complementary predictions. Age-dependent changes in telomere repeat length and accumulation of mutations and deletions in the mtDNA have been proposed as markers, but did not show the accuracy needed for human identification or forensic purposes (e.g. a standard deviation of 22 years for the telomere approach by Karlsson et al. [126,127]. The abundance of sjTREC molecules (single joint T-cell receptor excision circles) can act as an age indicator from blood sources, but the prediction shows a standard error of ± 9 years [128]. Thus far, the most promising DNA-based age predictions were derived from a model based on the analysis of three DNA methylation markers that showed age dependent changes in saliva samples from donors between 18 and 70 years old, with an average accuracy of 5.2 years [129]. The method used in this paper (bisulfite conversion before PCR amplification and detection by pyrosequencing) though, requires high amounts of template DNA and needs to be redesigned before it can be used in human identification analyses. When teeth of the deceased are available for examination, chemical analysis of the tooth dentin by aspartic acid racemisation can give age at death estimations with an overall absolute error of 5.4 ± 4.2 years, and even better estimations can be obtained from radiocarbon (^{14}C) analyses of the enamel with an overall absolute error of 1.0 ± 0.6 years [130]. Thus, advances in age at death estimations are being made and it might be worthwhile to assess the possibilities of a combinatorial approach (if enough sample is available) to obtain higher age estimation accuracies.

Another time-related aspect that can be of importance for human identification and forensic research is the post-mortem interval (PMI). Numerous approaches for PMI determination have been suggested of which most relate to the first hours or days after death (e.g. based on body temperature, metabolic changes or mRNA stability). Entomology can be used for PMI determination of weeks to months after death. However, for human identification purposes especially the longer time periods

in the range of months to years are of interest. Although DNA degrades with time, several studies have shown that there is no usable correlation between the amount of DNA or the DNA fragment length and PMI (e.g. Box 2.7 and [131]), which is probably due to the huge influence of environmental factors. A study into the nucleoside content of 5 to 40 year-old museum moth specimens showed promising results with a time-dependent loss of deoxyguanosine, measured by mass spectrometry [132]. We performed a pilot study with a similar setup using human tissue samples of 2 to 40 years old, but did not detect the decrease in deoxyguanosine as described in the study by Zimmermann et al. (Box 2.7). Thus far, no reliable method for late PMI determination has been described and further research into this subject is needed. A related forensic subject estimates the time since deposition of biological evidence. Many studies have examined the ageing of bloodstains using biochemical or physical methods [133]. For instance, UV-VIS spectrophotometric analysis and hyperspectral imaging of dried bloodstains showed a spectral shift with increasing stain age, and permitted to distinguish between bloodstains that were deposited minutes, hours, days and weeks prior to analysis [134,135,136,137]. The proposed methods are moving from a fully experimental phase to tests on simulated crime scenes, and by using a reference dataset the median relative error is reduced to 13 %. The effects of environmental factors (temperature and humidity) are being assessed, but when unknown it is possible to determine the order of formation of bloodstains [133]. Thus, these non-invasive/non-contact methods are moving slowly towards forensic practise. Not only time since deposition, but also clock time at the moment of deposition might provide forensically relevant information. A pilot study showed that the time of day (or night) that a bloodstain was deposited could be estimated in the order of about 4 to 5 hours, based on the 24-hour concentration fluctuations of circadian hormones [138]. For this study melatonin and cortisol were tested in fresh and aged bloodstains, and evaluation of additional circadian biomarkers in several body fluids might hold promise for a more accurate system to determine the time of deposition.

Should we do everything we technically can?

The introduction of novel markers and innovative technologies will bring new possibilities and opportunities for human identification. However, these advances also pose ethical and legal questions. For instance, when the analysis of large SNP arrays and whole genome sequencing becomes possible on low amounts of template DNA (such as usually present in human identification and forensic samples), possibilities open up to not only analyse the commonly accepted markers for identification, but also the predisposition for diseases and/or behavioural traits that may influence criminal behaviour. In some cases in the US and the UK, in court the defence has asked for

behavioural genetic analyses, which (in combination with other evidence) has led to a reduction of sentence, though not to acquittal, in a few cases. Although it is technically possible to type the behavioural genetic markers that are known, many factors (such as the effects of unknown genes, the interactions between genes and influences of nurture) remain unknown and other aspects (such as differences between population groups) may be socially unacceptable (regarded racism) [139]. These aspects make this kind of research delicate and complex at the very least.

