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Optimizing direct amplification of forensic commercial kits for STR determination

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ABSTRACT

Direct DNA amplification in forensic genotyping reduces analytical time when large sample sets are being analyzed. The amplification success depends mainly upon two factors: on one hand, the PCR chemistry and, on the other, the type of solid substrate where the samples are deposited. We developed a workflow strategy aiming to optimize times and cost when starting from blood samples spotted onto diverse absorbent substrates. A set of 770 blood samples spotted onto Blood cards, Whatman® 3 MM paper, FTA™ Classic cards, and Whatman® Grade 1 was analyzed by a unified working strategy including a low-cost pre-treatment, a PCR amplification volume scale-down, and the use of the 3500 Genetic Analyzer as the analytical platform. Samples were analyzed using three different commercial multiplex STR direct amplification kits. The efficiency of the strategy was evidenced by a higher percentage of high-quality profiles obtained (over 94%), a reduced number of re-injections (average 3.2%), and a reduced amplification failure rate (lower than 5%). Average peak height ratio among different commercial kits was 0.91, and the intra-locus balance showed values ranging from 0.92 to 0.94. A comparison with previously reported results was performed demonstrating the efficiency of the proposed modifications. The protocol described herein showed high performance, producing optimal quality profiles, and being both time and cost effective.

1. Introduction

Forensic genotyping is the result of a multi-step workflow, which includes sample collection, DNA extraction, quantification, short tandem repeats (STR) amplification, capillary electrophoresis (CE), genotype analysis, and interpretation. The obtained results from DNA-based evidence, as well as their interpretation, have major relevance at courtrooms to elucidate criminal and civil cases.

Intense research efforts have been made by scientists and companies to improve highly informative genetic markers, such as In-Del,[1–3] SNP,[4,5] rapidly mutating Y-STR,[6] and new generation techniques,[7,8] not only to improve and complement the identification power of the currently available marker set but also to simplify experimental procedures. Each development provided reliable, high-quality results with less time-consuming efforts. Over the years, methodologies have been developed that enable DNA amplification directly from biological samples such as blood, saliva,[9] and fingernails without previous DNA extraction.[10] Direct STR amplification kits reduced analytical time without pre-treatment of the sample, although the quality of the genetic profiles depends on the chemistry of these kits.[11] High-quality results from direct amplification were reported for AmpFℓSTR® Identifiler® Direct and PowerPlex™16,[12] AmpFℓSTR® Identifiler®,[9] AmpFℓSTR® SGM Plus™,[13] GlobalFiler®,[14] and PowerPlex™18D.[15] The development of a rapid forensic genotyping technique is the result of a combination and optimization of a high-speed thermal cycler, a rapid polymerase, and an enhanced buffer.[16,17]

Direct amplification from relevant forensic samples including fluids as semen[13] and blood stains on fabrics and diverse supports has also been reported[12,18–20]; however, sometimes without a high success rate.[11] In general, commercially available direct amplification kits require a pre-treatment of the sample, such as pre-PCR DNA elution, to obtain amplification.[21–23] Commercial buffers,

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e.g., Direct-N-Elute with AnyDirect buffer (*BioQuest, Seoul, Korea*)[9] complemented with enhanced amplification buffers (e.g., bovine seroalbumin), QIAGEN Multiplex PCR Kit (*QIAGEN, Silicon Valley, Redwood City, California, USA*), Prep-n-Go™ lysis buffer (*Life Technologies, Applied Biosystems, Foster City, CA, USA),*and/or engineered enzymes such as Phusion Flash polymerase (*Phusion, Leiden, The Netherlands*) are used to overcome PCR inhibitors.

