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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

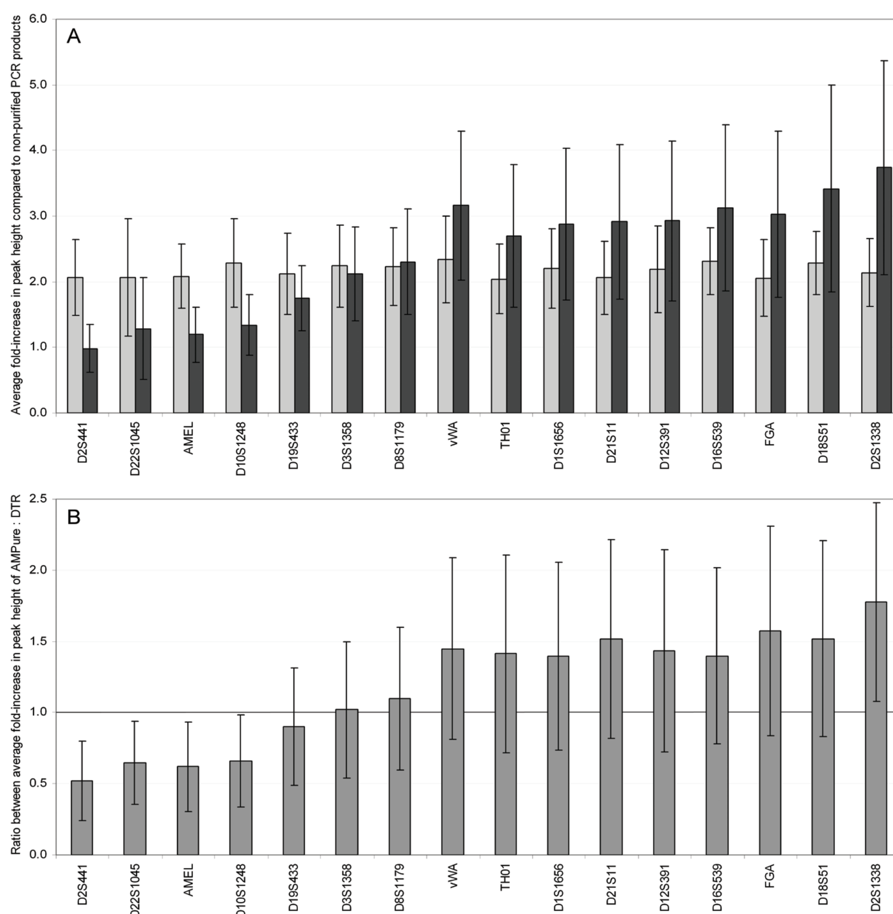

Fig. 1 (A) Average fold increase in peak heights when PCR products from moderately degraded samples are purified by either DTR gel filtration (light grey bars) or the in-house developed AMPure® protocol (black bars). This graph is based on a total of 522 alleles that were detected (i.e. above 50 rfu), 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

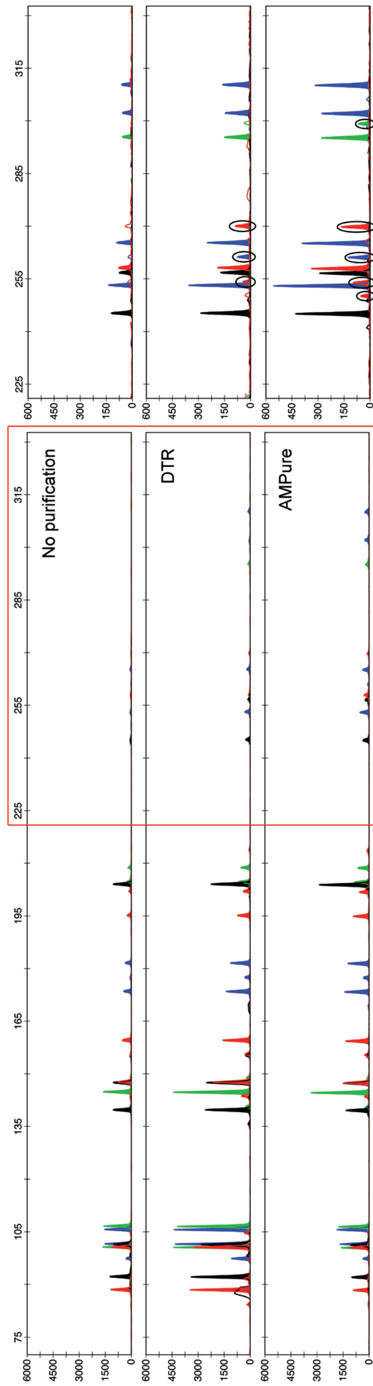

Fig. 2 Overlay electropherograms for a mixture with a minor/major ratio of 1:10 without purification (upper panes), after DTR gel filtration (middle panes) and after AMPure® purification (lower panes). The panes on the left show the full electropherograms, with the Y-axes scaled to 6,000 rfu; after DTR 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.