When markers became known for the analysis of someone's biogeographic ancestry, questions were raised on whether this information would be used rightfully to focus investigations and not misused to justify the targeting of certain racial groups [110]. In fact, an example of the opposite situation has been obtained in the (Dutch) case of the murder on Marianne Vaatstra, where asylum seekers (from Northern Africa and the Middle East) were initially suspected of the crime, while ancestry information pointed towards a perpetrator of Western European descent (and later on indeed a Caucasian suspect was matched to the evidentiary trace profiles). The Netherlands is currently the only country in which determination of biogeographic ancestry and externally visible traits (gender and eye-colour, at present) is explicitly allowed by law. Another research possibility that has recently been allowed by Dutch law is familial searching. In the aforementioned murder case, such a familial search within the national forensic DNA database did not provide new investigative leads, and based on tactic information the decision was made to perform a mass DNA screening (with the aim to find investigative leads towards the perpetrator through participating family members). 8080 men, who lived in the vicinity of the crime scene at that time, were asked to voluntarily participate in this research and the high attendance rate of 89 % shows the social involvement of people in that area. Based on a Y-chromosomal and autosomal match with biological material found on the crime scene, this research has led to the arrest of a suspect (and his subsequent confession, although the case has not yet been closed at the time of writing) thirteen years after the crime was committed, which shows the impact that changes in legislation can have.

The above-described case has renewed the discussion on whether everyone (in the Netherlands) should be in the national DNA database. Technically speaking this is possible since from almost every child (around 500 births per day [140]) FTA blood cards are sampled to test a variety of genetic diseases, and (in theory) it should be possible to collect one additional card for DNA databasing-purposes. Proponents of this idea bring forward that more crimes could be solved and that there is no reason for fear if you are innocent. Opponents refute that even if you are innocent there is a chance that your DNA matches the DNA found on a crime scene, which may put you in a position in which you need to prove your innocence (which is in sharp contrast with the presumption of innocence [141]). A similar situation occurs when during crime scene investigations unintentionally non-crime-related pieces of evidence are

collected as well. Another argument is that the people carrying out the research for the DNA database have access to your DNA without your consent, which could be seen as an intrusion of personal privacy [142]. Next to the considerable costs entailed with establishing a national DNA database, aspects like the storage time of such samples need to be considered; should they only be used to produce an STR profile and be destroyed afterwards, or should the samples be stored to make an upgrade to new markers possible in the future (the current legal storage time for certain samples in the DNA database is 80 years)? When the latter option should be chosen, misuse of such a database by future (malicious) politicians and health insurance companies might be a risk. On top of that, currently, the DNA profiles in the national DNA database are being exchanged daily with other European countries under the Prüm Convention, and it should be considered whether all DNA profiles are exchanged (which makes them also known to international authorities) or only those being crime-related.

In addition to the ethical and legal questions that may arise from marker development and technical advancements in DNA research, there can also be questions from a criminalistic point of view. Although techniques may become so sensitive that reliable DNA profiles can be made from single (or few) cells, an important question remains to be answered: "What is the criminalistic value of a single cell on a crime scene?" The mere fact that someone's DNA is found on a crime-related objected does not make him or her the perpetrator of the crime; biological material may be left at the scene before the crime was committed, or may have been deposited indirectly. In forensic literature, the latter is termed secondary (or even tertiary) transfer [143, 144]. Based on, for instance, presumptive testing or mRNA profiling, the cellular source of a sample can be elucidated [145], and it is evident that the finding of certain cell types (e.g. semen) may be more incriminating than others. Thus, when the sensitivity of forensic analyses is raised to the single cell level (assuming that advances have been such that single cell typing does not suffer from quality loss), the holistic or integrated interpretation of the evidence may become highly complex. Forensic scientists have the responsibility to not only point out the possibilities, but also the limitations of the available techniques irrespective whether these reside at the technical or interpretation level.

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Epilogue

Summary

The research described in this thesis was aimed at the development of additional or alternative methods to extract information from a person's DNA when standard DNA methodology is not sufficient for human identification.

