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Research Article

The molecular characterization of a depurinated trial DNA sample can be a model to understand the reliability of the results in forensic genetics

The role of DNA damage in PCR processivity/fidelity is a relevant topic in molecular investigation of aged/forensic samples. In order to reproduce one of the most common lesions occurring in postmortem tissues, a new protocol based on aqueous hydrolysis of the DNA was developed in vitro. Twenty-five forensic laboratories were then provided with 3.0 μg of a trial sample (TS) exhibiting, in mean, the loss of 1 base of 20, and a molecular weight below 300 bp. Each participating laboratory could freely choose any combination of methods, leading to the quantification and to the definition of the STR profile of the TS, through the documentation of each step of the analytical approaches selected. The results of the TS quantification by qPCR showed significant differences in the amount of DNA recorded by the participating laboratories using different commercial kits. These data show that only DNA quantification "relative" to the used kit (probe) is possible, being the "absolute" amount of DNA inversely related to the length of the target region ($r^2 = 0.891$). In addition, our results indicate that the absence of a shared stable and certified reference quantitative standard is also likely involved. STR profiling was carried out selecting five different commercial kits and amplifying the TS for a total number of 212 multiplex PCRs, thus representing an interesting overview of the different analytical protocols used by the participating laboratories. Nine laboratories decided to characterize the TS using a single kit, with a number of amplifications varying from 2 to 12, obtaining only partial STR profiles. Most of the participants determined partial or full profiles using a combination of two or more kits, and a number of amplifications varying from 2 to 27. The performance of each laboratory was described in terms of number of correctly characterized loci, dropped-out markers, unreliable genotypes, and incorrect results. The incidence of unreliable and incorrect genotypes was found to be higher for participants carrying out a limited number of amplifications, insufficient to define the correct genotypes from damaged DNA samples such as the TS. Finally, from a dataset containing about 4500 amplicons, the frequency of PCR artifacts (allele dropout, allele drop-in, and allelic imbalance) was calculated for each kit showing that the new chemistry of the kits is not able to overcome the concern of template-related factors. The results of this collaborative exercise emphasize the advantages of using a standardized degraded DNA sample in the definition of which analytical parameters are critical for the outcome of the STR profiles.

Keywords:
DNA depurination / Forensic genetics / PCR fidelity / STR typing
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Abbreviations: A–P, apurinic–apyrimidinic; **CL**, coordinating laboratory; **MW**, molecular weight; **PL**, participating laboratory; **rfu**, relative fluorescence units; **QSS**, quantitative standard sample; **TS**, trial sample

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1 Introduction

PCR analysis came into the field of "forensic genetics" more than 20 years ago. Since then, PCR-based techniques improved tremendously, growing from manual singleplex VNTR typing up to automated-multiplex STR analysis. In order to obtain as much genetic information as possible from casework samples, commercially available kits, designed with different configurations and combination of STR markers, are now available [1, 2]. In addition, the chemistry of PCR-based systems has also been improved. Therefore, forensic DNA typing results can be achieved even in presence of limited amount of DNA, and/or co-extracted inhibitors and/or DNA degradation [3].

Despite these significant technical developments, STR typing of many biological samples still provides partial and/or unreliable results, or no results at all. It is known, in fact, that the primary structure of the DNA recovered from aged/forensic samples can be significantly altered by enzymatic and nonenzymatic processes [3–10] that promote both PCR artifacts and PCR failure [3–9].

Out of the multitude of lesions occurring in forensic and ancient DNA samples, the formation of apurinic–apyrimidinic (A–P) sites, due to the hydrolysis of the *N*-glycosylic bond [11], is the most common [3–8] and this is well documented even by Next-Generation Sequencing analyses [12]. A–P sites, in fact, are known to promote misinsertions [6, 7, 9], frameshift errors [8], and block of the synthesis [5, 9], depending both on the extension of the lesion and the type of polymerase [6, 9].

In order to understand how consistent results can be obtained and what can be done to improve the reliability of the genetic typing from such samples, it is extremely useful to deal with standardized reference samples of degraded/modified DNA, since the study of the performance of routinely used PCR systems on problematic DNA samples is one of the targets of forensic genetic research and validation efforts. For this reason, DNA test samples were enriched in A–P lesions under controlled conditions in vitro in a pilot study [13]. Here, we report on a collaborative exercise aimed at investigating the strategies applied by the participating labs to optimize the analysis of a DNA sample exhibiting a known degree of damage (loss of about 5% of the bases and molecular weight (MW) $<$300 bp).

2 Materials and methods

2.1 Depurination protocol setup

The protocol described here follows our previous studies based on DNA depurination under mild acidic conditions [13] and represents an evolution toward its simplification. Depurination was performed in water at 70°C for 1, 2.5, 5, 7.5, and 10 h. Reagents, chemicals, and even the DNA sample here used (sample TSPV2, deriving from 500 mL of fresh blood, in water at concentration of 75 ng/μL) are the same as described elsewhere, as well as the methods employed to characterize the molecular features of the damaged DNA samples [13–15].