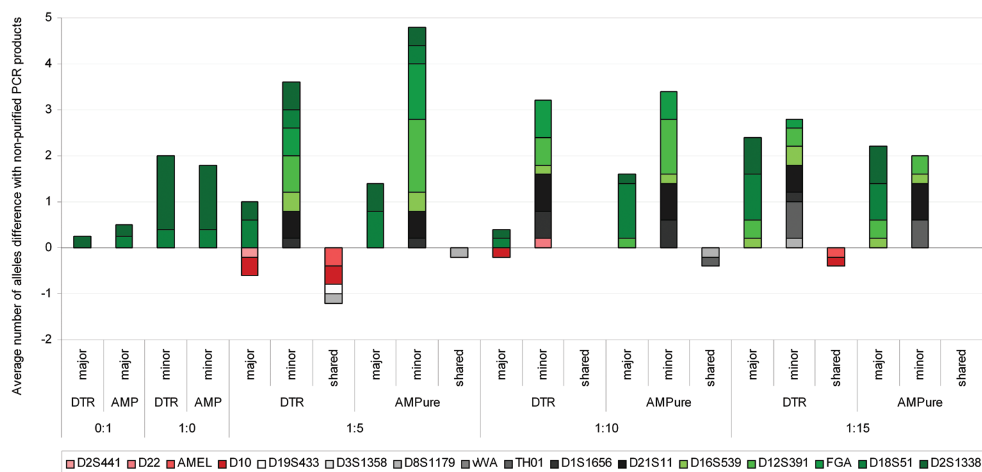

Fig. 3 Average number of allele difference between purified and non-purified PCR products. A comparison was made between DTR gel filtration and AMPure® post-PCR purification after differentiation between major, minor and shared alleles. Gained alleles are shown as a positive number, saturated alleles 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.

Acknowledgments

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Supplementary material

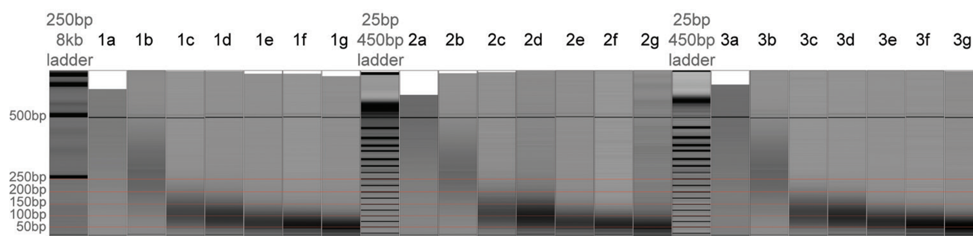
Supplementary Text 1

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).

