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1 Assessment of the Precision ID Identity Panel kit on challenging forensic samples

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1	Highlights
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3	The precision of the ID Identity Panel kit was assessed on a large set of challenging forensic
4	samples.
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6	A threshold of 50 reads for locus call reduces the frequency of sequencing errors.
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8	Replicate analyses assure a low/null rate of typing errors.
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10	The high number of markers of the kit assures a random match of probability $\leq 1.6 \times 10^{-13}$ even for
11	the most challenging samples.
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13	PCR-MPS of SNP markers is the ideal approach to the analysis of LCN and degraded DNAs.
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1 Abstract

The performance of the Precision ID Identity Panel (Thermofisher) was assessed on a set of 87 2 forensic samples with different levels of degradation for which a reference sample from the "same 3 donor" or from a "first degree relative" was available. PCR-MPS analysis was performed with DNA 4 input ranging from 1 ng to 12 pg and through 21-26 PCR cycles, in replicate tests, and a total number 5 of 255 libraries were sequenced on the Ion Personal Genome MachineTM (PGMTM) System. The 6 7 evaluation of the molecular data allowed to set a fix threshold for locus call at 50 x which suitably worked even when low amounts of degraded DNA (12 pg) were investigated. In these analytical 8 conditions, in fact, 25 PCR cycles allowed the genotyping of about 50% and 35% of the autosomal 9 and the Y-specific markers on average, respectively, for each single amplification with a negligible 10 frequency of drop ins (0.01 %). On the other hand, drop out artefacts reached 18-23 % when low copy 11 number and degraded DNA samples were studied, with surviving alleles showing more than 600 12 13 reads in 2.9 % of the cases. Our data pointed out that the Precision ID Identity Panel allowed accurate typing of almost any amount of good quality/moderately degraded DNA samples, in duplicate tests. 14 15 The analysis of low copy number DNAs evidenced that the same allele of a heterozygous genotype could be lost twice, thus suggesting that a third amplification could be useful for a correct genotype 16 17 assignment in these peculiar cases. Using the consensus approach, a limited number of genotyping errors were computed and about 37 % of the autosomal markers was finally typed with a 18 corresponding combined random match probability of at least 1.6 x 10⁻¹³, which can be considered 19 an excellent result for this kind of challenging samples. In the end, the results presented in this study 20 21 emphasize the crucial role of the expert opinion in the correct evaluation of artefacts arising from PCR-MPS technology that could potentially lead to genetic mistyping. 22

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1 1. Introduction

The current golden standard for DNA-based human identification is multiplex PCR followed by 2 capillary electrophoresis of the amplified products (PCR-CE) [1,2]. Short tandem repeats (STRs) are 3 4 the selected markers for genotyping a wide range of forensic specimens even if markers such as SNPs may be used in other scenarios [3]. In particular, the molecular structure of the SNPs (which, in case 5 6 of bi-allelic SNPs, is based either on a transition or a transversion of the wild type nucleotide) coupled 7 with the reduced molecular size of the amplicons makes this class of markers the best choice for the genetic identification of degraded samples [4,5]. It is also true, however, that PCR-CE may not 8 perform efficiently when low copy number and/or very degraded DNA is recovered from the samples 9 [1-3]. In these cases, in fact, even if several analytical approaches have been developed in the last two 10 decades, the resulting genetic profiles can be of scarce utility or even inconclusive [1-3,6,7]. 11

More recently, Massive Parallel Sequencing (MPS) technologies also entered the field of Forensic Genetics [8], allowing the customization of several kits. Even if MPS relies on PCR technology as well, the most important advantage over the conventional PCR-CE approach is that a huge number of markers with different molecular structure (e.g., STR and SNP) can be simultaneously multiplexed [8,9].

A few years ago, ThermoFisher company tested a new PCR-MPS panel, the HID-Ion AmpliSeqTM 17 Identity Panel kit, which allowed the genetic characterization of 103 autosomal SNPs plus 33 Y-18 markers [10]. After optimization of the prototypes [11,12], the ultimate kit format now allows the 19 20 simultaneous typing of 90 and 34 autosomal and Y-specific SNPs, respectively. The performances of this kit have been tested on control and reference samples (e.g., fresh samples) in several studies [13-21 23]; the resulting combined random match probabilities calculated in different populations are 22 between 1×10^{-34} and 1×10^{-37} [16-21,24,25]. However, only few studies focused on degraded samples, 23 that is samples whose analysis often shows partial or inconclusive genetic results. In particular, 24 Getting et al. [26] described the advantage of the use of this kit over conventional PCR-CE 25 commercial panels on a set of selected fragments eluted from sonicated DNA samples. More recently, 26 27 Salata et al. [27] reported the performances of this kit on a set of artificially degraded samples, among which a set of heat-treated bloodstains, and on a set of quite homogeneous forensic specimens (ten 28 29 bones and one tooth). Avila et al. [28] increased the set of forensic samples to 47 criminal specimens whose degradation indexes (DI) were however very similar to the ones of unmodified control samples 30 (DI < 1.5). Therefore, the performance of this kit should be investigated on more challenging 31 degraded/damaged real casework samples. It is worth mentioning that in two [27,28] out of these 32 33 three studies no threshold value was fixed for the locus call and a default coverage value of 6x was adopted for genotyping. The definition of calling thresholds has been questioned from the very 34

beginning of the PCR era [8,12], but currently the shared opinion is that the absence of threshold
values for a locus call would lead to unreliable results since background noise signals could be
erroneously mistaken as true alleles [8,12,14,15].

4 The aim of the present study is to assess the performance of the HID-Ion AmpliSeqTM Identity Panel

kit on the PGMTM platform starting from sub-optimal amounts (down to 12 pg) of DNA recovered
from degraded/damaged real casework forensic samples. In addition, the analytical condition leading

7 to a reliable locus call will be evaluated.

