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Technical Note

Use of the Investigator ESSplex SE QS Kit (QIAGEN) at Half PCR Reaction Volumes for the Analysis of Forensic Samples

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Abstract: The Investigator ESSplex SE QS Kit (Qiagen) is a next-generation polymerase chain reaction (PCR) kit that, in 60 min, amplifies 17 Short Tandem Repeat (STR) markers, including the five European Standard Set (ESS) loci (D10S1248, D12S391, D1S1656, D22S1045, D2S441), the SE33 marker, and the locus Amelogenin for sex determination. Two quality sensors (QS1 and QS2) are also co-amplified to check PCR performance. Since forensic laboratories carry out hundreds of DNA typings annually, we verified the kit's performance using half reaction volumes with the aim of improving the number of samples that may be amplified with a single kit and consequently reducing laboratory costs. In the present study, intended as a technical note rather than internal validation, some control samples (oral swabs) with known DNA profiles and 40 real casework samples were analyzed. We observed that reducing the total reaction volume, while keeping all component ratios unaltered, yields DNA profiles comparable to those obtained using standard reaction volumes and with allele peaks higher than those with regular volumes. Using half volumes for PCR amplification enables the analysis of a larger number of samples compared to the standard protocol, thereby reducing laboratory costs without compromising the quality of the analysis.

Keywords: DNA; STR; multiplex; reduced volumes

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1. Introduction

STRs are the first-choice markers for human identification and paternity tests because of their features and the good polymorphism degree. The ability to amplify multiple STR markers using fluorescent-labelled PCR primers together with laser detection by automated sequencers increases the amount of information collected from a sample. Additionally, it reduces the DNA quantity necessary to obtain results, and labor time and costs, because multiple loci are analyzed simultaneously in the same assay [1].

Over the years, commercial multiplexes were initially designed to amplify only a few STR loci, and later, they expanded to include all 13 markers from the CODIS database [2,3].

After the Treaty of Prüm (2009), it was decided to expand the 12 European Standard Set (ESS) markers including five new core loci to favor the amplification of degraded DNA samples.

In 2012, in the USA, the FBI proposed the expansion of CODIS core loci to 20 markers to increase international compatibility and discrimination power [4]. In order to simultaneously analyze European and USA core loci, new commercial multiplexes able to amplify up to 27 markers were introduced. These PCR kits are now commonly used in forensic laboratories due to their high discriminatory power and ease of use, facilitated by the inclusion of all reagents required for PCR amplifications (reaction buffer with polymerase, primer mix, positive controls, allelic ladder). Additionally, they have improved chemistry capable of overcoming inhibitors generally found in forensic samples [5,6].

As forensic laboratories conduct hundreds of DNA tests annually, it has become common to use reduced PCR reaction volumes, which are verified in-house, as demonstrated by several publications [7–11]. In our previous studies, we focused on testing the suitability of other commonly used forensic multiplexes for PCR amplification of forensic samples using half reaction volumes [12,13]. In the present study, intended as a technical note and not as internal validation, we verified the performance of the Investigator ESSplex QS on control samples and casework samples, using half of the standard prescribed reaction volumes. The aim was to improve the number of samples that may be amplified with a kit, consequently reducing analysis costs. The Investigator ESSplex SE QS is one of the new-generation PCR kits designed for forensic and paternity applications. It utilizes fast-cycling technology that allows simultaneous amplification of the following 16 STR markers in just 1 h: D1S1656, D2S1338, D2S441, D3S1358, D10S1248, D8S1179, D12S391, D18S51, D16S539, D19S433, D21S11, FGA, D22S1045, TH01, vWA, and SE33, and the locus Amelogenin for sex determination [14,15]. The kit co-amplifies two quality sensors named QS1 (71 bp) and QS2 (435 bp), which act as internal PCR controls providing information about PCR efficiency, the presence of PCR inhibitors, and the DNA degradation status.

2. Materials and Methods

2.1. Male Control DNA 9948 Analysis

According to the manufacturer's (Qiagen) protocol, a DNA input of 0.5 ng is recommended for amplification at regular volumes. To evaluate if this is the correct quantity when using half PCR volumes, we amplified with regular and half PCR reaction volumes, 0.1 ng, 0.5 ng, 1 ng of the Male Control DNA 9948 (0.1 ng/µL), available in the kit.

2.2. Sample Analysis

2.2.1. Control Samples

Saliva samples were collected using sterile oral swabs (FLOQSwabs, Copan, Brescia, Italy) from four unrelated volunteers (2 men + 2 women) with known DNA profiles. Collection was performed under controlled environmental conditions (25 °C), and then oral swabs were stored at 4 °C for 1 day until their use.