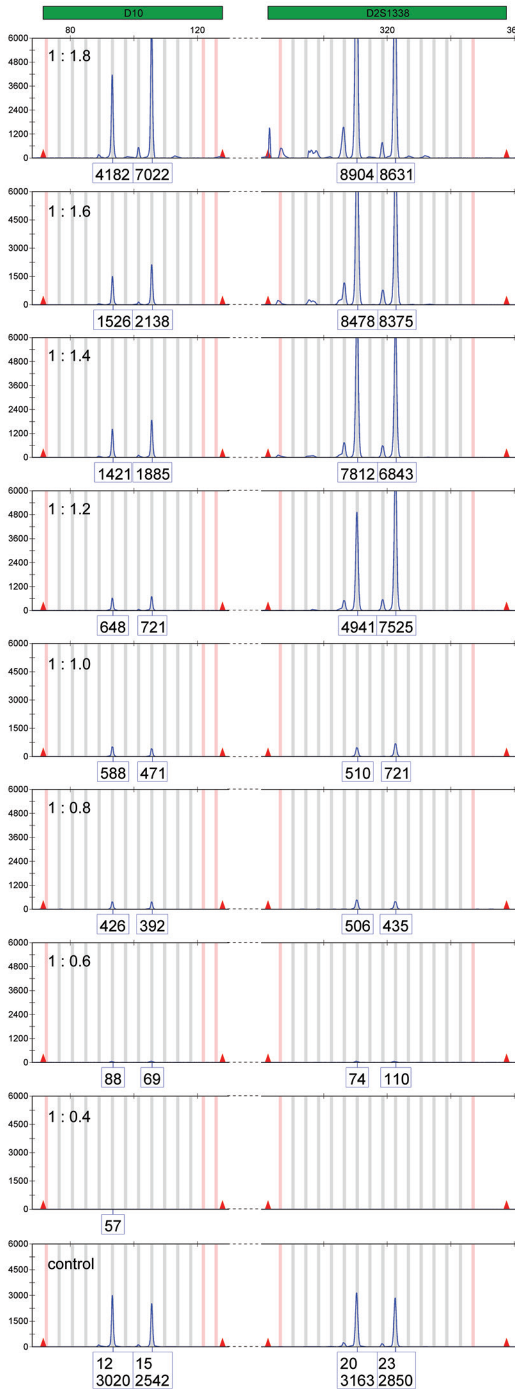
Capillary electrophoresis and DNA profile analysis

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

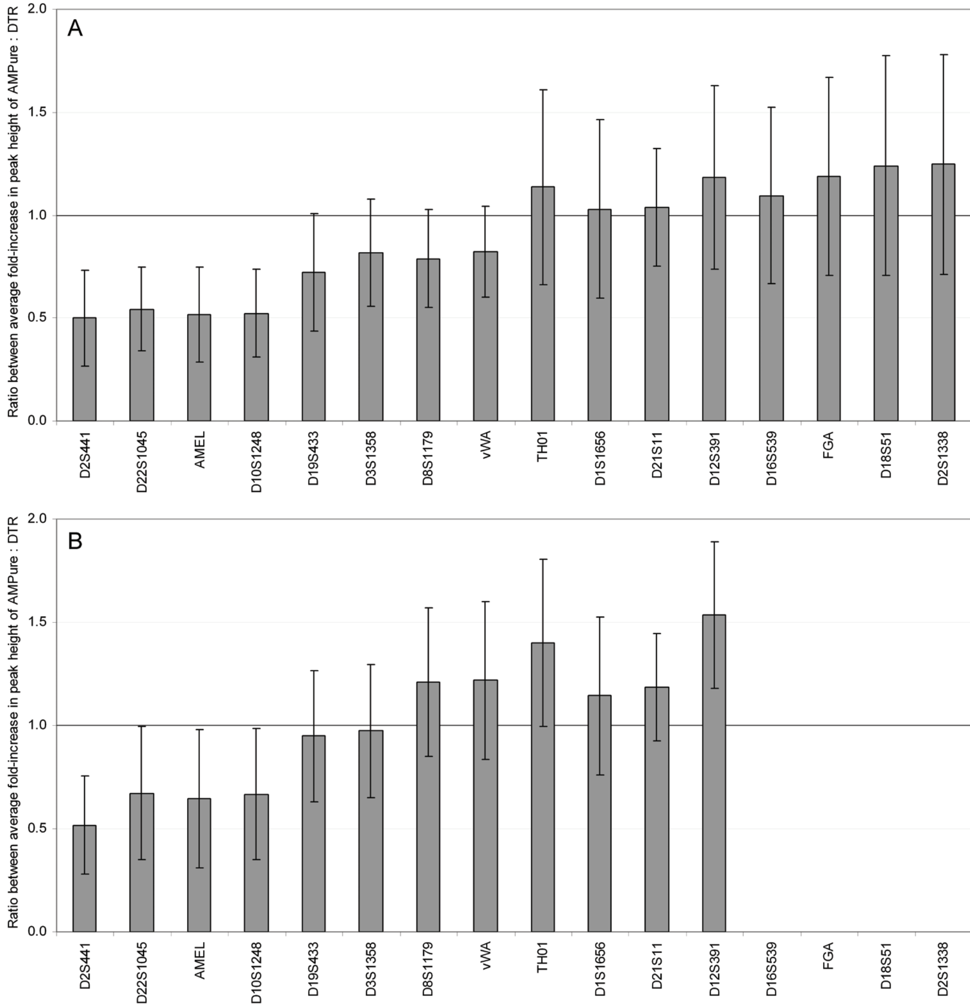


allele drop-outs start to occur. Size-selectivity of the AMPure® XP beads was also reported by David Edwards [16], although he observed slightly different recovery effects, as fragments below 150 bp and 200 bp were not retrieved with ratios of 1:0.8 and 1:0.6 respectively. The finding that in our study 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,

