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Technical Note

Comparative Analysis of Five Forensic PCR Kits in Duplets

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Abstract: In forensic DNA laboratories, it is important to conduct internal validations of the commercially available kits of short tandem repeat (STR) loci and to investigate their individual and combined effectiveness. This study aims to report on a comparative investigation of the forensic kits used in our laboratory and their combinations in analysing low-copy-number (LCN) human DNA samples. We used five partly overlapping multiplex kits with different marker configurations from different manufacturers: the NGM Select™ PCR Amplification Kit, NGM Detect™, the GlobalFiler™ Amplification Kit (Applied Biosystem™, Foster City, CA, USA), the PowerPlex® Fusion 6C System (Promega Co., Madison, WI, USA) and the Investigator® 24plex QS Kit (Qiagen GmbH, Hilden, Germany). The efficacy of the kits was scrutinised by specific criteria, such as allelic dropout rate, the individually calculated Likelihood Ratio (LR) of consensus profiles and the LR value of the composite profile produced by the combined profiles of two kits. According to the results, the pairing of PowerPlex® Fusion 6C System and Investigator® 24plex QS produced the lowest, while the pairing of the NGM Detect™ and GlobalFiler™ kits provided the highest LR value. In summary, our study is meant to aid the selection of the optimal kit combination for samples of different qualities.

Keywords: forensic STR kits; dual-amplification strategy; likelihood ratio; allelic dropout rates; experimental investigation; low-copy-number DNA samples; composite profile; consensus profile

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1. Introduction

Forensic genetics laboratories routinely conducting in-house investigations, such as internal validations and sensitivity tests, can effectively improve the accuracy of results and the efficiency of workflows.

DNA profiling by multiplex polymerase chain reaction (PCR) is still the primary tool in analysing short tandem repeats (STRs) and investigating forensic evidence. For human DNA profiling, there are several commercially available PCR kits that show differences between important characteristics, such as the number of dyes, loci, loci arrangement or the required DNA input. Accordingly, there are kits suitable for the simultaneous amplification of a high number of loci (>20), while smaller kits producing less and reduced-size amplicons were designed specifically to analyse degraded samples [1,2]. Since casework samples show a wide range of quality, the usage of different kits offers unique approaches to analyse forensic evidence. However, before processing such samples, selecting the right kit or kits is essential.

To overcome the limitations of using a single kit, the combined usage of complementary kits has proved to be an effective optimisation technique [3]. Later on, the term "dual-amplification strategy" was introduced by Parys-Proszek et al. [4]. The application of parallel PCR runs with different kits and marker configurations—occasionally from different manufacturers—provides an opportunity to increase the number of successfully typed loci and create composite profiles of the highest quality. Given the limited amount of

DNA that can be extracted by automated DNA isolation methods and the possible number of PCRs, choosing the most ideal approach to DNA profiling can be a challenging task.

To save time, money and DNA for further analyses, we aimed to conduct a comparative assessment of the five forensic STR kits used in our laboratory and combine them in the most effective way to analyse low-copy-number (LCN) samples often encountered in caseworks. The tests involved five STR kits that—according to the Prüm Treaty about cross-border information exchange—entirely meet the European Standard Set (ESS) and highly cover the original core loci set of the Combined DNA Index System (CODIS) (Table 1) [5–8]. The NGM Select™ PCR Amplification Kit (NGMS), the NGM Detect™ PCR Amplification Kit (NGMD), the GlobalFiler™ Amplification Kit (Applied Biosystem™, Foster City, CA, USA) (GF), the PowerPlex® Fusion 6C System (Promega Co., Madison, WI, USA) (PF6C), the Investigator® 24plex QS Kit (Qiagen GmbH, Hilden, Germany) (QI24) and their combinations were investigated and tested in dual amplification [9–13].

Table 1. STR kits and the included loci overlapping the ESS and/or the CODIS core dataset.