2.2.2. Forensic Samples

Forty forensic samples were selected to represent a wide range of forensic samples: 10 bloodstains (white cotton, blue denim, white paper, black wool, wooden stick, plastic cover, knife blade, leather, marble floor, FTA card), 5 semen stains (pink cotton, blue denim, beige cotton, toilet paper, condom), 8 saliva stains (water bottle neck, black balaclava, white plastic cup, white tissue, FTA card, cigarette butt, chewing gum, toothbrush), 7 contact traces (laptop keyboard, car wheel, knife handle, gun trigger, bullet, eyeglass temples, door handle), 3 femurs (time elapsed between death and sample collection: 1 year, 5 years, and 10 years), 2 molars teeth (time elapsed between death and sample collection: 1 year and 5 years), 1 paraffin-embedded tissue, 2 anagen hairs with roots, and 2 vaginal swabs. After collection, all samples were stored at 4 °C for 1 week.

Forensic Sample Pre-Processing

Biological stains on porous surfaces: a fragment of the stain (3 × 3 mm) was cut.

Biological stains on non-porous surfaces: the stain was collected using a sterile swab.

Bones and teeth were washed with distilled water, 80% ethanol, and 5% hypochlorite, and, after drying, powdered in liquid nitrogen using a using a cryogenic mill. They were then decalcified in 0.5 mol/L ethylenediaminetetraacetic acid (EDTA) for 5 days [16]. Remains collected after 10 years were interred in coffins within the cemetery grounds, and femurs gathered after 1 and 5 years were placed in a burial niche wall.

2.2.3. Informed Consent and Ethical Aspects

Control samples and forensic samples were taken with previously obtained informed consent signed by the donor's or by strict authorized relatives, according to Italian Law D.Lgs. 196/2003, and approved by SIMEF laboratory procedures. By signing the informed consent, the person authorized that laboratory, when the requested analysis was finished, to use a part of the remaining sample for research studies, unless otherwise indicated. According to the internal laboratory procedures, each sample was classified by a unique alpha-numeric code "00-N" that was used in all analytical steps for identifying the sample. This allowed us to guarantee anonymity, safeguarding the donors' privacy.

2.3. DNA Extraction

All samples were extracted, by the EZ1 Advanced XL DNA Investigator kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, using the biorobot EZ1 Advanced XL and the Trace protocol. Bones were extracted using the modified protocol developed by the Authors and previously published [17]. Positive (known sample) and negative controls were included.

2.4. DNA Quantification

DNA extracted from oral swabs and casework samples was quantified by the Investigator Quantiplex Pro RGQ Kit (QIAGEN) using the Rotorgene Q Real-Time instrument (Qiagen), which also allows estimation of the presence of inhibitors eventually co-extracted in the sample and the DNA degradation status. Data obtained were analyzed by the Q-Rex software v.1.0 together with the Quant Assay Data Handling Tool 3.3 according to the manufacturer's protocol [18,19]. Positive (DNA sample at a known quantity) and negative quantification controls were included to verify the efficiency of the method.

2.5. DNA Amplification and Typing

PCR amplification of all samples by the Investigator ESSplex SE QS was carried out using either the standard protocol recommended by the manufacturer (PCR total reaction volume of 25 µL containing 7.5 µL Master Mix, 2.5 µL Primer Mix, variable-volume Grade Water, and template DNA) or half the reaction volume, maintaining an unaltered volume ratio of each PCR component (PCR total reaction volume of 12.5 µL containing 3.75 µL Master Mix, 1.25 µL Primer Mix, variable-volume Grade Water, and template DNA). The amount of DNA used for PCR amplification (with regular and half volumes) of oral swabs and casework samples, was 0.5 ng, according to the manufacturer's protocol [20] and sensitivity test results (see Section 3.1). The amplification was performed using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems—Life Technologies, ThermoFisher Scientific, Waltham, MA, USA), at the following conditions: 3 cycles (98 °C for 30 s + 64 °C for 55 s, 72 °C for 5 s) + 27 cycles (96 °C for 10 s, 61 °C for 55 s, 72 °C for 5 s) + 1 hold (68 °C for 120 s) [21]. In addition, a negative control and the positive Male Control DNA 9948 (0.1 ng/µL) were amplified. All samples were amplified in duplicate, according to the laboratory internal procedures. PCR products were detected by capillary electrophoresis using the sequencer ABI3130 Genetic Analyzer (Applied Biosystems—Life Technologies, ThermoFisher Scientific, Waltham, MA, USA): the internal lane DNA standard BTO 550 was used for calibration. Allele assignment was carried out by comparison with the ESSplex SE QS Ladder using the GeneMapper ID 3.2 software (Applied Biosystems—Life Technologies). According to the internal laboratory guidelines, amplification was considered successful when DNA profiles showed the following characteristics: peak heights > 100 relative fluorescence units (RFUs), intra-locus peak height ratio > 70%, absence of extra peaks or artifacts (drop-in), absence of locus drop-out or allelic drop-out.