Despite technical improvements in routine casework, some pieces of evidence, as well as reference samples, may still be challenging. Different types of supports, sample distribution across the material, amount of material collected, and storage time and conditions could affect the success of PCR amplification. Likewise, large peak height variations can be observed when each sample is compared with a standardized input quantity of liquid DNA.[24,25] As recommended by manufacturer's instructions, failure to pre-treat non-FTA samples with Punch Solution™ kit *(Promega Corp., Madison, WI, USA)* may result in incomplete profiles.[22,23] Therefore, protocol standardization is required for reference samples from different sources such as buccal swabs and blood drops deposited onto different supports (e.g., cotton swabs or blood cards). A wide variety of such products is commercially available such as swabs from Bode Buccal DNA Collectors™ (*Bode Technology, Lorton, VA*), Whatman® EasiCollect™ Devices (*GE Healthcare Life Sciences, Buckinghamshire, England*), classic cotton swabs, brushes, and different types of papers such as FTA™ cards (*GE Healthcare Life Sciences*), Blood cards, and Whatman® 3 MM paper *(GE Healthcare Life Sciences*).

Aiming to reduce the re-amplification rate after direct PCR amplification we developed a workflow strategy for analyzing blood samples collected on different types of paper with different blood quantities as well as storage time and conditions. The processing strategy has been successfully used for the last five years in our laboratory and showed to be a suitable approach that allows time and costs reduction without compromising the quality of the genetic profiles.

2. Materials and methods

2.1. Sample collection

Samples were collected at the Department of Forensic Genetics and DNA Fingerprinting Service, School of Pharmacy and Biochemistry, Universidad de Buenos Aires (UBA), Argentina. The sampling included 770 voluntary donors, who participated in paternity testing. All donors read and signed a written consent statement form, approved by the Ethical Committee of the School of Pharmacy and Biochemistry, UBA. Approximately 50 μL to 2 mL of blood were spotted onto diverse types of paper including 115 (15%) on Blood cards, 539 (70%) on Whatman® 3 MM paper, 39 (5%) on FTA™ Classic cards, and 77 (10%) non-conventional filter paper for DNA preservation purposes such as Whatman® Grade 1 (*GE Healthcare Life Sciences*). These supports represent all the possible alternatives received at our DNA Fingerprinting Service as reference samples for routine casework requested by ten different country-wide Departments of Justice. Accordingly, the personnel who routinely obtain the reference samples and/or select the pieces of evidence apply different criteria for preservation and delivery of the items for analysis to the laboratory. The workflow proposed herein represents the result of the optimization of a one-round direct DNA amplification from blood samples spotted onto diverse supports used for preservation of reference samples.

2.2. Sample preparation

Five to six 2-mm discs per blood sample were cut with a saddler cutter, placed in a 2-mL Eppendorf tube, and washed twice with 1 mL of distilled water at 37 °C for 15 min with agitation (75 rpm) in a VorTemp (Labnet, USA). Although only one paper was employed in the PCR reaction, several papers were extracted in order to permit different marker systems to be used (e.g. Y-STRs, X-STRs or mt-DNA analysis). After discarding water, the paper discs contained within the tube were allowed to dry at room temperature (procedure A). Alternatively, a 100% ethanol wash step was introduced at the end of procedure A (assigned as procedure B). The effects of the drying time were evaluated at the two procedures by leaving the treated paper punches at 37 °C for 20 min, 1 h, or overnight.

2.3. PCR amplification

Three commercial direct STR amplification kits were tested, namely, PowerPlex® 18D (PP18D), PowerPlex® 21 (PP21), and PowerPlex® Fusion (PPF) Systems *(Promega)*. In all cases, a scale-down was performed leading to a 12.5 μL final volume including 2.5 μL reagent mix, 2.5 μL primer mix, 7.5 μL of distilled water, and one 2-mm punch. Alternately, ten samples without treatment and ten samples treated with Punch Solution™ reagent (*Promega*) were tested with PP21 and PPF *(Promega)* following manufacturer's protocol in order to compare the success rate and the PHR to the obtained in the strategy proposed herein. When the Punch SolutionTM reagent was incorporated to our Lab, the PP18D kit was replaced by PP21. Accordingly, the combination Punch Solution™ reagent and PP18D were not tested. As positive control, we used ~2–3 ng control DNA 2800 *(Promega)*, which was amplified under identical conditions as those of the punches. PCR cycling profile followed manufacturer's instructions. The thermal cycling was set at 27 cycles in all cases, and a final extension at 60 °C for 20 min was added to prevent ± A peaks, as recommended by the manufacturer. The PCR cycling time was approximately 1.5 h in all the cases. All reactions were performed in a Gene Amp® 9700 Thermal Cycler with max mode as the ramp speed (*Life Technologies*).