Loci			Kit				
			NGMS	**NGMD**	**QI24**	**GF**	**PF6C**
FBI Original CODIS Core STR Loci	ESS Loci	D19S433	✓	✓	✓	✓	✓
		D2S1338	✓	✓	✓	✓	✓
		D16S539	✓	✓	✓	✓	✓
		D1S1656	✓	✓	✓	✓	✓
		D12S391	✓	✓	✓	✓	✓
		D2S441	✓	✓	✓	✓	✓
		D22S1045	✓	✓	✓	✓	✓
		D10S1248	✓	✓	✓	✓	✓
		vWA	✓	✓	✓	✓	✓
		D8S1179	✓	✓	✓	✓	✓
		D21S11	✓	✓	✓	✓	✓
		D18S51	✓	✓	✓	✓	✓
		TH01	✓	✓	✓	✓	✓
		FGA	✓	✓	✓	✓	✓
		D3S1358	✓	✓	✓	✓	✓
		D5S818			✓	✓	✓
		D7S820			✓	✓	✓
		D13S317			✓	✓	✓
		CSF1PO			✓	✓	✓
		TPOX			✓	✓	✓
		SE33	✓	✓	✓	✓	✓
		Penta D					✓
		Penta E					✓

To each kit, we applied the metrics of allelic dropout rate and the individually calculated Likelihood Ratio (LR) based on a consensus profile from three replicates. The efficiency of the kit combinations was evaluated using the LR of the composite profile based on pooling the information of two DNA profiles (1-1) produced by two different kits. To represent LCN samples, this comparison was conducted only in the case of PCRs with 20 pg DNA input. The composite DNA profiles will hereafter be referred as duplets.

Although the composite and consensus profiles have not been the main interest of comparative evaluations since the introduction of continuous models, they are still the focus of databases and data exchange for pragmatic reasons.

Our study provides a practical approach to effectively identifying the best PCR kit combinations for analysing LCN samples using such profiles.

2. Materials and Methods

2.1. Samples Collection and DNA Profiling

One buccal swab sample was collected from one known female person with a sterile Whatman OmniSwab™ sample collector (Qiagen GmbH, Hilden, Germany). Informed consent including ethical approval was obtained for the research, and the DNA profile of the donor was determined before the tests.

DNA extraction from the buccal swab sample was performed on an EZ1 Advanced XL biorobot (Qiagen GmbH, Hilden, Germany) with a large-volume protocol using the DNA Investigator kit (Qiagen GmbH, Germany). DNA was eluted in 50 µL of Tris-HCL/EDTA (TE) buffer. The DNA quantity was measured on the ABI 7500 Real-Time PCR System (Applied Biosystem™, Foster City, CA, USA) using the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems™, Foster City, CA, USA). The extracted DNA was first diluted to a stock solution, then according the three parallel PCRs containing 80 pg, 50 pg and 20 pg template DNA (3 × 3 PCR). The concentrations of the diluted solutions were also confirmed by the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems™, Foster City, CA, USA).

The following five commercial PCR kits were involved in the analyses: the NGM Select™ PCR Amplification Kit (NGMS), the NGM Detect™ PCR Amplification Kit (Applied Biosystem™, Foster City, CA, USA) (NGMD), the GlobalFiler™ Amplification Kit (Applied Biosystem™, Foster City, CA, USA) (GF), the PowerPlex® Fusion 6C System (Promega Co., Madison, WI, USA) (PF6C) and the Investigator® 24plex QS Kit (Qiagen GmbH, Hilden, Germany) (QI24). The profile combination or duplet was the composite profile based on the information of two DNA profiles (1-1) produced by two different kits.