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9 2. Material and methods

10 2.1 Ethic statement

11 This study was approved by the Ethical Comity of the University of Trieste, Italy (101/04.12.2019).

12 Written informed consent was obtained from all living donors involved in the study.

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14 2.2 DNA Samples

Five laboratories collected a set of 87 forensic samples including saliva swabs (n=20), bone remains (n=19), cadaveric blood (n=7), cadaveric muscle (n=3), fingernails (n=2), FFPE tissues (n=15) and touch DNAs (n=16). The aging of the samples together with other relevant information on the samples are reported in **Table S1**. The availability of a reference sample (either "same donor" sample or "first degree relative" sample) was the recruitment criterion. In addition, DNA sample FM, already employed in other PCR-MPS studies [29], was used to produce a set of artificially degraded DNAs (n=5) following published protocols [30].

DNA quantification was performed by a single selected lab which was provided with six-ten 22 microliters aliquots of each sample in dry ice. DNA was quantified by duplicated tests on the Applied 23 Biosystems[®] 7500 real-time PCR System (Thermo Fisher Scientific) using the Quantifiler[™] Trio 24 25 DNA Quantification Kit (Thermo Fisher Scientific). In agreement with ref [27], when the amount of sample was below the Limit of Quantification (0.005 ng/µl), as assessed by the long target, the 26 degradation index (DI) was stated as "not computable" (nc). According to their DI, the samples were 27 pooled into three categories which were arbitrarily designed as follows: DI < 5 (not 28 degraded/moderately degraded), DI > 9 (degraded) and DI nc (very degraded), respectively. 29

The samples were finally sequenced in a selected laboratory. For sensitivity studies, the 2800M DNA
(Promega) was diluted appropriately.

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1 2.3 Library construction, template preparation, enrichment and MPS sequencing

Two hundred-fifty-five libraries were prepared by using the Precision ID Identity Panel and the Precision ID Library Kit (Thermo Fisher Scientific) according to the user guide [31]. Amplifications of genomic DNA target were performed with DNA input ranging from 1 ng to 12 pg and through a different number of PCR cycles (from 21 to 26), as outlined in Table S2. At least one negative amplification (no template) control was added in each round of PCR.

- 7 The total library-PCR system volume was 20 µL, which consisted of 4 µL of 5X Ion AmpliSeq[™] 8 HiFi Mix, 10 µL of Precision ID Identity Panel primer pool, DNA and nuclease-free water as needed to reach the total volume for each specific experiment. Thermal cycling was performed on the 9 VeritiTM 96-Well Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific) using the 10 following conditions: enzyme activation for 2 min at 99°C; denaturation, annealing and extension for 11 12 21-26 cycles of 15s at 99°C and 4 min at 60°C. After PCR, the amplicons were partially digested by adding 2 µL FuPa Reagent (Thermo Fisher Scientific) and incubated for 10 min at 50°C, for 10 min 13 at 55°C, and for 20 min at 60°C. Ligation of barcode adapters was performed by adding 4 µL Switch 14 Solution, 2 µL DNA Ligase and 2 µL diluted Ion Xpress[™] Barcode Adapters into 22 µL digested 15 16 PCR reaction, and followed by incubation for 30 min at 22°C and 10 min at 72°C. A negative control of ligation was also carried out by adding 4 µL Switch Solution, 2 µL DNA Ligase and 2 µL diluted 17 IonXpress_063 Barcode Adapters into 22 µL of water. After ligation, each library was purified with 18 AgencourtTM AMPureTM XP Reagent (Beckman Coulter) and then quantified by Ion Library 19 TaqMan[™] Quantitation Kit (Thermo Fisher Scientific) [31,32]. 20
- Six libraries (whose concentration ranged from 6.7 to 16.6 pM) were submitted to the optional step [31] of library amplification with 72 μ L PlatinumTM PCR SuperMix HiFi and 3 μ L Library Amplification Primer Mix using the following conditions: 2 min at 98°C, 8 cycles of 15s at 98°C and 1 min at 64°C. The amplified libraries were purified with AgencourtTM AMPureTM XP Reagent (Beckman Coulter) and then quantified as reported above.

All the libraries were diluted to 8 pM, pooled in equivolume and then submitted to emulsion PCR to 26 generate template positive Ion Sphere[™] Particle (ISPs) containing clonally amplified DNA. 27 Emulsion PCR (emPCR) was performed on the Ion OneTouch[™] 2 Instrument (Thermo Fisher 28 Scientific) with the Ion PGMTM Hi- QTM View OT2 Kit (Thermo Fisher Scientific). Template-29 positive ISPs were enriched with the Ion OneTouch[™] ES (Thermo Fisher Scientific) following the 30 manufacturer's recommendations. Sequencing was performed on the Ion Personal Genome 31 MachineTM (PGMTM) System using the Ion PGMTM Hi⁻ QTM View Sequencing Kit in 200-base read 32 mode. Sequencing primer and Control Ion SphereTM Particles were added to the enriched, template-33

positive ISPs. After the sequencing primer annealing, a sequencing polymerase was added and a final
 volume of 30 μL was loaded onto Ion 318TM Chip v2 (Thermo Fisher Scientific). Three libraries
 (namely #256, #257 and #258) were used in the fourth chip without dilution to 8 pM. In total, 255
 barcoded libraries were sequenced on four Ion 318TM Chip v2, as outlined in Tables S2.

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6 2.4 Sequencing data analysis

The alignment of reads against Homo Sapiens reference genome (GRCh37/hg19) was performed on
the Torrent SuiteTM v5.0.4 server. Coverage analysis was carried out by CoverageAnalysis v5.0.4.0
plugin [33], which provided statistics and graphs describing the level of sequence coverage produced
for Precision ID Identity targeted regions. Information about mapped reads, on-target percentage and
mean depth of coverage were downloaded for each sample library (Barcode Summary Report file).