2.4. Capillary electrophoresis (CE)

PCR products were prepared for subsequent analysis by adding 1 μL amplified product to 9 μL Hi-Di™ Formamide (*Life Technologies*) and 0.75 μL internal size standard ILS 500 (*Promega)*. CE and detection of PowerPlex® 18D amplicons were performed in a 3500 Genetic Analyzer using POP-7™ (*Life Technologies*), 50-cm capillary, 5 kV injection voltage, and 1.3 s injection time. Alternatively, PowerPlex® 21 and PowerPlex® Fusion amplicons were injected at 4 kV and 1 s injection time.

Samples were re-injected in two opposing situations. Firstly, in those cases in which the peaks did not reach the cut-off value of 100 relative fluorescence units (RFU), samples were re-injected increasing time or voltage. The cut-off value was previously determined by validation assays that allowed defining the standard operation procedure (SOPS) of our laboratory. Secondly, in those cases where the profiles showed excess of pull-ups, re-injections were carried out reducing injection time or voltage. The resulting electropherograms were analyzed, and the genotypes were assigned with the expert Gene-Mapper® ID-Xv 1.2 software (*Life Technologies*).[26]

2.5. Genotype concordance

To test the “PCR scale down at half volume”, 10 reference samples, previously analyzed as part of the proficiency test exercises organized by the Spanish and Portuguese Speaking Working Group of the International Society of Forensic Genetics (GHEP-ISFG) in

2015, were analyzed with PowerPlex® 18D, PowerPlex® 21, and PowerPlex® Fusion Systems to test genotype concordance.

2.6. Peak height evaluation

Aiming to evaluate the success of the amplification process, the electropherograms were divided into two zones based on the amplicon length, expressed in base pairs (bp). Low and high molecular weight zones were defined as follows: zone I (low molecular weight) between 85 and 250/300 bp and zone II (high molecular weight) over 250/300 bp, depending on the amplification kit used. Supplementary Fig. S1 depicts the markers' distribution and the zones of high/low molecular weight for each of the tested systems (a-PP18D, b-PP21, and c-PPF).

Peak height ratio (PHR) was determined for each locus considering all heterozygous genotypes and dividing the peak height, expressed as RFU, of the lower allele by the higher allele.

2.7. Evaluation of the genetic profile quality

In addition to the quality parameters included within the expert software used, a set of numerical indexes such as total peak height (TPH), mean local balance (MLB) (i.e., the mean of intra-locus balances or discrepancies between peak heights within a heterozygous STR marker), and inter-loci balance estimated by Shannon Entropy (SH) were calculated.[26,27] This analysis allowed an objective comparison of the genetic profiles. When analyzing PPF, the SH index was calculated separately for females and males as it depends on the number of markers (DYS391 is located on the Y chromosome and only appears in male samples).[26,27]

2.8. Statistical analysis

An ANOVA (ANalysis Of VAriance) analysis was performed in PHR obtained from treatments with Punch Solution™ reagent *(Promega)*, water washes and direct amplification. ANOVA analysis was also performed to test statistically difference between procedure A and B and different time of drying.

3. Results

The development of new methodologies requires adequate qualifying criteria for objective comparisons. Thus, to evaluate the proposed workflow we qualified amplification efficiency and heterozygous balance of the genetic profiles.