2.6. Data Analysis

All calculations and one-way ANOVA (analysis of variance) were carried out using a Microsoft Excel spreadsheet.

3. Results and Discussion

3.1. Male Control DNA 9948 Analysis

It is well known that using too much DNA for PCR amplification may produce off-scale data and incomplete A-nucleotide addition, while an extremely low quantity may result in unbalanced amplification. According to the manufacturer's developmental validation of the Investigator SE ESSplex, robust and balanced results may be obtained with up to 2 ng of DNA using the standard protocol. The DNA quantity recommended for PCR amplification is 0.5 ng [21]. In a first step of the present study the optimal DNA input amounts for PCR amplification at half volumes was established using different quantities of Male Control DNA 9948 (0.1 ng, 0.5 ng, 1 ng).

Peak heights obtained with full and reduced PCR volumes were compared for each locus using the 100 RFUs analytical threshold (Figure 1). Variation in RFUs was observed in the four dye channels depending on the DNA quantity. In general, it was observed that amplification using reduced volumes produced higher peak heights for all loci. The average standard deviation (SD±) for each dye channel for different DNA quantities has been evaluated using regular and half reaction volumes. The average standard deviation was comparable in both reactions: generally, the blue channel showed a higher SD. Since STR allelic peak heights are approximately proportional to the effective amount of amplifiable DNA, these findings appear more evident when using 1 ng of DNA input for PCR amplification, either with regular or with half volumes. This is probably due to the highest peaks being obtained especially for shorter-sized amplicons, and consequently, the main variability in RFUs (Figure 2). This is in accordance with data obtained by other authors [22].

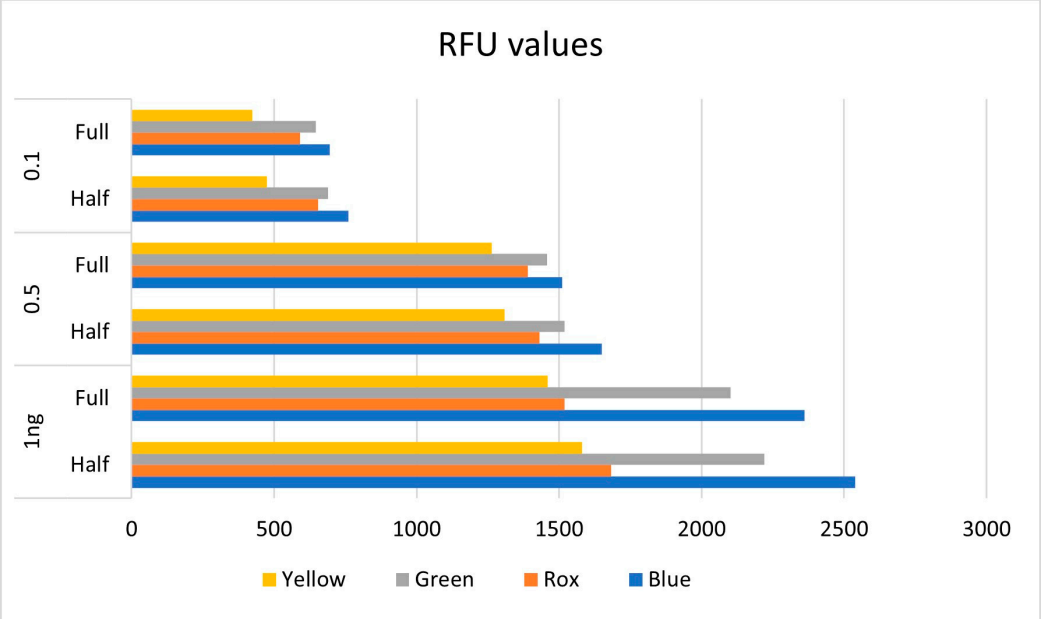

Figure 1. Average RFU values for each dye channel using different quantities of Male Control DNA 9948 (0.1 ng, 0.5 ng, 1 ng) amplified with regular and half PCR reaction volumes.