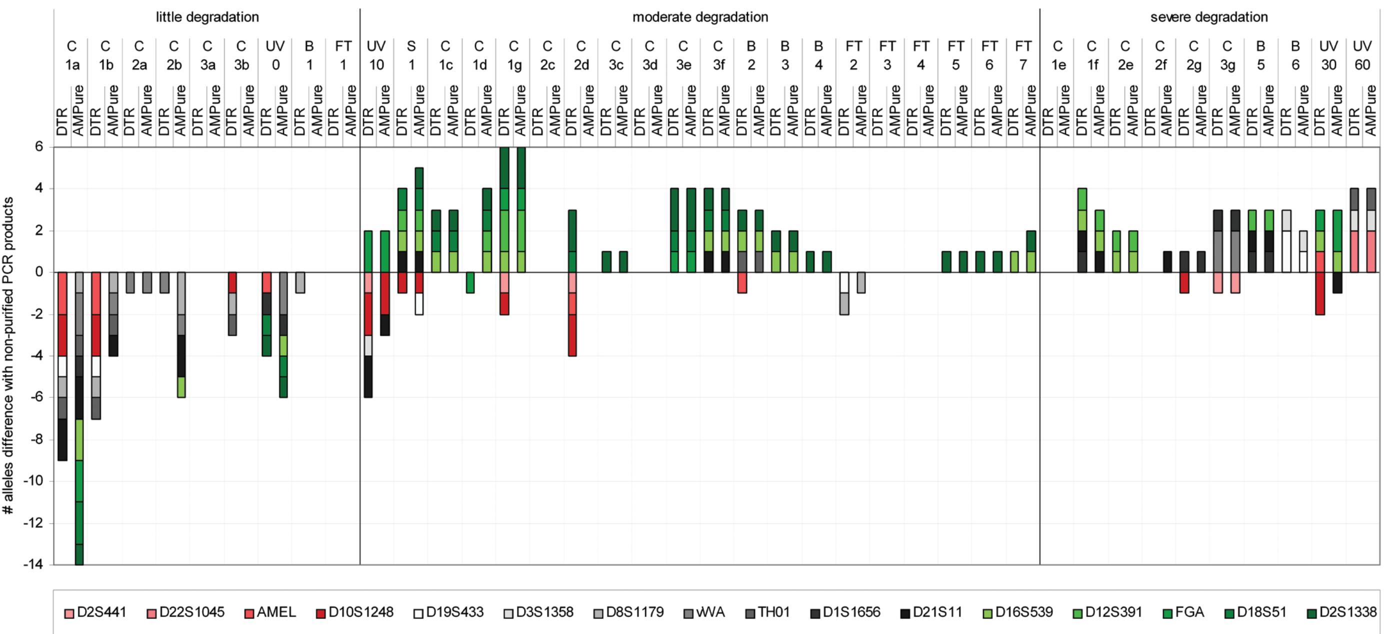
Supplementary Fig. 2 Post-PCR purification by AMPure® XP magnetic beads (according to the manufacturer's protocol) in various volume ratios of PCR product and beads (1:1.8 down to 1:0.4). These experiments are all based on 500 pg pristine DNA007 NGM™ PCR amplifications of which 24 µL were used during 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.