2.2 Samples provided for the collaborative exercise

Twenty-five laboratories participated in the collaborative exercise (23 Italian and two foreign labs). Each participating laboratory (PL) was provided with the two same DNA samples by the coordinating laboratory (CL) that shipped them in dry ice.

The first sample was the "trial sample" (TS) that was prepared from eight aliquots of sample TSPV2 [13, 14] incubated at 70°C for 5 h. The pooled aliquots were adjusted to the final volume of 2.4 mL with water and then carefully mixed. This solution was then subdivided into 40 aliquots of 60 μL each. Therefore, each tube contained a nominal amount of about 3.0 μg, corresponding to a concentration of about 50 ng/μL. All the samples were stored at –20°C until further use.

The second sample was a high-MW human DNA extracted from 5.0 mL of fresh blood collected from a volunteer who provided an informed consent. This sample was considered the "quantitative standard sample," and for this reason was named QSS. This sample was diluted in water at a concentration of about 35 ng/μL (as assessed by UV absorbance), divided in aliquots of 30 μL, and stored at −20°C until further use.

2.2.1 Quality controls performed before sending the samples and shipping procedures

To verify the outcome of the depurination protocol, the amount of released bases was assessed, in each of the eight samples, by MEKC as described previously [15]. This test showed that the mean percentage release of the purines was 5.8 ± 0.5 and 4.6 ± 0.3 for guanine and adenine, respectively (as calculated by the formula A*fX*/A*totX*, where A*fX* is the amount of a given free base *X* and A*totX* is the amount of the same base bound to the DNA before the treatment) [13].

To check the main molecular features of the TS and QSS, four randomly selected aliquots of each stock were analyzed as described elsewhere [13–15] in two different working sessions, the first one immediately after the sample preparation and the second one after a 2-month freezing storage.

The samples were shipped to the participants in dry ice. In addition, to minimize the possibility of further degradation of the samples, the participants were recommended to aliquot the samples into single-use tubes after the first thawing.

2.3 Documentation required to the PLs for data evaluation

In order to produce data describing the molecular features of samples TS and QSS, the participants could freely choose any

of the analytical methods routinely employed for casework analysis in their lab. Finally, each participating lab was asked to fill a questionnaire describing the methods used for the assessment of the DNA MW, DNA quantification, and STR characterization.

2.3.1 DNA evaluation and amplification of STR loci

In order to describe the MW of sample TS, it was suggested that the participating labs perform a 1.2% agarose gel electrophoresis loading a 5.0 μL aliquot of the sample. The MW had to be reported according to [13, 14].

After that, the participants were asked to quantify the amount of DNA in TS and QSS choosing physical and/or molecular assays.

Finally, each laboratory was requested to type the DNA samples choosing a single commercial kit and/or homemade PCR system (or any combination of two or more kits/systems) and following its analytical protocol as usually. In order to verify the quality of the results, each participant was requested to send all the electropherograms obtained from the samples to the CL as printouts. In a second step of the work, to normalize the results according to a defined amount of template, the participants were requested to carry out replicate PCRs (at least three) of a volume of 2.0 μL of TS.

As a control of the efficiency of the amplification, the participating laboratories were asked to analyze, in each PCR set, one microliter of a 1:70 dilution of QSS (corresponding to about 550 pg of DNA). Duplicate analyses of this sample were also recommended.

2.3.2 STR profile

The participants were requested to report the STR profile (complete or partial), if they were confident on the identification of the genotype of TS, by filling a form provided by the CL.

2.4 Data management and analysis

2.4.1 MW and DNA quantification

The results were pooled according to the scores and methods provided by the participating laboratories [13].

2.4.2 Evaluation of the STR profiles

The CL compared the STR profile obtained from sample TSPV2 (see Supporting Information Table 1) to the ones provided by the participants.

2.4.3 Establishment of the molecular database

Since five commercial multiplex STR kits were selected by the participating laboratories, five worksheets (one for each kit) were then created in Excel. Thus, the PCR data were pooled according to the commercial kit used as follows: AmpFLSTR Identifiler, AmpFLSTR NGM and NGM SElect, AmpFLSTR MiniFiler from Applied Biosystems (USA) and PowerPlex ESX and ESI 17 Systems from Promega (USA).

An initial classification of the PCR products (or amplicons) was based on the identification of an allele for a given locus, by comparison with the corresponding allelic ladder [16]. The cutoff for an unambiguous allele call was set up to 50 relative fluorescence units (rfu). Thus, for each multiplex PCR performed from TS, only the peak areas showing >50 rfu were entered in the corresponding worksheet. The same criteria were employed for entering the molecular data from QSS.

This database was then employed for the analyses described in the following paragraphs.