When collecting samples for human identification, it is important to minimise the risk of (cross-) contamination and to keep the samples in optimal condition, especially when working under mass disaster circumstances. In **Chapter 1** we proposed standard operating procedures for the excision of femur and rib samples and the extraction of molars or teeth. In addition, practical advice was given on 1) inexpensive and simple solutions for excision tools, such as the use of a hacksaw with disposable blades instead of an amputation saw that becomes blunt from the aggressive decontamination fluid, 2) preparation of decontamination fluid to clean instruments and body locations before sample excision in order to prevent cross-contamination from DNA from other victims, and 3) preservation of the samples, for instance by cooling the sample containers in ice-water baths, until the samples are genotyped in dedicated laboratories.

In **Chapter 2** we assessed whether we could obtain more short tandem repeat (STR) genotyping information from a sample with low DNA content by increasing the capillary electrophoresis (CE) injection voltage or extending the CE injection time. Changing the CE settings from 3 kV for 10 s to 9 kV for 15 s led to the best results, with good peak morphology, relatively low baseline noise and a six-fold increase in signal strength, resulting in the detection of additional alleles. Interestingly, compared to an increased number of PCR cycles (28 + 6 cycles, for SGM Plus™), the percentage of detected alleles for samples with 8 or 16 pg DNA input were similar for both methods, while the peak heights upon boosted injection were much lower. These lower peak heights are practical for the analysis of unequal mixtures, as these enable improvement of the genotyping results from the minor component, without over-amplifying or overloading the major component (as would be the case with performing additional PCR cycles). This method is regarded as a low template DNA technique and it is therefore recommended to perform replicate analyses. The method has been accredited for casework in our laboratory.

When not all STR information can be retrieved from a sample due to degradation of the DNA, different marker types that allow analysis in smaller amplicons can be used for identification. Short insertion/deletion polymorphisms and single nucleotide polymorphisms (SNPs) can be used for this purpose, but they are usually bi-allelic and thereby less efficient in detecting mixtures than STRs. In **Chapter 3** we explored the possibility of using a specific subclass of SNPs that exhibits three alleles (instead of the usual two) to improve genotyping results. We developed an algorithm to find these tri-allelic SNP markers in the NCBI SNP database and developed three multiplexes

that analyse a total of 16 tri-allelic SNPs. The tri-allelic SNPs showed the possibility to provide valuable genotyping information for samples in which the higher molecular weight STR markers are not detected anymore. In addition, mixtures with ratios between 8 : 1 and 1 : 8 could be successfully detected by the presence of a third allele on a locus. We determined allele frequencies for the tri-allelic SNP candidates in 153 Dutch and 111 Netherlands Antilles donors. Since not all candidates showed the three NCBI-described alleles in these two sample sets we searched for these alleles in 59 (YCC panel) samples from worldwide populations, but did not find them. Of the 16 candidate SNPs in the multiplexes 11 were detected to be tri-allelic, of which two were only tri-allelic in the Netherlands Antilles (and (South) African) and not in the Dutch samples. Therefore, certain tri-allelic SNPs seem interesting as ancestry informative markers as well.

In **Chapter 4** a comparison was made between several techniques to genotype artificially degraded (UV-irradiated) DNA. The following techniques were compared: STR genotyping using SGM Plus™ (which was the standard kit at the NFI at that time) or MiniFiler™ (with reduced size amplicons), DNA repair with the enzyme cocktails PreCR™ or Restorase™ followed by SGM Plus™, and SNP genotyping using bi-allelic (GenPlex™) or tri-allelic SNPs (as described in Chapter 3). For severely degraded samples, the average percentage of detected alleles after PreCR™ or Restorase™ DNA repair was on average slightly higher than for SGM Plus™ alone (15 %, 23 % and 13 %, respectively). However, the results were not consistent and for a single sample the results could be substantially worse after the repair procedure than without. MiniFiler™ showed much better results with an average of 60 % detected STR alleles. Even higher percentages were detected for tri-allelic SNPs (73 %) and bi-allelic SNPs (88 %). These results showed that the use of reduced size amplicons for mini-STRs and SNPs is effective for genotyping (severely) degraded DNA.