In our opinion and in accordance with the manufacturer's developmental validation of the Investigator SE ESSplex, also using half reaction volumes, the DNA quantity of 0.5 ng represents the optimal amount to be used in terms of peak heights, balance, and low signal noise [23].

In forensic DNA analysis, concordance between duplicate samples is required for the quality assurance of results [24]. In this case, allele assignment by Genemapper software 3.2 was identical in all duplicates, obtained either using regular or half volumes (Figure 3).

Per each locus, allele calls were the same as those reported in the reference table included in the Investigator® ESSplex SE QS Handbook [20].

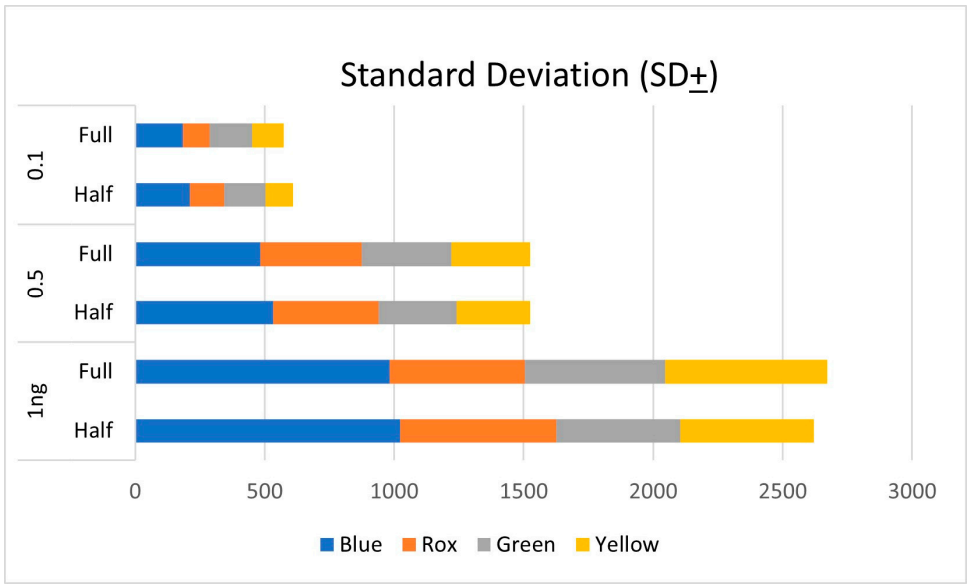

Figure 2. Average standard deviations (SDs±) for each dye channel using different quantities of Male Control DNA 9948 (0.1 ng, 0.5 ng, 1 ng) amplified with regular and half PCR reaction volumes.