2.4.4 Interpretation guidelines for genotyping the molecular data

In order to analyze the molecular database sets containing the data provided by each participating group, the CL defined interpretation guidelines according to the following criteria based on the "consensus" and the "composite" approaches described in [17]:

(i) The stochastic threshold level of 150 rfu was set to define a value above which it is reasonable to assume that allelic dropout cannot occur within a single-source sample [18, 19].
(ii) The genotype for a given locus was considered correct if more than 50% of the amplifications obtained by a single kit (or a combination of kits) showed the expected genotype.
(iii) No result for a given locus was defined when more than 50% of the amplifications obtained by a single kit (or a combination of kits) showed no PCR products.
(iv) When the genetic typing of a given locus produced different genotypes (among which the expected correct one was found in less than 50% of the amplifications) this result was considered unreliable, as it was not possible to unambiguously assign a genotype for that given locus.
(v) The genotype for a given locus was considered wrong if more than 50% of the amplifications obtained by a single kit (or a combination of kits) showed the same incorrect genotype.
(vi) If, in different amplifications, the same homozygous genotype for a given locus was observed and the corresponding peak heights fell in the "gray zone" between the analytical and the stochastic threshold (51–150 rfu), a possible dropout of a second allele cannot be excluded.

For this reason, this locus was not evaluated for comparisons to avoid misleading conclusions.

2.4.5 Evaluation of the PCR fidelity

In order to evaluate the PCR fidelity from TS, the molecular database was used to compare the data of each amplification to the reference genotype of TSPV2. The observed inconsistencies were grouped, for each locus, as follows:

(i) locus dropout: no alleles at a given locus;
(ii) allele dropout: absence of one of the two correct alleles for a given heterozygous genotype;
(iii) increased stutter product formation: alleles characterized by a –1 or +1 repeat compared to the correct allele, but with a stutter/correct allele area ratio exceeding 0.15;
(iv) allele drop-in: presence of additional incorrect alleles;
(v) heterozygous allelic peak imbalance: peak height ratio for an heterozygote genotype lower than 0.7;

The same criteria were adopted for creating reference data from QSS.

2.5 Statistical data analysis

All the data were collected in Microsoft Office Excel 2007 spreadsheets and analyzed with Excel and R version 3.0.1 (2013-05-16, Copyright 2013 The R Foundation for Statistical Computing).

3 Results and discussion

3.1 Development of the depurination protocol and molecular features of the damaged samples

The rate of depurination was calculated according to Fattorini et al. [13], as assessed by MEKC, and resulted in 2.75×10^{-6} for G ($r^2 = 0.9954$) and 1.96×10^{-6} for A ($r^2 = 0.9955$), respectively.

As described in Supporting Information Table 2, the main molecular features of the treated samples are in agreement with the extent of the induced damage [13, 20, 21]. In addition, our results are in excellent agreement with the data reported in previously published papers focusing on the role of DNA damage in PCR processivity [13, 21], while the role of DNA fragmentation in blocking the polymerase appears overall only marginal, at least in the range of MW considered here [22].

3.2 Collaborative experiment

The data provided by the 25 participating laboratories led to the following results.

3.2.1 MW of the samples

Twenty-four of 25 laboratories performed agarose gel electrophoresis as suggested by the CL while the remaining participant performed a chromatographic test using a 2100 Expert High-sensitivity DNA Assay. These assays show comparable results among the participant laboratories representing that TS is composed of DNA fragments whose mean MW is <300 bp. This simple test was requested in order to check if the degradation status of the TS could have been modified after shipping and further handling, although it is known that agarose gel electrophoresis provides an overestimation of the MW of the DNA samples [21].

3.2.2 DNA quantification

Three different methods were adopted by the participants to determine the amount of the TS: UV-spectrophotometry, qPCR, and fluorimetry. The results of the quantification are summarized and reviewed according to the method employed, as follows.

3.2.2.1 UV-based assays

This approach was used by nine laboratories. The results showed that no difference between the mean values of the participating laboratories and those produced by the CL was observed (paired *t*-test of the mean differences, $p < 0.05$).

3.2.2.2 qPCR

Seventeen laboratories used this method to determine the concentration of TS and quantitative standard (QSS) DNAs. Five different commercially available kits were chosen by the participating laboratories (see Table 1).

The results of the QSS quantification ranged from 26.7 ng/μL (for the autosomal nuCSF probe) [23] to 95.3 ng/μL (for investigator Q). It is interesting to note that both these values, varying almost four times for this unmodified high-MW DNA sample, were obtained disregarding the upper LOQs indicated for each kit. The same practice of just reporting DNA concentrations, even if beyond the upper LOQ of the kit, was carried out by two of three laboratories that used the autosomal probe of the Plexor HY kit. The mean quantification values provided by testing the other four probed regions did not differ significantly ($p < 0.05$) from those obtained by UV-spectrophometry (35.1 ± 1.5 ng/μL).