During in-house validation of the AmpF*l*STR® NGM™ kit we evaluated, amongst others, the aspects as described in **Chapter 5**. We found that the increased sensitivity, compared to previous AmpF*l*STR® kits, required elevation of the stochastic threshold. When regarding various CE injection settings, values of 175 rfu for 3 kV/5 s, 300 rfu for 3 kV/10s and 400 rfu for 3 kV/15 s are required to have 99 % of the single alleles on heterozygous loci below the stochastic threshold. Stutter peak heights appeared not to be normally distributed and therefore we determined the locus-specific stutter ratio thresholds empirically using the 99th percentile approach. Based on 2085 DNA profiles of Dutch volunteers, thirteen -1 stutter ratio filters could be lowered by up to 1.79 % compared to the ones provided by Applied Biosystems, and two had to be elevated slightly (with a maximum of 0.06 %). All loci showed +1 stutter peaks when high DNA inputs were used, and they were seen for locus D22S1045 with low inputs as well. For all loci a general +1 stutter filter was set at 2.50 %, except for D22S1045 for which it was determined to be 7.27 %. For low template DNA analysis 9 kV/10 s CE injection

settings can be used, and only for the very low DNA inputs (<31 pg) the use of 29 + 5 cycles is recommended. We advise to determine the abovementioned parameters in-house before introducing a new kit for (standard use in) forensic casework, in order to optimise the analyses of complex mixtures and low template DNA samples.

The Investigator™ HDplex™ kit contains nine STRs that are additional to the commonly used forensic markers. These STRs can be used to increase the discrimination power, which may for instance be useful in complex kinship analyses and when alleles are missing due to degradation of the DNA. In **Chapter 6** we evaluated whether the 30 markers in NGM™, Identifiler™ and HDplex™ (from which 17 syntenic STR pairs can be formed) can be regarded as independent. Based on 335 Dutch reference DNA profiles for these three kits, no linkage disequilibrium could be detected and we inferred that the product rule can be applied for profile probability calculations in unrelated individuals. Using five three-generation CEPH pedigrees we studied linkage between the syntenic STRs by determining their recombination fractions, and we compared these to the physical and genetic distances between the markers. The presence of (loose) physical linkage, as found for some of the assessed marker pairs, may influence the interpretation of genotyping data from (closely) related individuals. For an explanation on which type of pedigree could be affected by linkage and how to account for this effect using recombination fractions (as determined in our study), we referred to a paper by Gill et al. (Forensic Sci Int Genet 6:477-486, 2011). HDplex™ has many non-overlapping markers with NGM™ and Identifiler™, and the power of discrimination per marker is on average higher than for the other kits, which makes HDplex™ a good complementary STR kit that may aid complex kinship and degraded DNA analyses.

DNA profiles from degraded samples often suffer from information loss at the longer STR loci. Sensitising the reactions by performing additional PCR cycles or increasing the CE injection settings carries the risk of over-amplifying or overloading the shorter loci. In **Chapter 7** we explored whether the use of a size-selective post-PCR purification method, based on AMPure® XP beads, could increase the information obtained from the longer STR loci in degraded samples. This method was compared to unselective purification (DTR gel filtration) and no purification of the PCR products. Besides a set of 39 differently and serially degraded single source samples, unequal mixtures of degraded DNAs in the ratios 1:5, 1:10 and 1:15 (with $n = 5$ per mixture ratio) were analysed in order to extract more genotyping information for the minor contributor without overloading the major component at the shorter loci. DTR gel filtration resulted in an approximately two-fold increase in allele peak heights for all loci, while AMPure® purification showed a three- to fourfold increase at the longer loci and no increase at the shorter loci. Both post-PCR purification methods showed more detected alleles than the non-purified samples, with, on average, slightly more detected alleles (especially on the longer loci) after AMPure® purification.

In **Chapter 8**, several aspects of DNA-based human identification were discussed, with emphasis on low quality and/or quantity of the DNA, and choices both at a technical and an ethical level were reviewed. The focus of this general discussion lay on (disaster) victim identification, although human identification also applies to relationship testing and forensic analyses.

Curriculum vitae

Antoinette-Andrea (Toineke) Westen was born in Leiderdorp, the Netherlands on the 1st of July, 1980. She attended high school (Gymnasium-ß) at the R.K. Scholengemeenschap Alverna in Leiden and graduated in 1998. In the same year she started with her course on Biomedical Sciences at Leiden University. She did a combined seven-month research traineeship at the centres for Audiology and Clinical Genetics Leiden (KGCL), both connected to the Leiden University Medical Centre (LUMC). During this internship she set up a clinical trial and DNA-diagnostic method to link a specific type of hereditary hearing loss to defects in the connexin gene. An eleven-month research traineeship on improvement of the working of cochlear implants was performed at the LUMC department of Ear, Nose and Throat Surgery (ENT). Here, she developed a surgical method for implanting cochlear implants in guinea pigs (a common animal model in this field) and measured their nerve fibre responses. She graduated in August 2002. Thereafter, she started a course on Industrial Design at the Technical University Eindhoven, and she received her propaedeutic certificate in 2003.