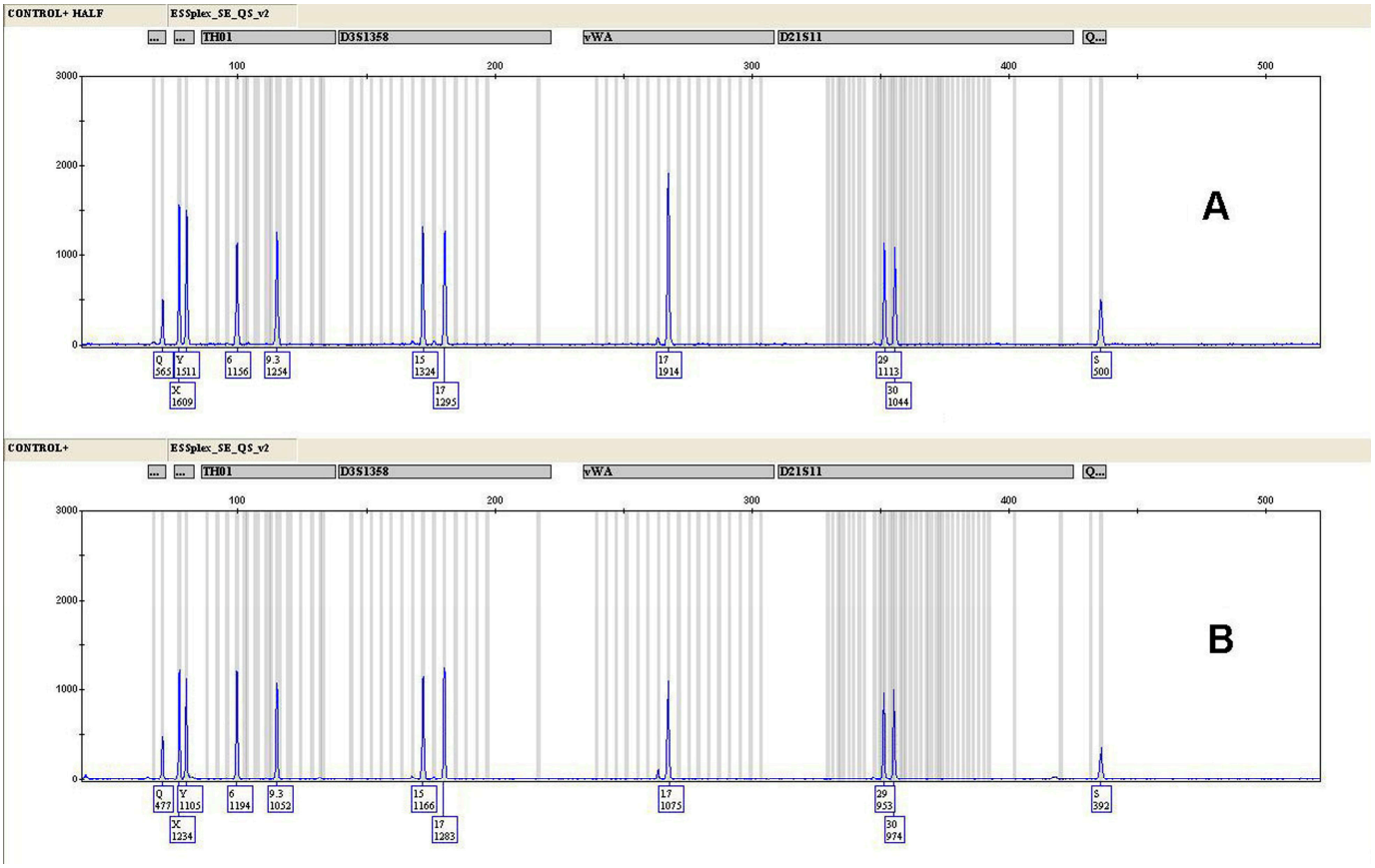

Figure 3. Electropherograms of 0.5 ng of Control DNA 9948 using half (**A**) and regular (**B**) reaction volumes. Example of concordance for the blue channel.

3.2. Control Samples

To evaluate the kit performance on good samples, four oral swabs belonging to volunteers with known DNA profiles were analyzed. Real-time quantification using the DNA extracted from oral swabs showed that the DNA extraction protocol allowed for the recovery of DNA of a high quantity/quality. Robust amplifications and full profiles were obtained using both regular PCR reaction volumes and half volumes. Balanced peaks were observed across all 17 loci. Amplification with half volumes resulted in higher peak heights. Allele assignment, using a 100 RFU threshold, was identical and correct in both reactions and across all replicates (Figure 4). In all cases, DNA profiles matched the reference profile of the donor.