Individual SNP genotypes were called by the HID_SNP_Genotyper v4.3.2 plugin with DNA target regions identified by the PrecisionID_IdentityPanel_targets.bed (Amplicon_Insert_IISNPv3) and known SNP variants listed in PrecisionID_IdentityPanel_hotspots.bed (iiSNP_FP_v2_hotspot) files. These resulting excel files were then used for data analysis.

16 Eighteen libraries related to ten different bone samples which showed above 10% of unmapped reads (range 13% - 82%; mean 43%) were submitted to further analysis in order to verify the occurrence of 17 out of target DNA amplification and sequencing. In these samples, unmapped reads were filtered by 18 using the "BAM filter" tool on Galaxy [34]. The derivative unmapped .bam files were then analyzed 19 on FastQC version 0.11.8 [35] in order to check the presence of overrepresented reads. They were 20 then downloaded and analyzed by Clustal W version 2.1 (Larkin, M.A 2007). The output of the 21 22 sequence alignment was visualized by Jalview 2.11.0 and the most represented sequences were compared on BLAST to finds regions of similarity between biological sequences. 23

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25 2.5 Data analysis

According to the DNA input used for PCR, the data of the selected libraries were pooled into three categories that were named as follows: OTQ (Optimal Template Quantity, e.g., 0.1-1.0 ng), STQ (Sub-optimal Template Quantity, e.g., 13-99 pg) and MTQ (Minimal Template Quantity, e.g., 12 pg). In addition, each group was further divided in three categories according to the degradation index (DI) of the samples. Therefore, nine classes of samples were in total defined (for example, the class OTQ/nc includes samples amplified with an amount of DNA from 0.1 to 1.0 ng and with degradation index not computable). The relative Depth of Coverage (rDoC) was calculated as the ratio between the coverage for a specific
 SNP locus and the overall coverage of the sample [37]. Comparison between the rDoC values was
 performed by r² test. Noise was calculated as reported elsewhere [14,15].

4 The presence of any of the four flags provided by the software (Major Allele Frequency, No Locus

5 Call, Percentage of Positive Coverage and Coverage) was scored and used for further elaboration.

6 Any replicate data were averaged.

7

8 2.6 Genotyping

9 The minimum coverage for locus call was empirically fixed at 50 x (see paragraph 3.6). All the 10 genotyping data were used irrespective both of their GQ (Genotype Quality) and the presence of flags. 11 The correctness of the results was checked by two operators visually. In particular, the results of each 12 test were compared with those of the corresponding reference samples and/or with those of the tests 13 performed with higher amounts of the same sample.

The frequency of amplification artefacts (both allelic drop outs and allelic drop ins) as well as the frequency of flags were given as the ratio between the total number of this kind of events out of the total number of markers with at least 50 reads of coverage.

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18 2.7 Consensus genotype

Standard methods (> (n/2)+1) [38] were used to build the consensus genotype by using the data of 19 two replicates. When analyzing the data of three replicates, a modification of the method proposed 20 by [27] was used. In particular, the homozygosity was assigned only if each of the three replicates 21 22 gave the same homozygous pattern. The heterozygosity (Ht) was assigned in the following four cases: 23 1) three identical heterozygous genotypes; 2) two identical heterozygous and one homozygous genotypes (in agreement with allelic drop out); 3) two identical heterozygous and one undetermined 24 25 genotypes (for example < 50 x); 4) one heterozygous and two homozygous genotypes for different alleles (both in agreement with allelic drop outs). In case of one heterozygous plus two identical 26 homozygous genotypes (for example, A/G + A/A + A/A), no result was assigned. 27

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29 2.8 Calculations and graphs

Microsoft Excel 2007 and Stata/SE version 12.1 (StataCorp) were used for calculations and graphs. For statistical analyses (*t*-test and ANOVA, when appropriate) significance was assumed with pvalues < 0.05.

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1 **3. Results and discussion**

2 3.1 DNA quantification

The samples were quantified in duplicate experiments using the Quantifiler[™] Trio DNA
Quantification Kit (Thermo Fisher Scientific). Quantifications were scored according to the Small
Autosomal (SA) Probe and reported in Table S1 together with the mean of the corresponding values.
In total, 59 samples showed a DI < 5 (average: 1.7; sd; 1.0; median: 1.3), 9 samples showed a DI > 9
(average: 24; sd; 35; median: 24; max: 110), while 19 samples showed a not computable DI. No
sample showed PCR inhibition as shown by the Ct values (< 26) of the Internal PCR Control (IPC)
probe of the kit.

10

11 3.2 The sequencing data: a general view

The main parameters of the four Ion 318 Chip run in this study are reported in Table S3. Out of the 13 11,287,275 addressable wells, 63.3 % showed Ion Sphere[™] Particles (ISPs) in average, with more 14 than 98.0 % of the ISPs represented by the libraries. The final ISPs libraries were 3,462,471 (48.9 % 15 of the total). These results fulfilled the recommended parameters for Ion 318[™] Chip v2 [31,33]. The 16 percentage of low quality and adapter dimer sequences was however high (up to 17.7 % and 15.7 %, 17 respectively) in the chips loaded with libraries built with low amounts of template [12].