References

1. Alaeddini R, Walsh SJ, Abbas A (2010) Forensic implications of genetic analyses from degraded DNA—a review. Forensic Sci Int Genet 4:148–157
2. Butler JM, Shen Y, Mccord BR (2003) The development of reduced size STR amplicons as tools for analysis of degraded DNA. J Forensic Sci 48:1054–1064
3. Wiegand P, Kleiber M (2001) Less is more—length reduction of STR amplicons using redesigned primers. Int J Leg Med 114:285–287
4. Hill CR, Kline MC, Coble MD, Butler JM (2008) Characterization of 26 mini-STR loci for improved analysis of degraded DNA samples. J Forensic Sci 53:73–80
5. Westen AA, Sijen T (2009) Degraded DNA sample analysis using DNA repair enzymes, mini-STRs and (tri-allelic) SNPs. Forensic Sci Int Genet Suppl Ser 2:505–509
6. Dixon LA, Dobbins AE, Pulker HK, Butler JM, Vallone PM, Coble MD, Parson W, Berger B, Grubwieser P, Mogensen HS, Morling N, Nielsen K, Sanchez JJ, Petkovski E, Carracedo A, Sanchez-Diz P, Ramos-Luis E, Brion M, Irwin JA, Just RS, Loreille O, Parsons TJ, Syndercombe-Court SH, Stradmann-Bellinghausen B, Bender K, Gill P (2006) Analysis of artificially degraded DNA using STRs and SNPs—results of a collaborative European (EDNAP) exercise. Forensic Sci Int 164:33–44
7. Tucker VC, Hopwood AJ, Sprecher CJ, McLaren RS, Rabbach DR, Ensenberger MG, Thompson JM, Storts DR (2012) Developmental validation of the PowerPlex(R) ESX 16 and PowerPlex(R) ESX 17 Systems. Forensic Sci Int Genet 6:124–131
8. Kloosterman AD, Kersbergen P (2003) Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci. J Soc Biol 197:351–359
9. Westen AA, Nagel JH, Benschop CC, Weiler NE, de Jong BJ, Sijen T (2009) Higher capillary electrophoresis injection settings as an efficient approach to increase the sensitivity of STR typing. J Forensic Sci 54:591–598
10. Forster L, Thomson J, Kutranov S (2008) Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle "low copy number" (LCN) method for analysis of trace forensic DNA samples. Forensic Sci Int Genet 2:318–328
11. Lederer T, Braunschweiger G, Betz P, Seidl S (2002) Purification of STR-multiplex-amplified microsamples can enhance signal intensity in capillary electrophoresis. Int J Leg Med 116:165–169
12. Butler JM (2011) Advanced topics in forensic DNA typing: methodology. Academic, Amsterdam
13. Hill CR, Butler JM, Vallone PM (2009) A 26plex autosomal STR assay to aid human identity testing. J Forensic Sci 54:1008–1015
14. Weiler NE, Matai AS, Sijen T (2012) Extended PCR conditions to reduce drop-out frequencies in low template STR typing including unequal mixtures. Forensic Sci Int Genet 6:102–107
15. Benschop C, Haned H, Sijen T (2011) Consensus and pool profiles to assist in the analysis and interpretation of complex low template DNA mixtures. Int J Leg Med. doi:10.1007/s00414-011-0647-5
16. Edwards D (2012) PCR purification: AMPure and simple. http://www.keatslab.org/blog/pcrpurificationampureandsimple
17. Rodrigue S, Materna AC, Timberlake SC, Blackburn MC, Malmstrom RR, Alm EJ, Chisholm SW (2010) Unlocking short read sequencing for metagenomics. PLoS One 5: e11840
18. Westen AA, Groen WJ and Maat GJR (2013) Human remains from the cloister garth of the 'Koningsveld' priory near the Medieval city of Delft - Ca. 1450–1572 AD. Barge's Anthropologica, Amsterdam

19. Westen AA, Matai AS, Laros JF, Meiland HC, Jasper M, de Leeuw WJ, de Knijff P, Sijen T (2009) Tri-allelic SNP markers enable analysis of mixed and degraded DNA samples. Forensic Sci Int Genet 3:233–241
20. Nicklas JA, Buel E (2006) Simultaneous determination of total human and male DNA using a duplex real-time PCR assay. J Forensic Sci 51:1005–1015
21. Scientific Working Group on DNA Analysis Methods (2010) SWGDAM Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories. http://www.fbi.gov/about-us/ lab/codis/swgdam.pdf
22. Westen AA, Grol LJ, Harteveld J, Matai AS, de Knijff P, Sijen T (2012) Assessment of the stochastic threshold, back- and forward stutter filters and low template techniques for NGM. Forensic Sci Int Genet 6:708–715
23. Benschop CC, van der Beek CP, Meiland HC, van Gorp AG, Westen AA, Sijen T (2011) Low template STR typing: effect of replicate number and consensus method on genotyping reliability and DNA database search results. Forensic Sci Int Genet 5:316–328