We evaluated the efficiency of the workflow strategy by analyzing the percentage of genotyping success in 770 blood samples. Although, a considerable degree of blood volume variation was reported by the labs that submitted blood samples, minimal differences were detected in the obtained profiles. In fact, the sample considered in our work is independent of the overall volume spotted onto de substrate. The amount of DNA bound to the substrate is mainly determined by the diameter of the punch and the thickness of the solid substrate. No major deviation was observed among the amplification success rate for the different type of solid substrate where the samples were spotted.

Table 1 summarizes the analytical efficiency of our workflow strategy, the use of Punch Solution™ and direct amplification without any treatment. In water procedure, most samples showed high-quality genetic profiles and the number of re-injections or failed amplifications were reduced to less than 5%. The results were similar for all commercial kits with over 95% high-quality profiles. PowerPlex® 21 showed the lowest re-amplification rate, whereas PP18D showed the highest. When direct amplification was tested with PowerPlex® 21 and PowerPlex® Fusion, we obtained an efficiency of 70% and 80%, respectively; lower than the 96.77% and 93.20% obtained for PP21 and PPF respectively using our workflow. When the samples were treated with Punch Solution™ kit, the efficiency was 100% and 90% for PP21 and PPF respectively (Table 1).

The quality of the genetic profiles obtained by the two DNA extraction methods (procedures A and B, with or without ethanol washes) was similar, amplifying in the 100% of samples. Decreasing the drying time had no detrimental effects on the quality of the genetic profiles, even when the paper was not completely dry at the time of setting the PCR reactions. ANOVA analysis reveals no significant statistical differences in terms on PHR between both methods with different drying time ($p > 0.05$).

Regarding peak heights, for both PP21 and PPF, higher peaks were observed in zone I compared to zone II, whereas for PP18D slightly higher peaks were observed in zone II, as shown in Supplementary Fig. S2. The average height obtained with a single 2-mm punch, processed as described, was 2700 RFUs, ranging from 2000 to 3500 RFUs for all the tested kits.

To evaluate and standardize measures, PHRs were plotted as shown in Fig. 1. For all markers and kits, a PHR over 0.6 was considered an acceptable value according to forensic community standards. The lowest PHR was obtained for D2S1338 in PP18D (0.85), Penta E in PP21 (0.87), and D22S1045 in PPF (0.89), The average PHR was 0.89 ± 0.07, 0.91 ± 0.06, and 0.92 ± 0.06 for PP18D, PP21, and PPF, respectively (Table 1). Regarding Punch Solution™ treatment as well as direct amplification, ANOVA analysis showed that the average PHR was statistically significantly lower ($p > 0.05$) than the PHR obtained by the proposed strategy (Table 1). Unlike the water strategy' PHR, a greater deviation was obtained employing any or Punch Solution™ or treatment which reflects that not all markers have the same amplification efficiency (Table 1).

Supplementary Table S1 shows the numerical indexes that qualify the electropherograms. The mean locus balance showed values ranging from 0.92 to 0.94 ± 0.02. The inter-loci balance estimated by Shannon Entropy showed average values that reached 97% of maximum for PP18D (2.81 over a maximum of 2.89), 95% for PP21 (2.89 over a maximum of 3.04), and 96% for PPF (3.04 over a maximum of 3.17 for males; 3.00 over a maximum of 3.13 for females) showing that the inter-loci balance is correct. The highest total peak height was obtained for PP21 and the lowest for PP18D. As expected, the total peak height values ranged from 49,741 to 64,742 RFUs, probably due to height variations within different markers.

Our results are in line with those reporting the efficiency of the main direct amplification commercial kits designed for human genetic profiling, published within the last five years (Table 2).