Table 1 shows the values of library concentration (l.c.), mapped reads (m.r.), mean depth of coverage 18 (m.d.c.) and percentage of on-target reads (% o-t.r.) assessed for 193 out of 255 libraries subdivided 19 according to each quantitative category based on the amount of DNA input (the data of the remaining 20 62 libraries were used for comparison and/or other purposes (see Table S4)). The results show a 21 progressive decay of the values for all these parameters directly related to the decrease of the DNA 22 amount used for the amplification steps (*p*-value $\leq 4.9 \ge 10^{-4}$). Figure S1 shows how the different 23 numbers of PCR cycles affected the picomolarity of the libraries whose concentration was directly 24 related to the number of PCR cycles (for OTQ from the 24th cycle and for the other two categories 25 for the 26th cycle; *p*-value $\leq 1.0 \times 10^{-5}$) [27]. Even if the pM of the MTQ libraries were higher than 26 the no-template control (p-value = 0.0007), almost half of the MTQ libraries (23 out of 51) showed 27 concentration values below 17.9 pM (that is, in the range of the mean value plus one standard 28 deviation of the no-template controls). Therefore, it is likely that the accuracy in diluting the MTQ 29 libraries down to 8 pM was poor. This provides an explanation for the low m.r. and m.d.c. values 30 found for 12 pg DNA samples amplified through 22-24 PCR cycles, as well as for the outliers of 31 Figures S2 and S3. 32

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1 3.3 Blank controls

A total number of 17 no-template controls were run together with the samples. The mean value and standard deviation of the libraries' concentration was 10.7 ± 7.2 (median value was 9.3; min= 2.0; max= 28.4), while the other sequencing parameters are shown in Table 1. In average, 0.5 markers per samples showed a coverage higher than 50 reads, with the exception of library #106. In that case, 14 markers showed a coverage > 50 x, consistent with a contamination occurred during library preparation.

8 The duplicate analysis of the negative control set up during the extraction of a set of FFPE samples
9 showed a minimal contamination (with 8 markers above 50 x) consistent with the following finding
10 of an excess of drop-ins in those samples (see paragraph 3.11).

11

12 3.4 Noise

Ten positive control samples (DNA from the 2800M cell line), amplified for 21-26 cycles with DNA amounts varying from 0.1 to 1 ng, were used for this calculation. The mean noise ratio for the autosomal and Y markers was 0.054 % and 0.038 %, respectively. In addition, the noise ratio was determined in 29 samples showing DI < 5, amplified for 21 PCR cycles starting from 1 ng of DNA template. The noise ratio was below 0.083% in all samples. The mean male noise ratio in 17 female samples out of the 29 described above, calculated as the number of Y-SNPs reads out of the total number of reads, was 0.038%.

Overall, the results showed that the noise ratio in the samples analyzed in this study is very low, even lower than the value found by [13,14]. Four single samples exhibited a remarkable high noise ratio

- 22 (until 11%) just for a very limited number of markers (which were however flagged by the software),
- which were probably originated by contamination issues [14].
- Finally, the noise ratio was tested even in 0.27-1 ng of nine degraded samples amplified through 21-

26 cycles of PCR. The results were similar to the values recorded for the samples described above
(*p*-value > 0.065).

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28 3.5 Locus balance

The locus balance among autosomal and Y-specific loci was evaluated by a parameter defined relative depth of coverage (rDOC), which is the ratio between the coverage of each locus and the overall coverage of the sample [37].

- 32 Twenty-six DNA samples with a DI < 5, amplified starting from 1 ng, and nine tests performed with
- 33 0.1-1 ng of the 2800M DNA were selected to this aim. The autosomal loci were covered by 84.1%
- and 84.2% of the reads of the two groups of samples, respectively, in agreement with the data of ref.

1 [13-15,27]. In addition, the mean rDOC of these two sets of samples was compared showing a good 2 coefficient of correlation $r^2 = 0.810$. Figures S4A and S4B show the rDoC of the 26 samples with a 3 DI < 5. Overall, as previously described [13-15], the locus balance did not correlate with the 4 molecular weight of the amplicons, but it rather reflected the molecular features of the primer sets 5 designed for the multiplex PCR.

In order to investigate the locus balance in degraded samples, five DNAs (namely S2, S21, S17, S40 6 7 e S43) with degradation indexes between 5 and nc were selected. The mean rDOC for each marker resulting from the analysis of 1 ng DNA was then compared to the one obtained from the 2800M 8 9 DNA samples. The results for the autosomal and Y-specific SNPs comparison are described in Figures S5A and S5B, respectively. The data show that, in degraded DNA samples, the rDoC depends 10 11 mainly on the molecular weight of the amplicons, as low molecular weight loci were preferentially amplified. The locus balance of the depurinated DNA samples showed a peculiar trend, different from 12 the one of the five "naturally" degraded samples described above as six autosomal (rs2292972, 13 rs576261, rs719366, rs430046, rs221956 e rs873196) and one Y-specific markers (rs372157627) 14 15 showed higher DoC than those of the 2800M control DNAs. The finding that a set of loci exhibited a peculiar behaviour in PCR/CE [30] and PCR/MPS analysis of depurinated DNA samples has been 16 17 already described elsewhere [27,29] and can be ascribed to the peculiar damaging pattern affecting the purine nucleobases in the DNA sequence. 18

Finally, to assess the intra and inter repeatability of the experiments, the rDOC of duplicate tests were compared both for the 2800M control DNA and the five above mentioned degraded samples. The coefficient of correlation remained good ($r^2 > 0.658$) for amounts of template corresponding to 270-300 pg while it significantly decreased when 12 pg of DNA were used ($r^2 < -0.004$). In conclusion, the repeatibility of the rDoC -in duplicate amplifications- depended mainly on the starting amount of template, for both the high molecular weight samples and the degraded ones.

25

26 **3.6** Threshold for locus call

The Coverage Analysis v5.0.4.0 plugin is set up to provide a genotype when the locus reaches a coverage ≥ 6 x. The use of a threshold for the "locus call" (e.g., its genotyping) has been debated from the very beginning of the application of PCR-MPS technologies in Forensic Genetics, and a minimum of 20 x has been suggested as a reliable threshold [12]. To date, a wide range of thresholds, from 6 x to 200 x, have been used in different studies characterizing the Precision ID Identity Panel [12-14,23,26-28] as well other SNP-based methods [39-41]. In the present study, the definition of

this threshold has been empirically defined as follows.