All the PCR reactions were performed on Applied Biosystems® GeneAmp® System 9700 instruments (Applied Biosystem™, Foster City, CA, USA) according to the manufacturer's protocol, with the only exception being NGM Detect. In this case, the number of PCR cycles was 30 and, for optimisation reasons, the final volume was 22 µL, contrary to the manufacturer's original recommendation of 25 µL. PCRs were carried out on a Hamilton Microlab® Autolys STAR biorobot (Hamilton Bonaduz AG, Bonaduz, Switzerland; Hamilton biorobot). Positive and negative controls were included in all steps. Additional PCR specifications are shown in Table 2.

Table 2. Number of autosomal loci, number of dyes and PCR specifications of the five involved PCR kits.

Kit	No. of Autosomal STR Loci	No. of Dyes	Input DNA (µL)	Total PCR Volume (µL)	No. of PCR Cycles
GF	21	6	15	25	31
NGMD	16	6	12	22	30
PF6C	23	6	15	25	31
QI24	21	6	15	25	31
NGMS	16	5	10	25	31

PCR products were analysed on an Applied Biosystems 3500XL Genetic Analyzer (Applied Biosystem™, Foster City, CA, USA), and the electropherograms were evaluated on GeneMapper ID-X 1.4 (Applied Biosystem™, Foster City, CA, USA) software.

2.2. Statistics

Individual statistics were calculated for each kit based on three PCR repetitions conducted with each DNA input (80 pg, 50 pg, 20 pg). In total, nine (3 × 3) PCRs were carried out for each of the five kits. Based on the data, we calculated allelic dropout (%) and LR.

To estimate the LR value of the duplets, a composite profile was created using the worst (the one with the most missing alleles) profile out of the three repetitions of each kit. Representing the worst cases and LCN samples, duplets were analysed only in the case of PCRs with 20 pg DNA input. In total, ten combinations were tested. To strengthen the results, we also included the values of Random Match Probability (RMP). This metric is used to evaluate the weight of evidence that an unknown person may have the same profile. The smaller the value of RMP, the greater the evidence against the defence.

LR and RMP values were determined by the LRmix Studio 2.1.3. Community Edition software (©2013–2016 Netherlands Forensic Institute) applying the database recommended by the International Society for Forensic Genetics (ISFG) [14].

3. Results

3.1. Dropout Rate

PCRs containing 80 pg, 50 pg and 20 pg template DNA were used to determine the allelic dropout rates of each kit.

In the case of PCRs with 80 pg DNA input, there was no detectable allelic dropout for any kits. For 50 pg DNA input, the QI24 and the PF6C showed low percentages of allelic dropout, 1.67% and 3.79%, respectively. The dropout tendency of each investigated kit became clearly noticeable among the PCRs with 20 pg DNA input. According to our results, the most susceptible to dropout was the PF6C kit (31.06%), and the least susceptible was the NGMD (10.11%). The results are shown in Table 3. The details of allelic dropout for each kit, DNA input and locus are provided in Table S1 in the Supplementary Material.

Table 3. Allelic dropouts calculated from the three replicates with each DNA input.

	Allelic Dropout (%)		
DNA Input/Kit	**80 pg**	**50 pg**	**20 pg**
NGMD	0.00%	0.00%	10.11%
GF	0.00%	0.00%	18.33%
PF6C	0.00%	3.79%	31.06%
QI24	0.00%	1.67%	23.33%
NGMS	0.00%	0.00%	13.33%

3.2. LR for Each PCR Kit

LR values for each kit were calculated individually based on the consensus profile of the three replicates. The calculation was also carried out separately for the PCRs containing 80 pg, 50 pg and 20 pg template DNA (Figure 1).

In the case of the 80 pg template DNA category, no dropout was detected, so the LRs were maximised in 1.24×10^{26} and 1.87×10^{20} for kits with 21 and 16 autosomal loci, respectively. Due to the Penta D and Penta E, the PF6C reached a higher LR of 1.54×10^{30}.

For the PCRs with 50 pg template DNA, the GF, the NGMD and the NGMS produced the same LR magnitude, while it decreased for the PF6C and the QI24.