4. Discussion

The aim of this work was to describe an optimized workflow for direct amplification of blood samples collected on different types of filter paper. To date, many protocols have described direct amplification strategies without pre-treatment or DNA extraction steps.[9,13] Nevertheless, in several cases, the amplification failure rates range from ~25% to ~73%,[28] being more than 50% for PP21[11] as it is schematically described in Table 2. The standardization process is difficult as several variables are involved, including different types of paper, drying conditions, and age of the stains,[15,18] which are beyond the control of the laboratory when samples are sent by third parties (e.g. criminalistic labs, police labs, etc). Our routine casework deals with at least ten different reference and evidence samples types from suppliers along the country. Moreover, the heterogeneity of the supports where the samples are deposited represents a challenge for an efficient standardization of the

Table 1
Comparison of the success rate of the workflow strategy proposed, the Punch Solution or the direct amplification.

Water washes	**PP18D (%) n = 270**	**PP21 (%) n = 248**	**PPF (%) n = 252**	**Average (%) n = 770**
High-quality profiles	93.70	96.77	93.20	94.5
Re-injected samples	2.20	3.22	4.40	3.20
Non-amplified samples	4.10	0.01	2.40	2.30
Average PHR	0.89 ± 0.07	0.91 ± 0.06	0.92 ± 0.06	0.91
Direct amplification	**PP18D (%) n = 270**	**PP21 (%) n = 10**	**PPF (%) n = 10**	**Average (%) n = 20**
High-quality profiles	–	70	80	75
Non-amplified samples	–	30	20	25
Average PHR		0.89 ± 0.12	0.89 ± 0.11	0.89
Punch Solution™ reagent	**PP18D (%) n = 270**	**PP21 (%) n = 10**	**PPF (%) n = 10**	**Average (%) n = 20**
High-quality profiles	–	100	90	95
Non-amplified samples	–	0	10	5
Average PHR		0.87 ± 0.19	0.86 ± 0.16	0.86

The acronyms: PP18D, PP21, and PPF stand for PowerPlex® 18D, PowerPlex® 21 and PowerPlex® Fusion Systems, respectively.