Using the PCR-MPS technology [13,15], in the 0.1-1 ng of high molecular weight DNA input range, 1 each locus is usually covered by hundreds of reads, whereas locus coverage drops when lower 2 amounts of degraded templates are analyzed [26,27]. Remarkably, the frequency of PCR artefacts 3 (mainly allele drop outs) also increases when low amounts of degraded samples are studied [27,29]. 4 Based on these findings, in order to set up the best analytical threshold for the kind of samples selected 5 for the present study, three different threshold values (20 x, 50 x and 100 x, respectively) were tested 6 7 and applied to the PCR-MPS data recovered from two very challenging samples (S2 and S21); to this aim, 12 pg DNA replicated analysis of the two above mentioned degraded samples and the same 8 9 amount of the control DNA 2800M were set up (see Table S5). As expected, the percentage of 10 markers with a coverage higher than the three selected thresholds decreased (from 59.9 % to 31.9 % 11 for sample S2; from 56.9 % to 34.9 % for samples S21) as well as the percentages of markers showing PCR artefacts was lower (from 14.7 % to 11.1 % for sample S2; from 24.7 % to 19.2 % for sample 12 13 S21).

When the 100 x cut off value was adopted, the percentage of available markers in S2 and S21 was 14 15 statistically reduced in comparison to the 20 x cut off (*p*-value of 0.0001 and 0.0139, respectively; see Table S6). The following comparison between the 50 x and 100 x threshold revealed that the latter 16 17 reduced statistically the frequency of available markers in S2 (p-value = 0.0035). A reduction of the percentage of errors was found only in sample S21 when the results of the 100 x analysis were 18 compared with those of the 20 x one (p-value= 0.0463). No statistically relevant differences between 19 the thresholds were recorded for 2800M DNA control samples. Therefore, a threshold of 50 x was 20 considered an acceptable agreement between the number of available markers for genotyping and the 21 frequency of errors such as drop outs. However, these artifacts could not be completely eliminated 22 even introducing the 100 x threshold (dropout frequencies were in the range of 11.1%, 19.2 % and 23 24 7.1 % in S2, S21 and in the 2800M DNA, respectively).

25

26 *3.7 Sensitivity*

The Precision ID Identity Panel kit is designed for the analysis of one nanogram of DNA through 21 27 28 PCR cycles even if additional cycles are recommended for lower amounts of template [31]. As reported in Table S7, to test the sensitivity of the assay, scalar amounts (from 1 ng to 12 pg) of the 29 2800M DNA were amplified through 21-26 PCR cycles. Figure 1A shows that when the amount of 30 input DNA decreased, the frequency markers with less than 50 reads of coverage increased. 31 Specifically, when 12 pg of the control DNA were amplified, 9.4 % and 28.4 % of the autosomal and 32 of Y-specific markers, respectively, showed less than 50 reads. The GQ (Genotype Quality) scores 33 of the markers were, in average, higher in the OTQ and STQ samples (mean value 97.4 and 94.6, 34

respectively) than in the MTQ ones (mean value 87.5). Interestingly, correct typing was provided by
the software even for markers whose GQ was as low as 3.

3 Drop out phenomena were observed only when 50 pg DNA (or less) were used for the amplification,

with an average of seven drop outs per sample (min 4; max 11) in the 12 pg tests. These typing errors
were observed even in markers whose GQ was optimal (e.g., 99), thus confirming that the GQ score
provides only information on the quality of the sequencing data but not on the correctness of the

- 7 genotyping [15].
- 8

9 3.8 Optimization of the number of PCR cycles

In a previously published paper reporting data on the prototype of the Precision ID Identity Panel [27], a control DNA sample was amplified down to 5 pg through 26 PCR cycles even if, in the same paper, it was speculated that 24-25 cycles could be sufficient to produce the same grade of results. However, in that study a systematic comparison was not performed between a different number of PCR cycles. Other data advise against the use of more than 25 cycles as stochastic effects are enhanced [14,39].

16 Considering that additional PCR cycles could increase the number of artefacts when Low Copy 17 Number DNA is typed [2,3,27], 12 pg DNA replicated amplifications through 23 and 25 cycles of 18 five degraded samples (S2, S21, S17, S43 and S74) were compared in the present study. The results 19 showed that the employment of 25 cycles increased the frequency of markers with more than 50 reads 20 -in average- from 42.7 % to 56.4 % (*p*-value =0.011) while the frequency of drop out remained the 21 same (16.8 % vs. 18.8%; *p*-value = 0.232).

The data obtained from duplicate analyses of these five challenging samples were also assembled to 22 generate eighteen consensus genotypes based on pairwise comparisons as shown in Table S8. This 23 approach showed that the number of markers that could be typed was always higher when 25 cycles 24 were performed (p-value = 0.031), while the mistyping error frequency was similar (p-value = 0.303) 25 through the PCR cycles. When the data of triplicate tests were used to generate consensus genotypes, 26 the samples amplified through 25 cycles provided a higher number of loci successfully typed without 27 any genotyping error (see Table S8). All these results support then the choice of performing 25 PCR 28 cycles when low quantity DNA amounts (12 pg) has to be characterized. 29 For higher amounts of template, 21-22 cycles and 23-24 cycles provided suitable coverages for OTQ 30

Tor higher amounts of template, 21-22 cycles and 25-24 cycles provided suitable coverages for OTQ

and STQ samples, respectively (see Supplementary Figures 2-3), in agreement with previous studies

32 [27]. However, when degraded/very degraded samples have to be characterized in those quantitative

categories, it is recommended to add one-two PCR cycles (up to a maximum of 25 cycles).

1 3.9 Samples genotyping

A total of 13,908 and 3,991 autosomal and Y specific markers showed at least 50 reads, respectively,
and were thus considered suitable for genotyping (see Table S9 for details).