The 20 pg DNA input category varied the most in terms of LR. The NGMD produced the highest, while the NGMS and the GF produced the lowest LR values. The lowest LRs were generated by the PF6C and the QI24. Based on our results, the order of the kits in terms of individual statistical power for LCN DNA analysis was as follows: PF6C < QI24 < GF < NGMS < NGMD.

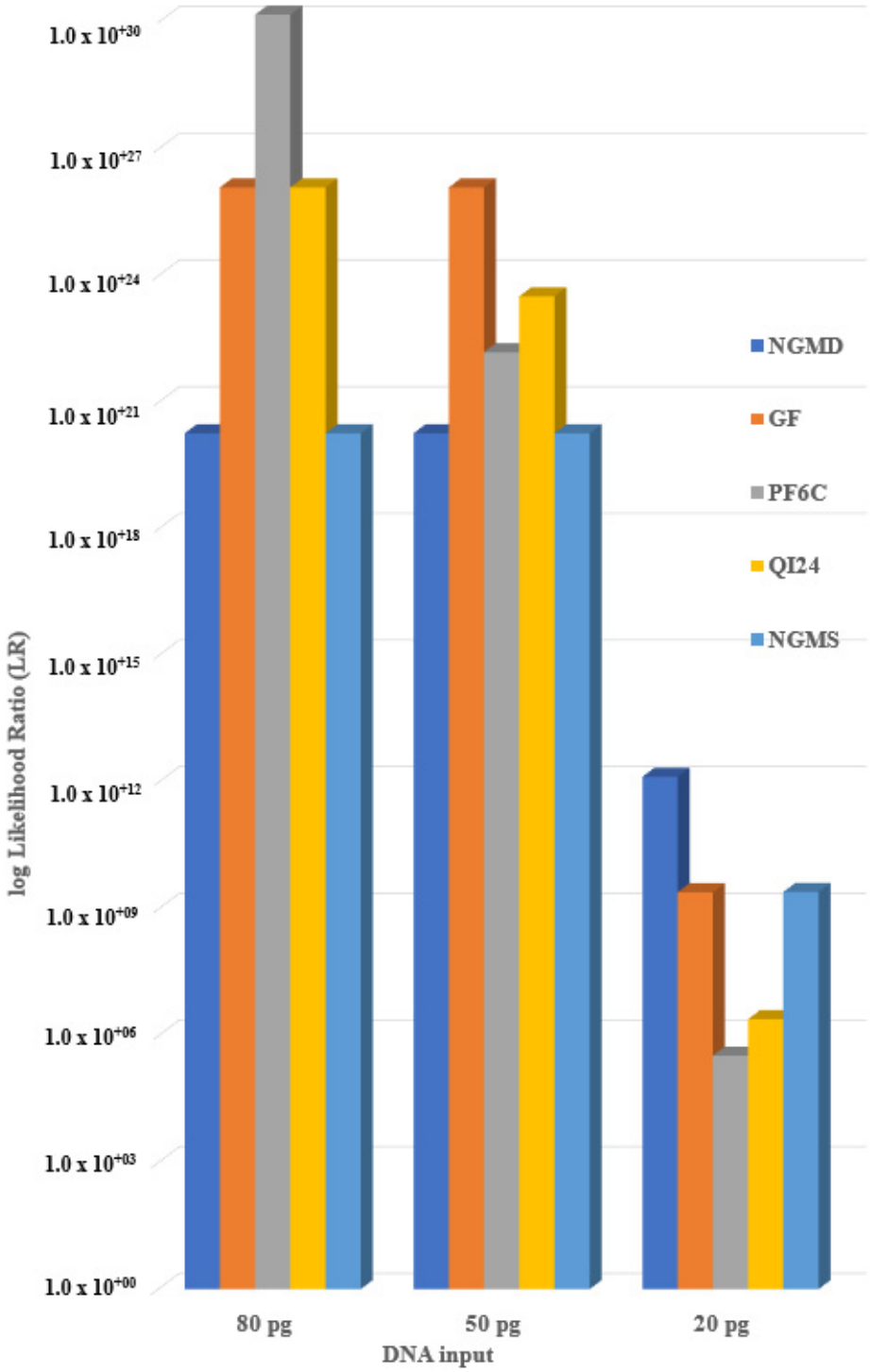

Figure 1. Individual logLR values for the consensus profiles of each kit and DNA input.