In 2003, she accepted a position as scientist at the ENT department of the LUMC to proceed with the research on cochlear implants, where she worked until 2005 under supervision of Dr Jeroen J. Briaire and Prof. Dr Johan H.M. Frijns. In the years 2004 and 2005, she additionally performed physical anthropological research on human remains from the cloister garth of the Koningsveld priory in the city of Delft at the LUMC department of Anatomy and Embryology, under supervision of W.J. (Mike) Groen and Prof. Dr George J.R. Maat.

Since 2006 Toineke is employed at the Netherlands Forensic Institute as a scientist in the R&D group of the Human biological traces department. Part of the results that she obtained, are described within this thesis. Most of this work was supervised by Dr Titia Sijen and chaperoned by Prof. Dr Peter de Knijff. In 2009 she was rewarded with a Young Investigators Award during the 6th ISABS conference in Split, Croatia. After completing her PhD, she will continue as a post-doctoral scientist within this R&D group.

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Nederlandse samenvatting

Humane identificatie & forensische analyses van afgebroken of minimale hoeveelheden DNA

Humane identificatie kan nodig zijn in situaties zoals (massa)rampen, terroristische aanvallen, vermiste personen en forensisch onderzoek. Deze identificatie kan gebaseerd worden op lichaamskarakteristieken (bijvoorbeeld gezichtsherkenning, vingerafdrukken en gebitsstatus) of DNA bewijs. Vooral deze laatste methode is zeer bruikbaar gebleken voor lichamen die niet meer compleet of onherkenbaar zijn.

Om een DNA profiel van een persoon te kunnen maken, moet het DNA van voldoende kwaliteit en kwantiteit zijn. Wanneer de tijd tussen het intreden van de dood en het ontdekken van het lichaam lang is en/of het lichaam is blootgesteld aan extreme omstandigheden (zoals vuur, onderwater, lucht met een hoge vochtigheidsgraad, aarde met een hoge zuurgraad, chemische middelen, etc.) kan het DNA sterk worden afgebroken of sterk teruglopen in hoeveelheid.

Het onderzoek dat in dit proefschrift staat beschreven, is gericht op het ontwikkelen van onderzoeksmethoden voor humane identificatiezaken waarin het DNA slecht van kwaliteit en/of laag in kwantiteit is. Het doel van dit onderzoek is om tot additionele of alternatieve methoden te komen om informatie vanuit iemands DNA te halen wanneer standaard DNA typeringsmethoden niet voldoen voor humane identificatie.

De DNA profielen die worden gebruikt bij humane identificatie zijn meestal gebaseerd op "short tandem repeats" (*STRs*): korte stukjes DNA die in een variabel aantal achter elkaar herhaald worden. Deze STR varianten (ook wel *allelen* genoemd; weergegeven als pieken in het DNA profiel) verschillen tussen personen. Een combinatie van allelen voor verschillende *markers* (locaties op het DNA die worden onderzocht) vormt een DNA profiel dat (vrijwel) uniek is per persoon wanneer voldoende markers worden gebruikt. Vooral in omstandigheden zoals die zich voordoen na een massaramp, worden lichamen (of monsters die daarvan genomen zijn) gemakkelijk gecontamineerd met DNA van andere slachtoffers. Om een DNA profiel van een zo hoog mogelijke kwaliteit te verkrijgen, is het van belang dat de uitgenomen monsters voor DNA onderzoek worden gehanteerd en opgeslagen onder de meest optimale condities, totdat ze worden geanalyseerd in een gespecialiseerd DNA laboratorium. In **hoofdstuk 1** is hiervoor een standaard werkvoorschrift beschreven. Instructies zijn gegeven voor het uitnemen van monsters uit het dijbeen, uit een rib of van tanden en kiezen. Daarnaast wordt praktisch advies gegeven voor goedkoop en eenvoudig gereedschap om monsters mee uit te nemen, voor het bereiden van decontaminatievloeistof en voor het preserveren van de monsters.