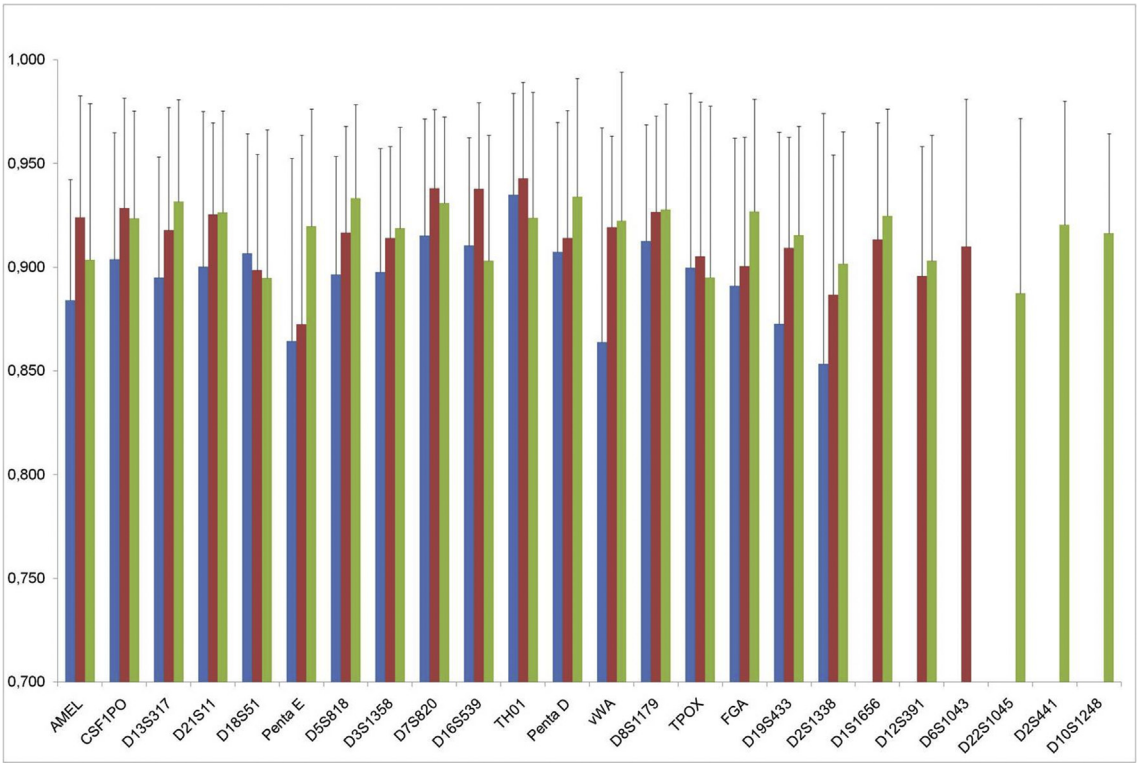

Fig. 1. Peak height ratios for all loci included in the three multiplex systems tested: PowerPlex® 18D System (PP18D) in blue, PowerPlex® 21 System (PP21) in red, and PowerPlex® Fusion (PPF) in green. Errors bars for each PHR are represented as a line on each box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

technical workflow to provide optimal results to the Departments of Justice.

The genotype concordance test not only demonstrated reliable performance when PCR was carried out in a reduced reaction volume but also showed full allele concordance when compared with results obtained from reference samples that were, previously typed with PowerPlex®16HS, PowerPlex® 18D, and PowerPlex® 21 during the 2015 edition of the proficiency testing exercise conducted by the Spanish and Portuguese Speaking Working Group of the International Society of Forensic Genetics (SPSWG-ISFG). With the exception of PowerPlex® 18D, these STR systems are routinely used in our laboratory.

The process described herein does not require the use of commercially available extraction kits or buffers like Punch Solution™ reagent for non-FTA papers as recommended by the manufacturer's instructions for PowerPlex® Fusion and PowerPlex® 21.[22,23] Additionally, distilled water is the only reagent used for sample pre-treatment; therefore, reducing the costs. The probable mechanism of action of the water on the blood spotted samples onto solid supports is the reduction of hemoglobin content. It is well known that hemoglobin is an efficient inhibitor of the DNA polymerase enzymes and two washes with distilled water seems to be enough to remove it as an inhibitor substance. Moreover, it was demonstrated that the reduction of the drying time -although the

Table 2
Comparison of the results reported herein and those summarized in literature on direct amplification.

Starting material	Sample/ pre-treatment	Kit/cycles/ final volume	Genetic analyzer	Evaluation	Reference
770 samples: 50 μL to 2 mL whole blood on different blood cards	2-mm punch/2 washes with water	PP18D, PP21 PPF/ 27cycles/12.5 μL	3500 Applied Biosystems Genetic Analyzer	Efficiency of approximately 95%	Present work
150 buccal swabs on FTA® cards	1.2-mm punch without treatment	IFD/25 cycles/15 μL	3130xl Applied Biosystems Genetic Analyzer	Efficiency of 93.3%	Stene et al. 2011[37]
18 blood samples on Whatman bloodstain cards 18 buccal samples on Whatman Sterile Omni Swabs	1.2-mm punch without treatment	IFD/ND/12.5 μL	3500 Applied Biosystems Genetic Analyzer	Efficiency of 80% for buccal samples and 100% for blood samples.	Brito et al. 2011[38]
66 blood and 84 buccal samples onFTA® cards	0.53-mm punch without treatment	IFD/26 cycles/10 μL PP16HS/27 cycles/ 10 μL	3730 or 3100 Applied Biosystems Genetic Analyzer	Efficiency of 97% for blood ID Efficiency of 94% for P16HS Efficiency of 89% for ID and P16HS for saliva	Laurin et al. 2012[12]
400 buccal samples on FTA® cards	Two 1.2-mm punches for PP18D and one for ID Direct without treatment	PP18D/27 cycles/25 μL IFD/27 cycles/25 μL	3130xl Applied Biosystems Genetic Analyzer	Efficiency of 96.25%	Myers et al. 2012[15]
340 blood samples on paper-based porous, fabric/textile porous/miscellaneous non-porous or hard porous	1-mm punch without treatment 1-2 mm of blood swabs without treatment	PP21/27 cycles/13 μL	3130xl Applied Biosystems Genetic Analyzer	Efficiency of 80%	Gray et al. 2014[28]
2° 3 from 8 each lab FTA, buccal swab or non-FTA card	Buccal swabs were extracted with Swab Solution™ reagent *(Promega)* Non-FTA card punches pre-treated with Punch Solution™ reagent *(Promega)*	PPF-6C/25 cycles/ 12.5 μL AmpSolution™ reagent (*Promega*) added in all cases	3500 3500xL 3130 3130xl Applied Biosystems Genetic Analyzers	Efficiency of 100%	Enselberg et al. 2016[39]