Figure 1B shows the frequencies of the markers displaying less than 50 reads, according to the three 4 quantitative and the three qualitative categories defined in this study. The data obtained from samples 5 6 with a DI < 5 were in agreement with those of the 2800 M DNA control sample, for each of the three 7 quantitative categories. As expected, to lower amounts of template corresponded to higher frequencies of markers with less than 50 reads of coverage. On the opposite side, an increase in 8 9 degradation indexes corresponded to an increase in the frequency of markers with less than 50 reads of coverage, in each of the three quantitative categories. Thus, while the finding of poorly covered 10 11 markers in the OTQ category with DI < 5 was unusual, the frequency of markers with less than 50 x of coverage increased dramatically in the MTQ category with high level of degradation. In 12 13 conclusion, the use of this threshold allowed the genotyping of more than 50 % and about 35 % of the autosomal and Y markers, respectively, when 12 picograms of very degraded DNA were 14 15 analyzed.

16

17 3.10 Allelic drop out

Out of the 479 allelic drop outs scored, 33, 112 and 334 occurred in OTQ, STQ and MTQ categories, 18 respectively, with frequencies ranging from 0.02 %, for the category OQT and DI <5, to 18-23 % 19 when 12 pg degraded/very degraded DNA were amplified (see Figure 2). One of the two alleles of 20 21 the heterozygous genotypes was totally missing in 446 cases (93.1 %) whereas in the remaining 33 tests it showed a low coverage. The median GQ (genotype quality) scores of these 446 markers was 22 38, 55 and 58 in the OQT, SQT and MQT categories, respectively, with the highest values in the last 23 two categories (*p*-value $\leq 1.5 \times 10^{-4}$). These data pointed out that false homozygous genotypes could 24 be assigned to a given locus as a consequence of acceptable/good GQ scores and without flags by the 25 software alerting the analyst [15]. The 33 alleles showing low coverage (in average 7.1-8.8 % of the 26 reads of the locus) were always highlighted with the MAF (Major Allele Frequencies) flag, meaning 27 28 an important allelic imbalance, and showed low GQ scores (median value= 18; min=8.3; max=20.0). In conclusion, the message emerging from the data analysis is that only a very limited percentage 29 (6.9%) of the total cases of real drop-outs could be speculated in the present study if the genotype of 30 the sample was not available. 31

Figure 3 shows the coverage of the surviving alleles, which ranged from 50 x to 300 x in about 86.8% of the cases, while in 13 cases (2.9 %) it was even higher than 600 x (up to 1,506 x). These data highlighted that there is no obvious stochastic threshold for locus call able to protect the expert from the occurrence of these amplification artefacts. A correlation coefficient $r^2=0.559$ was found between the molecular weight of the amplicons and the frequency of allelic drop outs (see Figure S6). As expected, the highest frequencies of allelic drop outs were scored for high molecular weight markers, even if it is well known that some low molecular weight loci are more prone to allelic imbalance than others [13-15], situation that can frequently turn out in the complete loss of the allele.

6

7 3.11 Allelic drop in

Only autosomal markers showed this kind of artefacts, while in ref. [27] they were described to occur 8 9 for Y-SNPs as well. In total, 184 allelic drop-ins were scored. Out of them, 179 markers which were actually homozygous showed an additional allele instead (for example, $A/A \rightarrow A/G$), while in the 10 11 remaining five cases the original homozygous pattern was replaced by the new one (for example, $A/A \rightarrow G/G$). Supplementary Table 10 shows the main features of the markers affected by this artefact 12 13 in the present study. In general, the median values of GQ ranged from 69 to 99 while the median values of the MAF was always high (> 76.3). In addition, about 96 % of these drop ins were flagged 14 15 by the Major Allele Frequency alert.

It has to be noted, however, that these drop-ins were not randomly distributed among the whole set 16 samples, but rather clustered in two sub-sets of samples. The first is a set of eleven FFPE samples 17 and the second originated from nine palmar swabs covering overall about 96 % of these artifacts. In 18 addition, the drop-ins occurred at the same loci of the same sample in replicate analyses, in both sub-19 sets. For this reason, minimal contamination issues in the DNA extraction step or minimal amounts 20 of exogenous human DNA transferred to the samples were likely involved rather than spurious reads 21 at high coverage. The remaining samples (including one 2800M control of 12 pg) showed a single 22 drop-in in one of the replicate analyses likely originated from PCR, sequencing errors, or, 23 alternatively, to a minimal contamination occurred in the pre-sequencing steps (target enrichment 24 PCR and library building). In conclusion, drop-ins seem to be a quite rare event in the samples 25 analyzed in this study. 26

27

28 3.12 Analysis of the flags

The Coverage Analysis v5.0.4.0 plugin provides four alerting flags: MAF (Major Allele Frequency),
NoC (No Locus Call), PPC (Percentage of Positive Coverage) and COV (Coverage). These flags are
evidenced for markers above the selected interpretation threshold, which in the present study is set to
≥ 50 reads. The detailed analysis of these flags is reported as follows.

33

34 3.12.1 MAF (Major Allele Frequency)

A MAF flag is assigned by the analysis software to heterozygous or homozygous genotypes. In the
first case, the reads of the two alleles are unbalanced (< 35 % or > 65 %) while, in case of homozygous
typing, the reads of the minor allele are < 10 % compared to the reads of the more represented one
[33].

A total of 769 MAF flags were scored for heterozygous genotypes. These flags occurred mainly in 5 the MTQ category and in DNAs with higher degradation indexes (see Table S11), in agreement with 6 7 what it is expected for PCR amplification of Low Copy Number DNA [1-3]. However, it is interesting to note that specific markers are more prone to allelic imbalance than others, as already reported 8 9 [14,15]. In the present study, about 8.6 % of these flags were recorded for the rs7520386 marker 10 which was systematically flagged even in the 2800M control DNA (other markers involved are rs987640, rs1077376, rs876724, rs914165, rs891700 and rs1048871). No correlation was found with 11 the molecular weight of the markers (r^2 = -0.208). In conclusion, no genotyping error was scored in 12 13 the 769 MAF flagged heterozygous markers, independently from their GQ (median value: 53; min 3; max 99). 14

15 A total of 47 MAF flags were scored for markers genotyped as homozygous by the software instead of the correct heterozygous genotype. In 33 cases (70.2 %) the genotyping error was originated by 16 17 the software which did not consider as true alleles the small residual number of reads (< 10 % compared to the reads of the more represented allele). These situations have been already discussed 18 in paragraph 3.10, whereas in the remaining 14 cases the flags originated by high noise ratio issues. 19 20 The final operative consideration is that a marker typed as homozygous and highlighted with a MAF flag must be evaluated carefully since a drop out artefact cannot be ruled out. In this situation, the 21 22 only possibility to clarify the real genotype is to perform a replicate test.