As reported in Table 1, the coefficients of variation (CVs %) of the assessments performed by the same kits in different labs are low (no more than 19%). This finding confirms that the poor concordance of the qPCR assays, using different kits, is mainly due to the different chemistry and bio-molecular design of the kits themselves (e.g., multicopy vs. single-copy methods, sequence and length of the probes, sequence and length of the target sequence) [24–26]. In addition, also differences in the human DNA calibration

Table 1. Quantification of the QSS and the TS by qPCR

Kit/system[a)]	*n*[b)]	Target[c)]	QSS[d)]	TS[d)]
Quantifiler (0.024–50 ng/μL)	3	62 (Aut.)	37.7 ± 1.4 (*3*; *n* = 7) 42.4 ± 1.9 (*7*; *n* = 12)**	4.4 ± 0.5 (*12*; *n* = 8) 3.7 ± 0.2 (*10*; *n* = 12)**
Quantifiler Duo (0.024–50 ng/μL)	8	140 (Aut.) 130 (Y-spec.) [Aut]/[Y][e)]	41.9 ± 1.1 (*5*; *n* = 18) 39.3 ± 1.4 (*7*; *n* = 18) 1.07	0.082 ± 0.006 (*19*; *n* = 26) 0.35 ± 0.02 (*13*; *n* = 26) 0.23
Plexor HY (0.004–50 ng/μL)	3	99 (Aut. §) 133 (Y-spec.) [Aut]/[Y][e)]	56.8 ± 6.0 (*11*; *n* = 7) 45.5 ± 8.3 (*19*; *n* = 7) 1.21	5.22 ± 0.96 (*17*; *n* = 7) 0.203 ± 0.015 (*7*; *n* = 7) 25.75
Investigator Q (0.004–20 ng/μL)	2*	146 (Aut. §)	95.3 ± 27.7 (*18*; *n* = 4) 33.8 ± 7.1 (*8*; *n* = 12)***	0.251 ± 0.072 (*18*; *n* = 4) 0.074 ± 0.006 (*4*; *n* = 12)**
Fast M. M. (0.004–20 ng/μL)	1	67 (nuCSF)	26.7 (*n* = 1)	5.67 (*n* = 1)

a) Kit/system: The LOQ defined for each kit by the manufacturers is specified in brackets. Fast M. M.: Taqman Universal Fast PCR Master Mix

b) *n*: number of PLs that used that specific kit; * indicates that the two PLs performed the assay simultaneously, in the same round of qPCR

c) Target: MW in base pairs of the target qPCR probe (Aut.: autosomal single-copy sequence; Aut. §: autosomal multicopy sequence; Y-spec.: Y-specific single-copy sequence); nuCSF is an autosomal single-copy sequence

d) QSS and TS: the DNA amount is given as mean ± CI at the 95% probability level and expressed in nanograms per microliter; The CV% of the assessments (in italic characters) and the number (*n*) of replicated analyses are reported in the brackets. ** and *** indicate the data obtained by the CL from an undiluted sample and from a dilution 1:3 in water (then normalized for the dilution), respectively. The quantitative results reported in this table were obtained from calibration data with $r_2 > 0.99$ in 22 of 26 cases and $r_2 > 0.97$ in four cases. The PL that used the Fast M. M. kit diluted DNA 2800 M (Promega) for calibration

e) [Aut]/[Y]: ratio between the results for DNA concentrations as assessed by the autosomal and the Y-specific probes of the same qPCR kit.

standards included in the commercial kits are certainly involved [25, 26]. Therefore, as already claimed [26–28], a stable human DNA certified reference material could help forensic scientists to reduce within- and among-laboratory quantification variability.

The molecular quantification of the TS DNA (45.7 ± 5.0 ng/μL at NanoDrop assay) confirmed that it is rather refractory to qPCR amplification. Interestingly, this lack of sensitivity to qPCR produced quantification results significantly different among the seven probed regions accounting for a 64-fold discordance. In addition, discordance was observed also in the TS quantification results using those kits that include both autosomal [Aut] and Y-chromosome [Y] probes in a duplex-qPCR format. In fact, although the expected [Aut]/[Y] ratio for an unmodified male DNA sample was close to 1 [29, 30], the [Aut]/[Y] values shifted from 25.75 to 0.23, for Plexor HY and Quantifiler Duo, respectively. Therefore, these values may represent a potential source of confusion, leading to erroneous conclusions on the composition of the sample.