PowerPlex® 18D (PP18D), PowerPlex® 21 (PP21), PowerPlex® 16HS (PP16HS), PowerPlex® Fusion (PPF), PowerPlex® Fusion 6C PPF-6C, Identifiler®Direct (IFD), ND: not declared.

papers treated only with water were not completely dried - or the addition of ethanol to accelerate the drying process, do not alter the quality of the genetic profiles. Regarding peak heights, the results obtained for punches were comparable to approximately 2–3 ng of the commercially available control DNA 2800. This feature allows PCR standardization for liquid samples, e.g., buccal or blood swabs/ stains processed by semi-automated robots such us Maxwell®16 (*Promega)* or EZ1 Advanced XL (*QIAGEN*). For extraction robots, the range of DNA quantity obtained depends on the amount of starting material on the swabs as well as the robot's capacity. Therefore, an optimized DNA dilution for PCR amplification would usually be useful to obtain reliable genetic profiles while omitting the sample quantification step.

More than 95% of the samples were successfully genotyped with the three commercial kits tested using the strategy described herein. Only ~4% of the samples were re-amplified by PP18D, 2.4% by PPF, and less than 1% by PP21. The amplification failure could be attributed to diverse factors including (a) exposure of the blood spotted onto paper cards to >40 °C before sample preparation, (b) whether the paper punches were totally or not submerged in the reaction mix during the PCR reaction, (c) the age of the stain, and (d) the storage environment. The impossibility to remove denatured proteins from the cards that might inhibit amplification can be circumvented by using a regular DNA extraction method (e.g., liquid phase).

The DNA profiles considered in the analysis were those with peak heights ranging between 100 and 20,000 RFUs. The average height for all the tested kits was approximately 2750 RFUs, which is the recommended height for ABI 3500 Series Genetic Analyzer,[29] and it is comparable with that of previous studies.[14,30] Additionally, the ABI 3500 Genetic Analyzer instrument has a much higher off-scale limit than the 31XX Series Genetic Analyzers, and it was shown that even peaks higher than 30,000 RFUs did not produce associated pull-ups. Moreover, the ABI 3500 Genetic Analyzer works in a wider range than the 310 or 31XX Analyzers, making it more robust and reducing the number of re-injections.[29,31] However, in some cases, using short injection times, for the three commercial kits tested, disabled ILS automatic labeling requiring manual labeling.[15]