23

24 3.12.2 NoC (No call)

In total, 108 NoC flags were assigned by the software to 83 and 25 autosomal and Y markers, respectively, meaning that it was not possible to unambiguously assign a genotype to these loci. They occurred in more than 98 % of the cases in samples that showed a minimal contamination (see paragraph 3.11).

29

30 3.12.3 PPC (Percentage of Positive Coverage)

31 The software assigns this flag to a given marker if the forward and reverse sequencing strands are

unbalanced (< 30 % or > 70 %). In total, 389 flags were scored representing a frequency of 2.18 %.

The autosomal markers rs436205, rs1463729, rs2111980 and rs354439 and the Y-marker rs2032595

34 were mostly involved, thus suggesting a locus-specific susceptibility [13-15]. No correlation with the

amount of template was found. Genotype mistyping due to allelic drop outs was scored in 20 cases
for which minimal amounts of degraded DNA was used for PCR amplification. The corresponding
GQ of these 20 markers ranged from 11 to 99 (median value= 87).

4

5 **3.12.4** COV (Coverage)

6 The software flags a marker when its coverage is less than two standard deviations the average 7 coverage of the sample and, according to the instruction manual [33], this flag should be able to detect 8 potential allelic drop out artefacts. In total, 70 COV flags were scored from the analysis of good 9 quality samples amplified with optimal and sub-optimal amounts of template. Two autosomal 10 (rs2342747 and rs214955) and two Y markers (rs372157627 and rs4141886) were mainly flagged, 11 thus assuming a locus-specific predisposition [14,15]. At the end, only a single allelic drop out 12 artefact was identified.

13

14 3.13 Inhibited samples

Sixteen samples (4 bones, 11 touch DNAs and 1 buccal swab) displayed low sequencing depth 15 compared to what emerged from the sensitivity studies described above, even if a suitable amount of 16 DNA (ranging from 90 pg to 1 ng) was added for library reparation. In order to check the presence 17 of any PCR inhibitor in these samples, new libraries were built amplifying diluted volumes (down to 18 1:20) of input DNA and then submitting them to sequencing runs. This approach allowed a 19 remarkable improvement of all the sequencing parameters (see Figures S7A and S7B) as well a 20 successful typing in 15/16 samples. In conclusion, these results strongly support the hypothesis that 21 22 PCR inhibition [42,43] occurred in the first round of amplifications.

23

24 3.14 Experiments with undiluted libraries

25 Three libraries displaying quantification values ranging from 21.1 to 23.0 pM were selected in order to check if an increase in the library concentrations during the emulsion PCR step could improve the 26 genotyping efficiency, when low-level DNA is sequenced. To this aim, replicate analyses were 27 performed by adding either the above mentioned libraries as such or diluted to the concentration 28 suggested by the manufacturers (8 pM) (see Table S12). The tests displayed a high level of 29 repeatability of the rDoC ($r^2 \ge 0.979$). As expected, the use of undiluted libraries increased the values 30 31 of mapped reads, mean depth of coverage and number of markers with coverage ≥ 50 x but, on the other hand, this caused an increment of the number of genotyping errors as well. The error frequency 32 between the two sets of samples remained however statistically the same (p-value = 0.280). These 33

results showed that this approach could be considered a further tool for improving the genotyping of
 the most challenging samples (see below).

3

4 3.15 Experiments with additional PCR cycles after library preparation

5 Six amplified libraries were submitted to an optional step of re-amplification, by adding eight extra 6 amplification cycles (see Table S13). Libraries amplified for 23 cycles and re-amplified with additional cycles (23 + 8 cycles) were loaded and sequenced in two different chips. The tests 7 displayed a high level of repeatability ($r^2 \ge 0.983$) and showed an improvement of the values of the 8 9 main sequencing parameters (see Figure S8) together with the number of markers with a coverage 10 above 50 reads. Similarly, even if an increment of the number of the genotyping errors was scored, the error frequency remained statistically the same in the two sets of samples (p-value = 0.332). In 11 conclusion, although further studies are needed, our results confirm that the use of eight additional 12 cycles of amplification could be a useful tool for improving the results from low concentration 13 libraries [31]. 14

15

16 3.16 Assessment of non-human DNA sequencing in bone samples

Thirty-three libraries bam files originated from the analysis of eighteen bone samples were checked 17 in order to verify the presence of non-human DNA sequences (see Table S14). Out of them, twenty 18 libraries coming from eleven bone samples showed a percentage of unmapped reads above 10% and 19 poor genotyping results, and were thus selected to be assessed for the presence of non-human DNA 20 sequences. This analysis revealed the presence of a 50 nucleotides long sequence in all these libraries, 21 representing up to the 10 % of the unmapped reads; the following check on BLAST® detected 22 similarity between this sequence and Mycolicibacterium smegmatis. Sample S36 revealed the 23 24 presence of one group of 50 nucleotides long sequences which found similarity with Pseudomonas extremaustralis. Similarly, in sample S38 a group of 55 nucleotides long sequences was found, which 25 26 revealed similarity with Lysobacter enzymogenes. These data confirmed that bacterial DNA can be efficiently ligated into the libraries and sequenced [27], and provided further data on the bacterial 27 28 species which colonize skeletal remains [44]. Few samples showed a 26 nucleotides long sequence, 29 which did not return any result on BLAST®. It is interesting to note that this 26 nt sequence (5'CCAGAGGCTTCGGAGGAGGGCTGGCC3') was also found in no-template controls in 30 31 remarkable amounts. The origin of this sequence remains unknown as it does not match either with 32 those of the adaptamers or with other sequences used for the library construction.