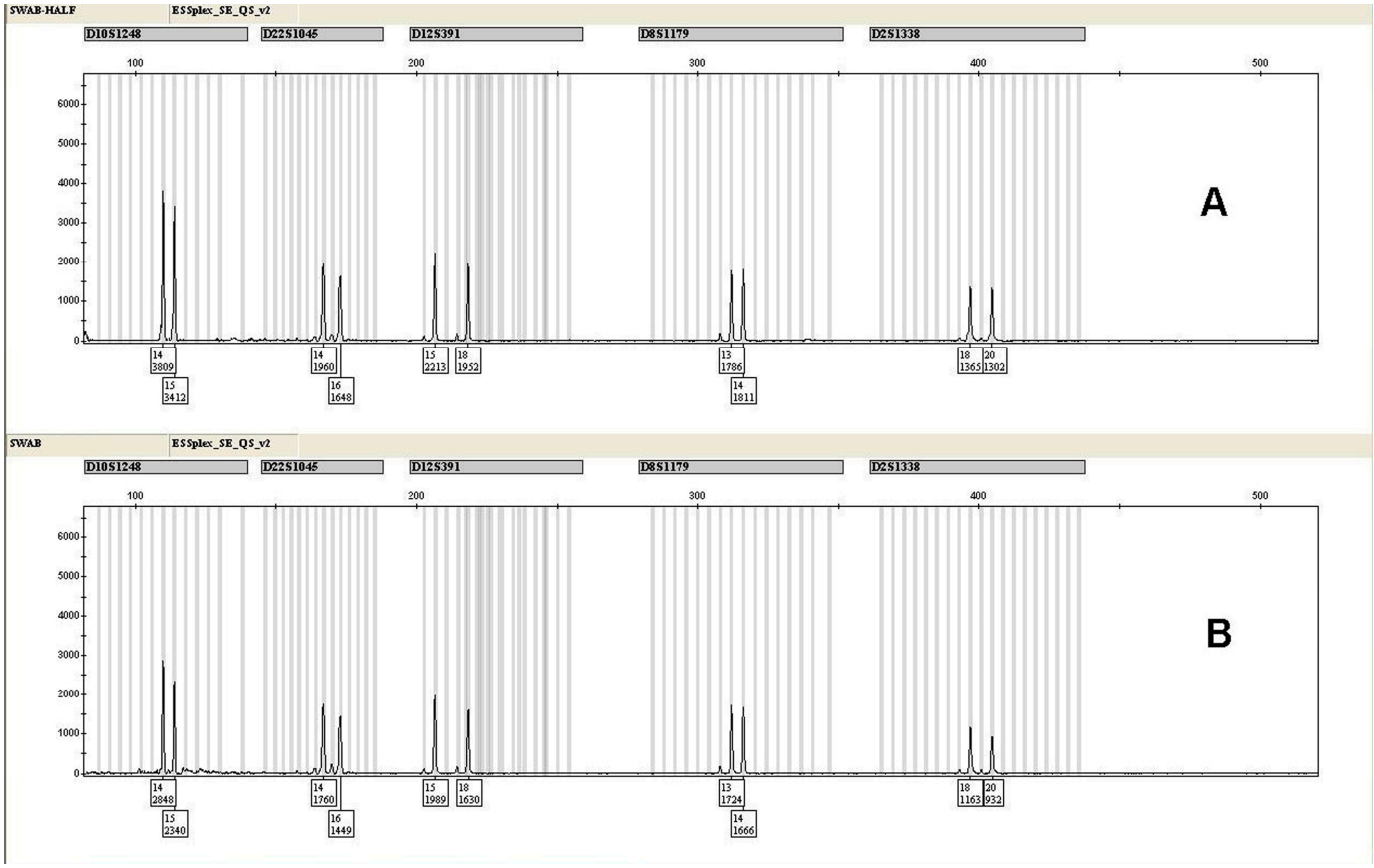

Figure 4. Electropherograms of 0.5 ng DNA extracted from an oral swab belonging to a female donor and amplified using half (**A**) and regular (**B**) PCR volumes. Example of concordance for the yellow channel.

3.3. Forensic Casework Samples

Challenges in the analysis of casework samples arise from the wide array of biological samples, variations in sample amounts, the high heterogeneity of substrates containing samples, exposure to factors favoring DNA degradation (such as environmental conditions and microorganisms), and the presence of PCR inhibitors. All these factors may impact the quantity and quality of DNA recovered from forensic samples [21,25]. In the present study, 40 casework samples (bloodstains, vaginal swabs, semen stains, cigarette butts, saliva stains, contact traces, bones and teeth, hairs, tissues) were selected to represent real forensic samples found under different conditions.

Various strategies are employed in dealing with forensic samples: the selection of an effective DNA extraction method is crucial to remove inhibitors and maximize DNA recovery [26]. The use of new-generation real-time quantification kits and PCR multiplexes with robust chemistry and resistance to inhibitors allows for the accurate evaluation of

DNA quantity/quality and STR detection, even in challenging samples [27,28]. In this study, to alleviate issues related to the DNA extraction step, we utilized the EZ1 automated DNA extraction protocols, known for their high efficiency in DNA extractions from a diverse range of casework samples [29,30].

Quantification is a crucial step in forensic sample analysis, providing essential information about the DNA available in unknown samples exposed to various conditions that may damage DNA. In this study, DNA extracted from casework samples was quantified in real time using an alternative quantification kit: the EZ1 Quantiplex Pro RGQ Kit. In all samples, DNA extraction efficiently recovered enough DNA and removed PCR inhibitors. Quantification data analysis, using the Q-Rex software along with the Quant Assay Data Handling Tool, revealed a Mixture Index (MI) > 2, indicating a female/male mixture for specific samples like a bloodstain on white cotton, contact trace on a laptop keyboard, door handle, vaginal swab, saliva on a bottle neck, and a cigarette butt [5].

The quality of the DNA profile obtained using half PCR reaction volumes was evaluated in comparison with that resulting from regular volumes, considering the number of alleles detected (drop-in or drop-out), peak height, stutter ratio, intra/inter locus balance, and inhibitor tolerance.