Om een DNA profiel te kunnen maken, worden specifieke markergebieden van het DNA vermenigvuldigd door middel van een "polymerase chain reaction" (*PCR*).

Wanneer de kwantiteit van het DNA laag is, hebben DNA profielen lagere piekhoogtes en kunnen stochastische vermenigvuldigingseffecten optreden, zoals disbalans in piekhoogtes, het uitvallen van allelen en/of markers (wat resulteert in incomplete DNA profielen) en het detecteren van extra allelen of verhoogde stotterpieken (dit zijn artefacten die lijken op echte allelen). Deze effecten bemoeilijken de interpretatie van de DNA profielen en kunnen de identificatie van een persoon daardoor belemmeren. Om meer informatie over de DNA donor te verkrijgen, werd een techniek ontwikkeld om de DNA detectie (die plaatsvindt door middel van capillaire electroforese (*CE*)) gevoeliger te maken en deze is beschreven in **hoofdstuk 2**. Deze techniek is gebaseerd op het verhogen van het injectievoltage en het verlengen van de injectietijd tijdens CE om daarmee hogere piekhoogtes en meer informatie te verkrijgen uit DNA monsters van één of meerdere (ongelijk gemengde) donoren. De beste resultaten werden verkregen na de CE instellingen te hebben veranderd van 3 kV voor 10 seconden naar 9 kV voor 15 seconden. Met deze instellingen behielden de signalen een mooie piekvorm, ontstond relatief weinig ruis in de basislijn, waren de pieken zesmaal hoger en werden meer allelen gedetecteerd. Deze methode is universeel toepasbaar op diverse DNA marker systemen, die gebruikt worden in forensische laboratoria. Aangezien de methode gebruik maakt van het overgebleven deel van het PCR product (dat anders enige tijd na standaard DNA analyse zou worden weggegooid) hoeft er geen extra DNA extract te worden verbruikt. De methode wordt gezien als een "*low template DNA*" techniek, een techniek waarmee DNA profielen worden gemaakt van zeer weinig DNA, en daarom wordt aanbevolen om meerdere onafhankelijke analyses uit te voeren. Deze techniek is geaccrediteerd voor gebruik in zaakwerk binnen het Nederlands Forensisch Instituut (NFI).

De *amplicons* (DNA fragmenten die tijdens de PCR worden vermenigvuldigd) van de STR kits die gebruikt werden in het eerste gedeelte van dit proefschrift variëren in lengte van 100 tot ongeveer 400 *basenparen* (basen zijn de bouwstenen van het DNA). Wanneer de kwaliteit van het DNA door (sterke) afbraak laag is, kunnen de DNA fragmenten korter worden dan sommige van de STR amplicons. In zulke gevallen zullen de pieken die de langere STRs representeren in het DNA profiel lager worden of zelfs afwezig zijn. Een ander soort DNA marker is de "single nucleotide polymorphism" (*SNP*). SNPs die worden toegepast bij humane identificatie hebben meestal ampliconlengtes van 55 tot ongeveer 115 basenparen, wat hen interessant maakt voor de analyse van afgebroken DNA. In **hoofdstuk 3** is het onderzoek beschreven aan een speciale subklasse van SNPs die drie verschillende allelen kunnen hebben (in plaats van de gebruikelijke twee). Deze eigenschap maakt hen zeer interessant voor toepassing in humane identificatie en forensisch onderzoek, omdat de detectie van mengsels (die kunnen worden herkend door de aanwezigheid van een derde allel binnen één marker) veel gemakkelijker is voor tri-allelische dan voor bi-allelische SNPs (die alleen herkend kunnen worden aan verschillen in de balans tussen