It is known that damaged templates are scarcely sensitive to polymerization [5, 9, 21]. Therefore, to test whether our results could be ascribed to the modification of the primary structure of the TS DNA, a correlation between the length of each target region and the corresponding quantification data was investigated. To this aim, qPCR data were used to calculate the *UV*/*RT* ratios [13, 14], where *UV* is the amount of the sample, as assessed by UV-spectrophotometry, while *RT* is the mean DNA concentration as estimated by probing each different target region. The $\log_{10}$ of the *UV*/*RT* data and

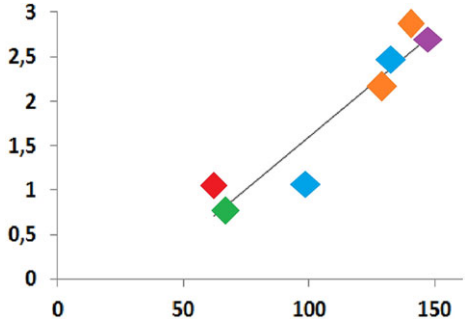

Figure 1. Relation between the length of the target regions and the *UV*/*RT* ratios in the TS. *X*-axis, MW (bp) of the target regions; *Y*-axis, $\log_{10}$ *UV*/*RT*. The coefficient of determination was $r^2 = 0.891$. The plots are shown in red for Quantifiler, green for nuCSF, blue for Plexor HY, orange for Quantifiler Duo, and purple for Investigator Quantiplex.

the MW (in base pair) of each qPCR target probe were then compared. The results are shown in Figure 1 where an $r^2 = 0.891$ between the two parameters was found.

These results definitely show that the quantification of damaged DNA samples by qPCR provides apparently scattered data but very comparable, if clustered according to the length of the probe used. The molecular explanation for these data accounts for an inversely proportional correlation between the length of the probe and the amount of amplifiable DNA molecules, while a minor contribution of kit chemistries is however expectable. Therefore, it is not possible to achieve an "absolute" quantification of the DNA

recovered from forensic degraded/modified DNA samples, but at most a quantification "relative" to the qPCR probe selected is possible.

3.2.2.3 Fluorimetry

This method was selected by four laboratories. These results were too heterogeneous to be further analyzed.

3.2.3 STR analysis

As shown in Table 2, five commercial multiplex STR kits were selected by the participants. All the 25 participating laboratories submitted the electropherograms as printouts with the indication, for each amplicon, of the corresponding allele call, peak height and area. Only 19 of the 25 PLs filled out the form where the TS STR profile had to be reported. Each group indicated the analytical threshold used for the allele identification, varying from 35 to 150 rfu, but most of the labs indicated 50 rfu as the standard analytical threshold.

Each participant performed at least two multiplex PCR amplifications adding variable volumes of TS (from 0.04 to 10.0 μL, corresponding to a total amount of DNA of 1.8 and 457 ng, respectively, as assessed by Nanodrop) and/or the fixed volume (2.0 μL, corresponding to 91.4 ng, always as assessed by Nanodrop) in replicate analyses. In addition, all the participants performed at least one multiplex PCR from 550 pg of QSS, as requested by the CL.

The 212 and 69 multiplex PCRs performed for TS and QSS, respectively, generated the database containing a total number of 6373 amplicons, defined by the corresponding peak heights and areas, mostly (4445) originated from the TS analyses and the remaining from amplifications of QSS (see Table 2 for details).

3.2.3.1 STR typing provided by the participating laboratories

Nineteen laboratories reported what they consider the STR profile for TS, in the tabular results form, not filling the cell for a given locus if they considered it as missing, and indicating as unreliable a genotype that the participant could not assign unambiguously. The provided genotypes were then compared with the STR profile already obtained analyzing the unmodified sample TSPV2 (see Supporting Information Table 1). This comparison allowed the CL to assign the genotypes provided by the participating laboratories to the four categories: correct genotype assignment; no results, meaning a locus dropout; unreliable results; incorrect genotype assignment (see Table 2).

Four participants recorded 16 of 16 correctly assigned STRs while five laboratories reported incorrect genotypes, mainly for the high-MW markers.

3.2.3.2 Evaluation of the electropherograms provided by the participating laboratories

The data so far collected give an interesting overview of the different analytical approaches used by the participating laboratories to perform the genetic typing of a degraded/modified DNA sample but are too heterogeneous regarding the criteria the participants used to define which markers of the STR profile should be considered reliable or not. In addition, six laboratories did not report the TS genetic profile, even if they performed extensive experiments, therefore omitting all information on the evaluation criteria adopted in interpreting the molecular results. Thus, in order to include the data produced by all the 25 participants in the course of the collaborative exercise, the CL decided to harmonize the results evaluating the electropherograms according to the arbitrary parameters described in Section 2.4.4.

The results of such analysis are reported in the last four columns of Table 2. The only three laboratories that correctly scored 100% (16 of 16) of the tested STRs used the combination of AmpFLSTR Identifiler and MiniFiler, and performed at least three amplifications with the first kit and two for the second one, respectively.

The lowest performances, in terms of correctly assigned alleles, belong to two participating laboratories that identified only 55% of the alleles (12 of 22 STRs, for both participants), with four and nine loci dropped out, respectively, and a number of unreliable loci varying from 1 to 6. These two groups (PL 13 and PL 19) performed three and eight amplifications with the combination of Identifiler and NGM.