3.3. LR for PCR Kit Duplets

The duplet's efficacy was evaluated based on the LR using the composite profile created from the worst profiles of two different kits. The main interest in this study was to check the efficiency of the duplets when the possible DNA input was low, such as 20 pg DNA. According to our calculations, duplet formed by the NGMD and the GF produced the highest LR, providing the most effective solution for genotyping low template samples. The worst combination was the QI24 and the PF6C, which contained a higher number of loci. Based on the LR values, the ranking order of the duplet statistics was QI24+PF6C <

PF6C+NGMS < QI24+GF < NGMD+PF6C < QI24+NGMS < PF6C+GF < NGMD+QI24 < NGMD+NGMS < NGMS+GF < NGMD+GF. The results are shown in Figure 2.

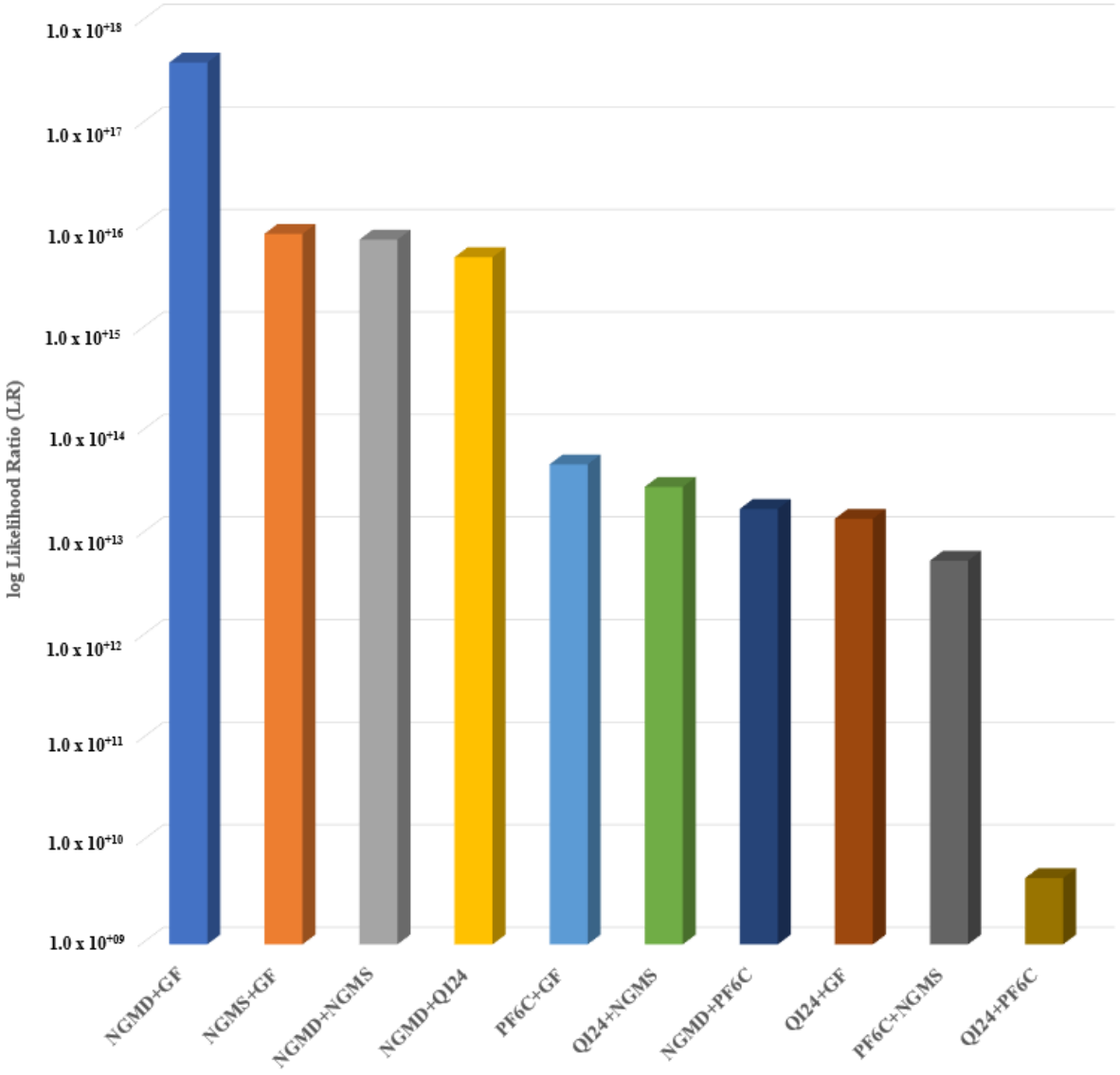

Figure 2. LogLR values for the duplets in the case of 20 pg DNA input.

The RMP values reflected the LR values (Table 4).

Table 4. LR and the corresponding RMP values for the duplets in the case of 20 pg DNA input.

Kit Combination	LR	RMP
NGMD+GF	3.97×10^{17}	2.52×10^{-18}
NGMS+GF	8.48×10^{15}	1.18×10^{-16}
NGMD+NGMS	7.46×10^{15}	1.34×10^{-16}
NGMD+QI24	5.03×10^{15}	1.99×10^{-16}
PF6C+GF	4.80×10^{13}	2.08×10^{-14}
QI24+NGMS	2.89×10^{13}	3.46×10^{-14}
NGMD+PF6C	1.76×10^{13}	5.67×10^{-14}
QI24+GF	1.42×10^{13}	7.06×10^{-14}
PF6C+NGMS	5.52×10^{12}	1.81×10^{-13}
QI24+PF6C	4.43×10^{09}	2.26×10^{-10}

4. Discussion

Following the latest developments in forensic genetics, laboratories must regularly use optimisation techniques to work efficiently. The wide selection of rapidly developing STR kits offers many approaches for analysing samples of different natures. To achieve the ideal investigation strategy, it is crucial to assess the efficacy of the commercially available kits and to determine their optimal combination.