1 **4. Final remarks**

The data presented in this study addressed the central role of the threshold value for locus call in 2 molecular analysis. It is well known that the minimum depth of coverage to assign a genotype depends 3 on the sequencing technology and on the end-point of the study [9,12]; in forensic genetic studies 4 based on PCR-MPS of SNPs, it widely ranged from a minimum of 6 [27,28] up to 200 reads [21,39] 5 depending on the set of specimens considered and the frequency of errors that it was intended to 6 7 accept in response to the increased number of scorable markers [9,41]. In this paper, the threshold for 8 the analysis of the data was fixed for all the markers at 50 x of coverage after having tested three 9 different values (20 x, 50 x and 100 x) on a set of LCN-DNA and degraded samples. Our evaluation 10 was that the 50 x threshold represented a proper balance between the two issues above mentioned, 11 allowing to type in average, for each amplification, more than 50 and 35% of autosomal and Yspecific markers, respectively, and to limit the number of errors when challenging samples were 12 13 examined. In addition, according to the data presented in this study, the amplification for 25 PCR cycles of the Precision ID Identity Panel kit represented the most appropriate experimental condition 14 15 in order to obtain a set of markers displaying valuable molecular data even from the most challenging samples. In conclusion, however, the results here reported clearly pointed out the need of setting up 16 17 a consensus profile [38] when LCN-DNA and degraded samples are tested in order to minimize the risk of mistyping. This consensus approach, in fact, allowed the typing -in average- of 33 out of 90 18 autosomal markers (see Table S8) and provided a good statistical support to the genetic evidence. In 19 detail, assuming 0.41 as the average random match probability (RMP) of each autosomal SNP marker 20 [24,25], the combined RMP was of at least 1.6×10^{-13} . Thus, even if only 37 % of the autosomal loci 21 22 were typed, the high number of markers which can be analysed by PCR-MPS provided however a high discriminatory power that underlined the usefulness of the SNP markers in the analysis of 23 24 degraded samples [4,5,26,27,44,45].

A critical issue emerged from the quantification of low concentrated libraries. In fact, about 45 % of 25 the libraries built with 12 pg of template showed picomolarities overlapping those of the no-template 26 controls thus leading to an overestimation of the real concentrations of those samples; this caused the 27 28 typing of only a limited number of markers (20-40 % of SNPs above 50 reads). In order to increase the percentages of markers above the 50 x threshold for these LCN-DNAs, two strategies were 29 adopted. The first considered to add eight PCR cycles to the standard 23 as suggested by the 30 manufacturer [31] and the second one to employ undiluted, not normalised, libraries. Both approaches 31 produced an increase in the number of markers with \geq 50 reads but no statistical significant increment 32 in the error rates. Although both these approaches seem promising, they require extensive evaluation 33 34 and validation before routine use in forensic genetics.

A further critical issue emerged from the analysis of a set of samples (mainly bones and palmar swabs) 1 which gave no or very poor coverage when 0.2-1 ng of DNA (in high volumes as much as 10 µl) 2 were tested. These samples were then diluted down to 1:20 and tested again thus providing good locus 3 coverages with the expected number of markers above the threshold. These results led us to speculate 4 that the Precision ID Identity Panel was sensitive to inhibition [41,42]. This is a tricky situation to 5 prevent as these samples showed IPC values < 26 Cq, not pointing out any inhibition, when quantified 6 7 with the QuantifilerTM Trio DNA Quantification Kit, which was however performed using a limited 8 volume of DNA (2 µl).

9 Finally, non-human DNA sequences belonging to different bacterial species were characterised analysing libraries bam files originated from bone samples buried in soil showing a percentage of unmapped reads above 10% and poor human-specific coverage. PCR-MPS technology offers the possibility to study these unmapped reads thus revealing the microorganism to which they belong. This can potentially lead to the definition of a soil microbiome profile, peculiar of that specific bone sample, which can be further compared with that of the soil where the bones were recovered or with different soil samples if there is the suspect that the bones were transferred from another burial site.

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	OTQ (0.1-1.0 ng)	STQ (13-99 pg)	MTQ (12 pg)	NT-CTRL
n	92	50	51	17
Library concentration (pM)	132.4	57.8	20.3	9.3
Mapped reads	70,929	52,374	23,475	4,415
Mean depth of coverage	494.5	252.7	121.3	0.8
% of on-target reads	94.0	85.9	76.4	7.7

Table 1. Pooled main sequencing parameters of the samples analysed in this study according to the

10 three quantitative categories OTQ, STQ and MTQ, respectively (the values refer to median values).

11 NT-CTRL: no template control; n= number of tests.

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Figure 1A. Frequencies of markers showing less than 50 x of coverage in the 2800 M cell line DNA.
The results are pooled according to each of the three quantitative categories based on the amount of
input DNA (in brackets the number of tests). Y-axis: frequency (x 1,000).

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Figure 1B. Frequencies of markers showing less than 50 x of coverage in the samples employed in this study. The results are pooled according to the nine classes obtained from the combination of the three quantitative (OTQ, STQ, MTQ) and the three qualitative categories (based on the DI). In brackets the number of tests. Y-axis: frequency (x 1,000).





Figure 2. Percentage frequencies of allelic drop outs. The results are pooled according to the nine
classes obtained from the combination of the three quantitative (0.1-1.0 ng, 13-99 pg and 12 pg) and
the three qualitative categories (based on the DI). Y-axis: frequency (x 100). nda: no data available.

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2 Figure 3. Coverage of the 479 surviving alleles scored in this study, according to the three

³ quantitative categories (OTQ, STQ, MTQ).