The combined use of the PowerPlex ESX and ESI 17 Systems, characterizing the same loci in different configurations (in terms of amplicon sizes), produced apparently heterogeneous results. Two participants performed multiple amplifications but correctly scored only 11 of 17 STRs while one laboratory, just with a single amplification for each kit, properly typed 13 of 17 markers. Nine participants amplified the TS using only a single kit. The results showed that the best performing multiplex was NGM, allowing the correct typing of most of the STR profile (14–15 loci of 17). The combinations of three or more kits were very efficient in profiling the TS, as they allowed the correct genetic typing of more than 85% of the markers with a very limited amount of dropped out loci and unreliable results.

The overall evaluation of the performances of the participating laboratories in retrieving the TS profile allowed the CL to identify three laboratories that correctly identify all the markers tested, six laboratories that reported partial profiles with variable success in STR typing (57–95% correctly genotyped loci and the rest dropped out), four showing partial profiles (69–95% correctly genotyped loci) characterized by 1–5 loci showing unreliable results and eight (55–92% correctly genotyped loci) showing partial profiles with unreliable and dropped out loci.

Four apparently incorrect genotypes were detected analyzing the molecular results provided by four participants. For a given locus, in fact, instead of the correct heterozygous genotype, the electropherograms showed just a single

Table 2. Results of TS and *QSS* STR characterization and evaluation of the genotypes

PL	STR multiplexes kit[a]									MW[b]	Results provided by the PLs[c]				Results revised by the CL[d]			
	IF		*NGM*		*ESI*		*ESX*		*MF*		*C*	*NR*	*U*	*W*	*C*	*NR*	*U*	*G*
	T	R	*T*	R	*T*	R	*T*	R	*T*									
1	1	5					1		1	82–353	21/22	1			21/22	1		
2	6		5*	5						101–344	16/21		4	1	13/21	6	2	
3	4	5			2				2	82–394	15/22	7			15/22	7		
4	3								3	101–344	16/16				16/16			
5					12	3	12			82–394	16/17		1		16/17		1	
6	6									107–344	16/16				12/16		4	
7					6		5			82–394					11/17	5	1	
8					7	5				82–394					11/17	6		
9	3	4			3		3		3	82–394	19/22	3			20/22	1	1	
10	2									107–344	13/16		2	1	10/16	5		1
11	4	5							2	101–344	16/16				16/16			
12	1		1							101–393	12/22	8	1	1	14/22	2	5	1
13	2		1							101–393	12/22	7	2	1	12/22	4	6	
14	4	5	3						2	101–393	18/22	4			19/22	1	2	
15	1		2*						1	101–344	19/21	2			19/21	1		1
16	5	5							2	101–344	16/16				16/16			
17					1		1			82–394	13/17	4			13/17	4		
18						5				82–394					10/17	7		
19		5	3							101–393					12/22	9	1	
20			1	5						101–393					15/17		2	
21	5									107–344	10/16	6			10/16	6		
22	2	7								107–344	11/16	5			9/16	6	1	
23		5								107–344	12/16		4		11/16		5	
24					3			3		82–353					11/17	3	3	
25			3							101–393	15/17		1	1	14/17		2	1
tot[e]	49	46	19	10	34	13	22	3	16	–	–	–	–	–	–	–	–	–
Amp[f]	2059		744		896		474		272	–	–	–	–	–	–	–	–	
QSS[g]	28 (784)		12 (341)		13 (403)		10 (310)		6 (90)	82–394	–	–	–	–	–	–	–	

a) STR multiplexes: *IF* (Identifiler), *NGM*, *ESI*, *ESX*, *MF* (Minifiler). The number of PCRs performed using each specific kit are subdivided in the following: *T* reporting the tests carried out with a variable number of cycles (28–36) and with the following ranges of TS volumes: *IF*: 0.1–10.0 μL; *NGM*: 0.1–10.0 μL; *ESI*: 0.04–8.0 μL; *ESX*: 0.04–8.8 μL; *MF*: 0.5–5.0 μL; R, reporting the tests performed by adding a fixed volume (2.0 μL) of TS, and using the number of cycles suggested by the manufacturers (*NGM kit did not contain the SE33 system).

b) MW: MW range of the alleles studied

c) Results provided by the PLs: evaluation of the STR profiles provided by the PLs; *C:* ratio of the number of loci correctly typed to the total number of analyzed loci; *NR:* number of loci for which no result was provided; *U:* number of loci showing ambiguous results; *W:* number of loci for which wrong genotypes were scored

d) Results revised by the CL: the STR profiles were edited by the CL according to the criteria described in Section 2.4.4; *C*, *NR*, and *U*: see above; *G:* number of loci characterized by alleles in the "gray zone" between the analytical and stochastic threshold, and not evaluated in the present study

e) tot: total number of multiplex PCRs

f) Amp: number of amplicons entered in the molecular database

g) QSS: total number of multiplex PCRs performed adding 550 pg of QSS DNA and, in brackets, the number of amplicons entered in the molecular database.

allele (see Supporting Information Table 3). Since all these loci were characterized by high-MW amplicons and almost all the homozygous peaks showed an rfu value in the gray zone (e.g., between the analytical and the stochastic threshold), the presence of a second allele that dropped out could not be excluded. For this reason, the genotypes suggested by the electropherograms were not considered as wrong results, according to the parameters defined by the CL, and were not evaluated for comparisons. However, a common feature of these experiments was the limited number of amplifications with the same kit that the laboratories carried out (one to three PCRs), which appears to be clearly inadequate to define the correct genotypes from damaged DNA samples.