piekhoogtes). Een zoekalgoritme werd ontwikkeld om tri-allelische SNPs te kunnen vinden in de NCBI SNP databank. Vervolgens werden drie *multiplex-assays* (testen waarin meerdere markers tegelijkertijd kunnen worden vermenigvuldig door middel van PCR) opgezet om in totaal 16 SNPs te kunnen analyseren. Met behulp van deze assays bleek waardevolle DNA informatie verzameld te kunnen worden, wanneer de langere STR markers niet meer konden worden gedetecteerd (door afbraak van het DNA). Daarnaast konden mengsels in de verhoudingen 8 : 1 tot 1 : 8 succesvol worden herkend door de aanwezigheid van een derde allel op één marker. Allelfrequenties voor de tri-allelische SNP-kandidaten werden bepaald voor 153 Nederlandse en 111 Nederlands-Antilliaanse donoren. Omdat in deze twee populaties niet voor alle kandidaat-SNPs de drie allelen (zoals beschreven in de NCBI SNP databank) werden gevonden, is hier naar gezocht in 59 monsters van wereldwijde populaties, maar dit bleek tevergeefs. Van de 16 kandidaat-SNPs werden er 11 aangemerkt als tri-allelisch, waarvan twee alleen tri-allelisch waren in de Nederlands-Antilliaanse (en (Zuid-) Afrikaanse) en niet in de Nederlandse monsters. Deze laatste vinding maakt bepaalde tri-allelische SNPs ook interessant als markers voor geografische herkomstbepaling.

In **hoofdstuk 4** werd een vergelijking gemaakt tussen een aantal verschillende technieken om artificieel afgebroken (UV-bestraald) DNA te analyseren. De volgende technieken werden met elkaar vergeleken: STR analyse met behulp van SGM Plus™ (de standaard multiplex-assay die op dat moment op het NFI gebruikt werd) of MiniFiler™ (een STR assay met verkorte amplicons), DNA reparatie door gebruik van de enzymcocktails PreCR of Restorase gevolgd door SGM Plus™ en SNP analyse met behulp van bi-allelische (GenPlex™) of tri-allelische SNPs (zoals beschreven in hoofdstuk 3). Voor sterk afgebroken DNA monsters lag het percentage gedetecteerde allelen na PreCR™ of Restorase™ DNA reparatie gemiddeld iets hoger dan voor SGM Plus™ alleen (respectievelijk 15 %, 23 % en 13 %). De resultaten waren echter niet consistent en voor een afzonderlijk monster konden de resultaten soms aanzienlijk slechter zijn na de reparatieprocedure dan zonder reparatie. MiniFiler™ gaf veel betere resultaten met een gemiddeld percentage gedetecteerde STR allelen van 60 %. Nog hogere percentages werden gedetecteerd voor tri-allelische SNPs (73 %) en bi-allelische SNPs (88 %). Deze resultaten tonen dat het gebruik van verkorte amplicons voor mini-STRs en SNPs effectief is voor het analyseren van (sterk) afgebroken DNA.

Nadat de Europese Raad besloten had om vijf extra STR markers toe te voegen aan de Europese standaard STR set, werden nieuwe STR assays met 15 STRs of meer ontwikkeld door verschillende bedrijven. In deze kits werden zo veel mogelijk mini-STRs (met ampliconlengtes vanaf 70 basenparen) opgenomen om meer informatie te kunnen verkrijgen uit afgebroken DNA; de gevoeligheid van de assays werd verhoogd door het gebruik van geoptimaliseerde buffers en één of meer extra PCR vermenigvuldigingscycli. Het NFI besloot om met de AmpF*l*STR® NGM™ assay te gaan werken. Deze assay werd vervolgens intern gevalideerd en specifieke aspecten

van deze validatie staan beschreven in **hoofdstuk 5**. Wanneer een DNA profiel allelen bevat met piekhoogtes onder de *stochastische drempelwaarde*, moet men rekening houden met het uitvallen van allelen en stochastische vermenigvuldigingseffecten. Deze stochastische drempelwaarde is bepaald voor gebruik bij verschillende CE instellingen. Daarnaast is per marker bepaald op welke waarde de filters ingesteld moesten worden om tenminste 99 % van de stotterpieken (één van de stochastische vermenigvuldigingseffecten) weg te filteren, op basis van 2085 DNA profielen van Nederlandse vrijwilligers. Voor de analyse van low template DNA bleek een CE instelling van 9 kV voor 10 seconden optimaal te zijn en alleen voor zeer lage DNA starthoeveelheden (minder dan 31 picogram) wordt het gebruik van extra PCR vermenigvuldigingscycli aanbevolen. Wij adviseren om voornoemde parameters per laboratorium vast te stellen, voordat een nieuwe assay wordt geïntroduceerd als (standaard) assay voor forensisch zaakwerk, om de analyse van complexe mengsels en low template DNA te optimaliseren. De voor NGM™ bepaalde stotterfilters en stochastische drempelwaarden zijn binnen het NFI ingevoerd voor al het zaakwerk sinds 2011.