DNA profiles obtained from casework samples using half reaction volumes were reliable in the two replicates and comparable to those produced using standard reaction volumes. In all samples, all 17 loci of the Investigator ESSplex SE QS Kit were successfully amplified. No significant differences in terms of peak balance, preferential amplification, allelic drop-out, or PCR inhibition have been observed. However, the reduced volume produced higher peak heights, likely due to increased signal intensities (Figure 3). Although differences between the heights of peaks obtained using regular and half reaction volumes have been observed, no significant statistical differences (p-value > 0.05) were found.

The heterozygous (Het) locus ratio was > 0.7 (Table 1, Figure 5) and the stutter ratio was < 10%, using standard and half reaction volumes (Table 2, Figure 6).

Table 1. Heterozygosity (Het.) Ratio and SD (±)—Average values for each locus.

Locus	HALF	SD (±)	REGULAR	SD (±)
Amel.	0.85	0.10	0.84	0.11
D10S1248	0.89	0.07	0.86	0.07
D12S391	0.87	0.09	0.88	0.08
D16S539	0.88	0.10	0.9	0.11
D18S51	0.82	0.12	0.81	0.11
D19S433	0.89	0.07	0.9	0.07
D1S1656	0.90	0.07	0.88	0.06
D21S11	0.91	0.11	0.89	0.12
D22S1045	0.88	0.08	0.87	0.07
D2S1338	0.85	0.12	0.86	0.11
D2S441	0.91	0.08	0.9	0.09
D3S1358	0.86	0.09	0.88	0.10
D8S1179	0.87	0.09	0.88	0.09
FGA	0.86	0.11	0.87	0.10
SE33	0.85	0.09	0.83	0.08
TH01	0.90	0.05	0.91	0.06
vWA	0.91	0.09	0.9	0.10

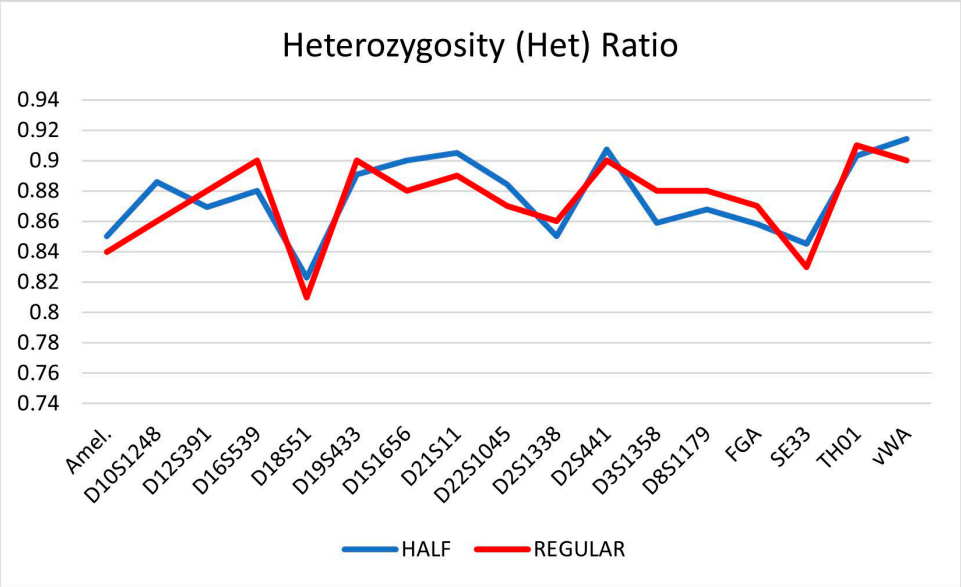

Figure 5. Heterozygosity ratio—average values for each locus.

Table 2. Stutter % and SD (±)—average values for each locus.

Locus	HALF	SD (±)	REGULAR	SD (±)
D10S1248	9.7	0.01	9.5	0.01
D12S391	8.5	0.02	8.6	0.02
D16S539	9	0.02	8.9	0.03
D18S51	9.4	0.03	9.2	0.02
D19S433	7	0.01	7.1	0.01
D1S1656	9.3	0.02	8.9	0.02
D21S11	8.8	0.01	8.2	0.01
D22S1045	9.5	0.01	9.6	0.02
D2S1338	9.3	0.02	9.2	0.02
D2S441	4.7	0.01	4.9	0.01
D3S1358	9.6	0.02	9.8	0.01
D8S1179	8.5	0.02	8.6	0.02
FGA	9.7	0.01	9.6	0.01
SE33	9.2	0.04	9	0.03
TH01	5.9	0.008	6	0.009
vWA	8.6	0.02	8.8	0.02

When comparing the heterozygosity ratio and stutter rates at regular and half reaction volumes, no significant differences (p-value > 0.05) were observed.