3.3 PCR fidelity

The molecular database was analyzed in order to understand how the performance of each kit was influenced by the molecular features of the TS, in terms of locus amplification rate and PCR artifacts. To this aim, the STR loci of Identifiler, NGM, ESI, and ESX kits were subdivided in four classes, each one containing 4–5 loci, clustered according to the MW of the TSPV2 alleles (see Supporting Information Table 1). The PCR artifacts considered were "locus dropout, allele dropout, allele drop-in, stutter bands, and allele peak imbalance," as defined in Section 2.4.5.

The frequency of each artifact was checked in the 72 replicate amplifications performed with different kits, using the fixed volume of 2.0 μL of TS, and in the remaining 124 PCR tests carried out in different analytical conditions (number of PCR cycles varying from 28 to 36, and volume of TS sample added to the PCR reaction varying from 0.04 to 10.0 μL). As control, the presence of these artifacts was also verified in the 63 multiplex PCRs carried out using 550 pg of the high-MW QSS DNA. Table 3 shows the incidence of such PCR artifacts, for each category of STR markers assigned on the basis of the MW of the alleles, displayed for each single kit.

For each kit, the increase of the locus dropout rate from the low- to the high-MW categories of STR markers becomes easily apparent. This condition is clearly due to the chemical damage of the DNA template that exhibited, in mean, the loss of one base out of 20, and a MW below 300 bp.

This finding shows that not even the enhanced sensitivity of the new STR forensic kits allowed to overcome the template-related features affecting the PCR processivity [3, 5, 9, 31], although huge amounts of template are amplified.

The incidence of allele dropout artifacts increased for each kit with the same trend shown by locus dropout, reaching top frequencies varying from 15 to 23% for the third categories of markers.

The presence of additional alleles to the expected ones (i.e., allele drop-in) was observed mainly in the first categories of markers, especially for the Identifiler (mainly at the D19S433 marker) and NGM kits. In addition, we observed that the presence of additional alleles seemed to be directly related to the amount of template used.

Stutter bands were artifacts producing a very limited effect on the STR profile outcome. These artifacts affected mainly the low-MW markers, in agreement with the model representing that efficient syntheses are needed, in the first cycles of the PCR, to produce them [9, 32].

While all the PCR artifacts so far described were absent in pherograms of QSS, allelic peak imbalance was observed from the analyses of that high-MW DNA sample, with frequencies varying from 6% (for ESI) to 13% (for Identifiler). Differences in the amplification efficiency of the alleles of a heterozygous genotype were instead clearly visible in the pherograms of TS, especially in the first and second low-MW categories of STR markers, probably reflecting the heterogeneous distribution of undamaged/damaged templates that affected PCR amplifications in their first cycles [9, 33].

Finally, PCR fidelity was investigated also in 16 and 6 MiniFiler amplifications performed on TS and QSS, respectively. Only a higher incidence of allelic peak imbalance in the depurinated samples compared to the high-MW controls (28 vs. 11%) was observed. This result confirms the advantages offered by the mini-STR over the conventional STR approach to the analysis of degraded DNA samples [3].

4 Concluding remarks

A simple method to produce damaged DNA samples in a controlled way has been established. Hydrolysis in water at 70°C has been confirmed to be an inexpensive and time-dependent procedure whose outcome can be monitored by checking the UV/RT ratio. This value, in fact, clearly points out a correlation with the "state of depurination" of the DNA samples, confirming that the integrity of the template plays the major role in PCR processivity [5, 9, 14, 21, 31].

Twenty-five participating laboratories were then provided with 3.0 μg of a TS whose extent of depurination (loss of one of about 20 bases) is known to promote both PCR artifacts and PCR failure [9, 13] and therefore modeling a problematic forensic sample.

The assessment of the amount of TS was found to be the first critical step of this collaborative exercise. qPCR analysis, in fact, was carried out using five different commercial kits showing high interlaboratory precision but with a high discordance (up to 64-fold) among the results of the kits themselves. The findings provided by the participants suggest that reliable and comparable DNA quantitation results can be achieved only using the same commercial kit and procedure.