Aiming to achieve the highest level of discrimination and the highest quality of DNA profiles, this study measured and compared the efficacy of the kits used by our laboratory in the dual-amplification strategy. To find the best resolution of forensic multiplex PCR kits, we combined the PCRs of five kits and calculated dropout rates and LR values. Individual analyses were based on consensus profiles created from three PCR repetitions carried out by the same kit, while the duplets—that were meant to represent LCN samples—were evaluated using composite profiles made of the worst profiles of two different kits.

The individual statistics of the investigated kits included the calculation of dropout rates and LR.

Regarding allelic dropout, the lower the DNA input, the higher the dropout. When a sufficient amount of 80 pg DNA was used, no dropout was detected. When 50 pg DNA was added to the amplification reaction, the kits containing the highest number of loci (PF6C and QI24, but not the GF) failed to amplify 3.79% and 1.67% of the loci. In the case of 20 pg template DNA, the dropout was between 31.06% and 11.11%. The NGMD kit could amplify the most, while the PF6C kit could amplify the fewest of its alleles. It is worth noting that the PF6C kit is also the one that investigates the most loci and enables us to provide the most information about a profile. The result of the NGMD kit confirmed, again, that this kit was successfully designed for degraded samples and—in line with our latest study—suitable for PCRs with low DNA input [4,15]. Out of the bigger kits, the GF kit presented a relatively low 18.33% of dropout.