While peak height is proportional to the DNA concentration, peak height ratio is an objective standardized measure to evaluate electropherogram quality. Although low PHRs are associated with low amounts of DNA, low heights are not necessarily associated with low PHRs. In this work, the average PHR was 0.91, higher than the 0.89 obtained for Punch Solution™ treatment, 0.86 obtained for any treatment and much higher than the 0.6 usually considered as indicator of good profile quality. The average PHR value for each kit increased according to the time when they were released in the market, being those most recently released the most efficient one (e.g., PPF). The lowest PHR was obtained for D2S1138 in PP18D, Penta E in PP21, and D22S1045 in PPF. In all cases, the values exceeded 0.6. One possible explanation could be attributed to the high molecular weight zone where the markers are located (defined as zone II in the Results section). It is known that the markers with high molecular weight are less efficiently amplified and more likely to show imbalance.[32] This is also reflected in Supplementary Fig. S2 where PP21 and PPF show lower peak heights for amplification fragments longer than 250bp. The peaks height evaluation is represented as TPH, which is a straightforward but misleading measure of the profile quality as a consequence of the signal intensity. As expected, the standard variation in TPH was large due to the variation in peak heights for the different markers along the electropherogram as a consequence of working with different samples and starting DNA quantities. In cases of high fluorescence, saturation due to template DNA overload and artifacts like dye blobs, bleed-through peaks or odd peaks produced by non-linear matrix effects might appear. On the contrary, extreme low peaks heights could mask stochastic effects such as allelic or genotypic drop-out and heterozygote imbalance lowering the profile quality.[33] The commercial kits tested showed to be robust;

therefore, high-quality profiles could be obtained even without working within a strict range of DNA amounts. Moreover, the use of the 3500 Genetic Analyzer as the analytical platform for profile detection enables the analysis of a wide range of peak heights and, therefore, a wide range of DNA quantities without artifacts.

To standardize quality measures, we calculated other indexes based on two quality aspects, which together describe the DNA profile quality, namely, balance within a locus (MLB) and balance between loci (SH). Both numerical indexes depend on the number of markers, and MLB also depends on PHR. For all the kits tested, this index was beyond 0.9 (±0.02) showing an excellent balance within a locus. The MLB value increased with the evolution and technological improvement of the commercial kits, reaching a value of 0.94 for PPF. The balance between loci was measured in terms of Shannon entropy. The maximum value of SH depends on the number of markers (n), being SH = ln (n). SH showed a maximum value of 2.89, 3.04, 3.14, and 3.18 for PP18D, PP21, PPF females, and PPF males, respectively. The SH obtained in all cases represents more than 95% of the maximum value showing high amplification efficiency for all markers regardless of the peak height.

Although there are reports of similar or higher percentage of direct amplification success, the results are not comparable since they either start from other DNA sources using other commercial kits or do not scale down the reaction volume, as described in Table 2. Likewise, other publications have evaluated different commercial kits, e.g., GlobalFiler® Express,[14,34] PowerPlex® Fusion,[25] PowerPlex® 21,[24] PowerPlex® ESI 16/17 Fast,[35] or PowerPlex® 18D.[36] In these reports, the authors evaluated parameters like sensitivity, accuracy, precision, stutter studies, number of cycles, annealing temperature, intra-locus imbalance, peak height ratio, and peak height. However, they have not clearly reported the efficiency of the proposed strategy. On the contrary, we used PowerPlex® 18D, PowerPlex® 21, and PowerPlex® Fusion starting from diverse supports, simply washing with distilled water, and carrying out the PCR reaction in a 12.5 μL final volume as described. Therefore, the strategy proposed herein is a valid alternative. When direct amplification was tested using Punch Solution™ reagent, the results showed excellent success rate at PowerPlex® 21 and PowerPlex® Fusion kits, but the PHR was statistically significant lower than our strategy which reduced the electropherogram quality. However, the use of the strategy proposed in this study results in an excellent amplification efficiency and avoids the use of commercial reagents.

5. Conclusion

We present a workflow strategy which includes a low-cost sample pre-treatment, the omission of DNA quantification, a scale down of the reagents for PCR amplification, and the use of the 3500 Genetic Analyzer as the analytical platform. Although the success of the presented strategy is comparable to that of other strategies, this proposal has been showed to be highly effective, producing optimal quality profiles and being cost-effective. The standardization of working conditions allowed overcoming the issues of having blood samples on different supports as starting material.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jflm.2017.01.003.

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