Soms hebben de 15 STRs die aanwezig zijn in de huidige STR assays niet voldoende onderscheidend vermogen voor de analyse van complexe verwantschapszaken, of wordt het onderscheidend vermogen verminderd doordat incomplete profielen zijn verkregen uit afgebroken DNA. Een redelijk nieuwe assay op de forensische markt (HDplex™) bevat 9 STRs die niet standaard worden toegepast in forensisch DNA onderzoek en analyse van deze markers kan het onderscheidend vermogen vergroten. Met de komst van nieuwe markers wordt het onvermijdelijk dat meerdere markers op hetzelfde chromosoom worden onderzocht. Om de vraag te beantwoorden of deze markers als onafhankelijk van elkaar kunnen worden beschouwd, is onderzoek verricht aan de 30 verschillende STRs die aanwezig zijn in de NGM™, HDplex™ en Identifiler™ assays, zoals beschreven in **hoofdstuk 6**. Op basis van 335 Nederlandse referentie DNA profielen voor deze drie assays zijn geen aanwijzingen gevonden dat twee of meer markers gekoppeld overerven op populatieniveau. Om genetische koppeling binnen families te onderzoeken, werd het DNA van vijf stambomen bestaande uit drie generaties (elk met vier grootouders, twee ouders en zeven tot elf kinderen) onderzocht. Voor een aantal STR paren werd een zwakke genetische koppeling gevonden die effect kan hebben op de interpretatie van DNA gegevens van (sterk) verwante individuen (voor een methode om hier rekening mee te houden bij berekeningen wordt verwezen naar een artikel van Gill en collega's (Forensic Sci Int Genet 6:477-486, 2011)). HDplex™ heeft veel niet-overlappende markers met NGM™ en Identifiler™ en het onderscheidend vermogen per marker ligt gemiddeld hoger dan voor de andere assays; dit maakt HDplex™ een goede, complementaire STR assay die van nut kan zijn in complexe verwantschapszaken en de analyse van afgebroken DNA.

In DNA profielen van afgebroken monsters ontbreekt vaak informatie over de langere STR fragmenten. Het gevoeliger maken van de reacties door middel van het uitvoeren van extra PCR vermenigvuldigingscycli of het verhogen van de CE instellingen brengt het risico met zich mee dat de kortere STR fragmenten te veel vermenigvuldigd of overladen worden tijdens de PCR of de CE. In **hoofdstuk 7** is uitgezocht of meer informatie uit de langere STR fragmenten kan worden verkregen door het gebruik van een lengtespecifieke *post-PCR* (uitgevoerd na de PCR) zuiveringsmethode. Deze methode, die gebaseerd is op AMPure® XP bolletjes, is vergeleken met een niet-selectieve zuivering (door middel van DTR gel filtratie) en geen zuivering van de PCR producten. Naast een serie van 39 verschillend afgebroken enkelvoudige DNA monsters werden ook ongelijke mengsels in de verhoudingen 1:5, 1:10 en 1:15 geanalyseerd om meer informatie over de DNA kenmerken van de nevendonor te kunnen verzamelen zonder dat de kortere STR fragmenten van de hoofddonor overladen werden. Na DTR gel filtratie werd een ongeveer tweevoudige toename in piekhoogte gevonden voor allelen op alle markers, terwijl AMPure® zuivering een drie- tot viervoudige toename in piekhoogte veroorzaakte voor de langere maar geen toename voor de kortere STR fragmenten. Beide post-PCR zuiveringsmethoden leverden meer gedetecteerde allelen op, met gemiddeld iets meer gedetecteerde allelen (vooral voor de langere fragmenten) na AMPure® zuivering.

Humane identificatie op basis van DNA vindt toepassing in zowel (massaramp) slachtofferidentificatie, als verwantschaps- en forensisch onderzoek. In **hoofdstuk 8** worden verschillende aspecten van humane identificatie bediscussieerd, met nadruk op de gevolgen van lage kwaliteit en kwantiteit van het DNA. Daarnaast worden de keuzes aangaande humane identificatie op zowel technisch als ethisch niveau besproken.

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