When 80 pg template DNA was used, the highest individual LR value was produced by the PF6C kit containing the most autosomal loci (23), reaching the 10^{30} magnitude. The QI24 and the GF kits containing 21 autosomal loci reached a 10^{26} magnitude, while the smaller kits with 16 autosomal loci reached a 10^{20} magnitude. When 50 pg template DNA was used, out of the three 'big' kits, only the GF was able to keep the same LR, similarly to the NGM kits. Nevertheless, in the case of 20 pg template DNA, the NGMD kit provided the highest LR value of 10^{12}, while the GF could still provide the same LR as the NGMS that contains only 16 autosomal loci. Although we expected the kits containing fewer loci to perform better when a small amount of DNA was available, the GF kit still showed satisfying results. The 20 pg DNA input category varied the most in terms of LR, possibly partly due to the different number of loci included in the kits.

The duplet investigation was planned regarding the limitations of casework samples and PCRs. Since degraded and/or LCN samples and the final reaction volume also limit the possible number of PCRs, we decided to take only one (the worst) profile out of the three PCR replicates of each kit and combine them to create a duplet. This consideration had a technical reason behind it. As it was mentioned before, our samples were eluted in 50 µL buffer that—using robotic DNA isolation methods—allows only two repetitions on full reaction volume, using the common amount of a 15 µL template. After the minimum two repetitions of the same kit required in our laboratory, the remaining approximately 20 µL allows only one more PCR, which could be more informative if it is carried out by another, overlapping kit. Representing more extreme, but not so rare situations, the analyses were conducted only for the PCRs with 20 pg input DNA.

In our study, we combined five multiplex PCR kits, and the best combination of kits providing the most versatile solution for the LCN samples was the GF+NGMD, although this combination was not the one with the maximum number of overlapping loci among the possible combinations. This finding is also consistent with our results regarding the dropout rates and the individual LR values based on the three PCR repetitions. Parys-

Proszek et al. also compared the efficacy of the GF+NGMD and the NGMS+NGMD kits in combination and confirmed the efficiency of these kits [4].

The NGMS+GF, the NGMS+NGMD and the NGMD+QI24 duplets also produced high LRs.

Interestingly, the combination of the two 'small' kits (NGMD, NGMS) containing only 16 autosomal loci performed better than the combination of the two biggest kits, the PF6C and the QI24, which produced the lowest LR value. The PF6C kit generated weaker results in all combinations that participated, even with the NGMD kit that was particularly designed to maximise the information recovery from degraded samples. In addition, it is clearly visible that the NGM family—although it contains less loci—is able to efficiently contribute to DNA profiling when challenging samples are analysed. The supportive properties of the NGMS and NGMD kits in terms of analysing degraded samples and using them as supplementary kits was also confirmed by Burch et al. [16].

Summarising our results, the dual-amplification (duplet) strategy is an efficient approach to maximise the LR values and minimise the allelic loss when the PCRs and the available DNA input are limited. Out of the tested kits, we recommend using the combination of the GF and the NGMD kits, which provided a remarkably high LR value, even for PCRs with highly limited DNA input (20 pg). Our results can offer further assistance to other forensic laboratories in selecting optimal dual-amplification strategies to each kind of situation.

Based on recent publications about the efficiency of reduced-volume PCRs, we believe that future studies should include half-volume PCR reactions to further expand the options for analysing challenging samples and increase the efficiency of information recovery [17–19].

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/dna4030014/s1: Table S1. Number of missing alleles for each kit, DNA input and loci, based on three repetitions.

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Data Availability Statement: All relevant data are contained in the article. Should you be interested, the corresponding author would be glad to provide further information.

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Conflicts of Interest: The authors declare no conflicts of interest.

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