In addition, an even more significant discordance was observed using those kits that included both autosomal [Aut] and Y-chromosome [Y] probes in a duplex-qPCR format, recording differences up to 112-fold in the *[Aut]/[Y]* ratios. However, all this was shown to be due to the chemical damage of the template that was less prone to the polymerization of longer target regions [9, 21, 31]. DNA quantitation of a degraded DNA samples can thus be considered only relative

Table 3. Frequency of the STR-PCR artifacts in the TS

Artifact	Identifier				Artifact	NGM			
	MW	QSS (28)	R (46)	*T* (49)		MW	QSS (12)	R (10)	*T* (19)
LDO	107–140	0	0.01	0.01	LDO	85–109	0	0	0
	143–204	0	0.07	0.08		136–187	0	0.05	0.05
	221–276	0	0.39	0.30		193–250	0	0.38	0.30
	281–344	0	0.65	0.57		252–328	0	0.51	0.43
ADO	107–140	0	0.01	0.01	ADO	85–109	0	0	0.03
	143–204	0	0.08	0.05		136–187	0	0.08	0.04
	221–276	0	0.18	0.16		193–250	0	0.05	0.24
	281–344	0	0.12	0.12		252–328	0	0.13	0.18
ADI	107–140	0	0.26	0.10	ADI	85–109	0	0.23	0.28
	143–204	0	0.02	0.02		136–187	0	0.43	0.07
	221–276	0	0.01	0.01		193–250	0	0.03	0
	281–344	0	0	0		252–328	0	0	0.01
PI	107–140	0.05	0.27	0.32	PI	85–109	0.04	0.43	0.47
	143–204	0.11	0.36	0.33		136–187	0.13	0.20	0.22
	221–276	0.15	0.15	0.24		193–250	0.10	0.23	0.17
	281–344	0.09	0.03	0.08		252–328	0.02	0.16	0.09
Artifact	ESI				Artifact	ESX			
	MW	QSS (13)	R (13)	*T* (34)		MW	QSS(10)	R(3)	*T*(22)
LDO	82–127	0	0	0	LDO	82–113	0	0	0
	141–191	0	0	0.04		127–173	0	0.33	0.14
	220–310	0	0.73	0.65		215–247	0	0.17	0.68
	308–394	0	0.86	0.75		295–353	0	0.25	0.83
ADO	82–127	0	0	0	ADO	82–113	0	0	0.05
	141–191	0	0.02	0.06		127–173	0	0.13	0.13
	220–310	0	0.14	0.15		215–247	0	0.58	0.16
	308–394	0	0.09	0.12		295–353	0	0.41	0.09
ADI	82–127	0	0.06	0.01	ADI	82–113	0	0	0.09
	141–191	0	0.02	0.01		127–173	0	0.27	0
	220–310	0	0.02	0		215–247	0	0	0
	308–394	0	0	0		295–353	0	0.08	0
PI	82–127	0.05	0.14	0.23	PI	82–113	0.13	0.42	0.41
	141–191	0.08	0.31	0.30		127–173	0.08	0.2	0.31
	220–310	0.08	0.06	0.10		215–247	0	0	0.09
	308–394	0.02	0.02	0.06		295–353	0.10	0.08	0.03

LDO: locus dropout; ADO: allele dropout; ADI: allele drop-in; PI: peak imbalance; MW: MW of the amplicons; R: data from a fixed volume (2.0 μL) of the TS at standard number of cycles; *T*: results of other PCR tests performed on the TS (see legend of Table 2 for more details); QSS: results from the QSS. The numbers in the brackets indicate the PCR tests performed by the PLs.

to the specific target region or probe investigated in a specific commercial kit as well as the absolute amount of DNA is shown to be inversely related to the length of the target region.

The final point about DNA quantitation in forensic genetics is that there is a necessity for a stable certified reference material such as the one offered by NIST [29], since commercial kits include different human DNA calibration standards. Moreover, qPCR assay results disregarding the kit's LOQ were sometimes reported by few laboratories, confirming that Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines are sometimes ignored [34] even in forensic laboratories.

The STR typing results provided by the participants showed that a broad range of molecular approaches were selected. Most of the laboratories tried to effectively characterize the TS, by testing the reliability of the DNA profiles in multiple amplifications (by the same or by two or more different kits). The STR typing results show that more than three repetitive amplifications of the same degraded DNA sample are required to obtain a reliable profile and that, in order to get at least 85% correct results from the TS, at least three different kits are required.

Besides these results, even if it appears trivial that a limited number of repetitive amplifications can potentially lead to a high incidence of unreliable and incorrect genotypes, there are still (few) labs that consider that testing a sample such as the TS just by two or three PCRs can be sufficient to define its genetic profile, not being apparently aware of the risks of mistyping the sample. Therefore, the data collected in the present collaborative exercise clearly point out the need of standard operating procedures for the definition and interpretation of DNA typing results, as suggested by the SWGDAM (SWGDAM, Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories, http://www.fbi.gov/about-us/lab/biometric-analysis/codis/swgdam.pdf., 2010) especially when difficult DNA samples have to be analyzed [35].

In conclusion, the employment of a degraded DNA standard sample such as the one reported in this study is believed to be extremely helpful in identifying analytical parameters important for achieving a reliable STR profile among different laboratories, which use a wide variety of forensic DNA analytical protocols for quantitation and qualitative assessment of PCR amplifiable products.

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