Concordance was evaluated between duplicates of the same sample using regular and half volumes: allele assignment by Genemapper 3.2 software was identical for each locus in each duplicate. Furthermore, we compared allele calls for each sample obtained using regular volumes with those obtained with half volumes. DNA profiles obtained using the manufacturer's protocol were considered as reference profiles, and genotypes obtained were identical with both protocols.

Regarding mixed samples (bloodstain on white cotton, contact trace on a laptop keyboard, door handle, vaginal swab, saliva on a bottle neck, a cigarette butt), detection

of full profiles for the minor contributor was possible in all analyzed samples with both PCR volumes, using an analytical threshold of 100 RFUs, in accordance with the laboratory internal guidelines.

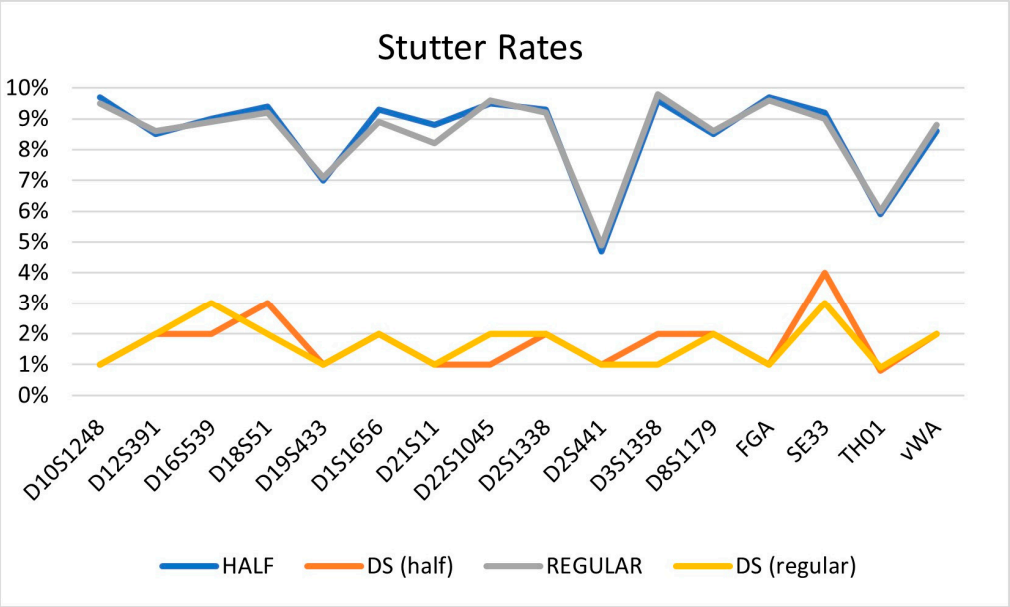

Figure 6. Stutter % and SD (±)—average values for each locus.

Full concordance was observed in the allele assignment by the GeneMapper 3.2 software for every sample analyzed with regular and half reaction volumes and in all its replicates. (Figure 7). In all cases, the quality of DNA profiles obtained varied due to the different types of samples, the nature of the substrates, exposure to different environmental conditions, and sample aging.

The efficiency of quality sensor (QS1 and QS2) amplifications was evaluated in all analyzed samples. According to the kit manual, the successful amplification of QS1 indicates that the PCR reaction was performed correctly, regardless of the DNA presence, while the absence of detection indicates that the PCR did not work correctly. DNA inhibitors can interfere with PCR amplification, causing drop-outs of long loci and artefacts. This is in accordance with the longer quality sensor being the most inhibitor sensitive of the two quality sensor fragments: a drop-down of the QS2 signal below 20% of the QS1 signal indicates inhibition of the PCR reaction. In the case of DNA degradation, longer STR fragments may fail to amplify, or they may show decreasing peak heights (ski-slope profile). In this case, the amplification of QS1 is more efficient than the amplification of the QS2 larger target fragment and drop-outs of QS2 may be observed [20].

In all analyzed samples, the quality sensors were successfully amplified in control and casework samples, even if the peak heights varied slightly between different samples. This observation aligns with the manufacturer's indications [20]. We observed that the use of half reaction volumes had no impact on the detection of quality sensors, and the QS1–QS2 ratio was comparable in both PCR reactions (regular and half volumes) of the same samples (regular volumes: minimal value 0.89 (SD 0.25±)—maximum value 2.24 (SD 0.33±)—average 1.25 (SD ± 0.15); half volumes: minimal 0.93 (SD 0.27±)—maximum 2.25 (SD 0.31±); average 1.26 (SD ± 0.17)).

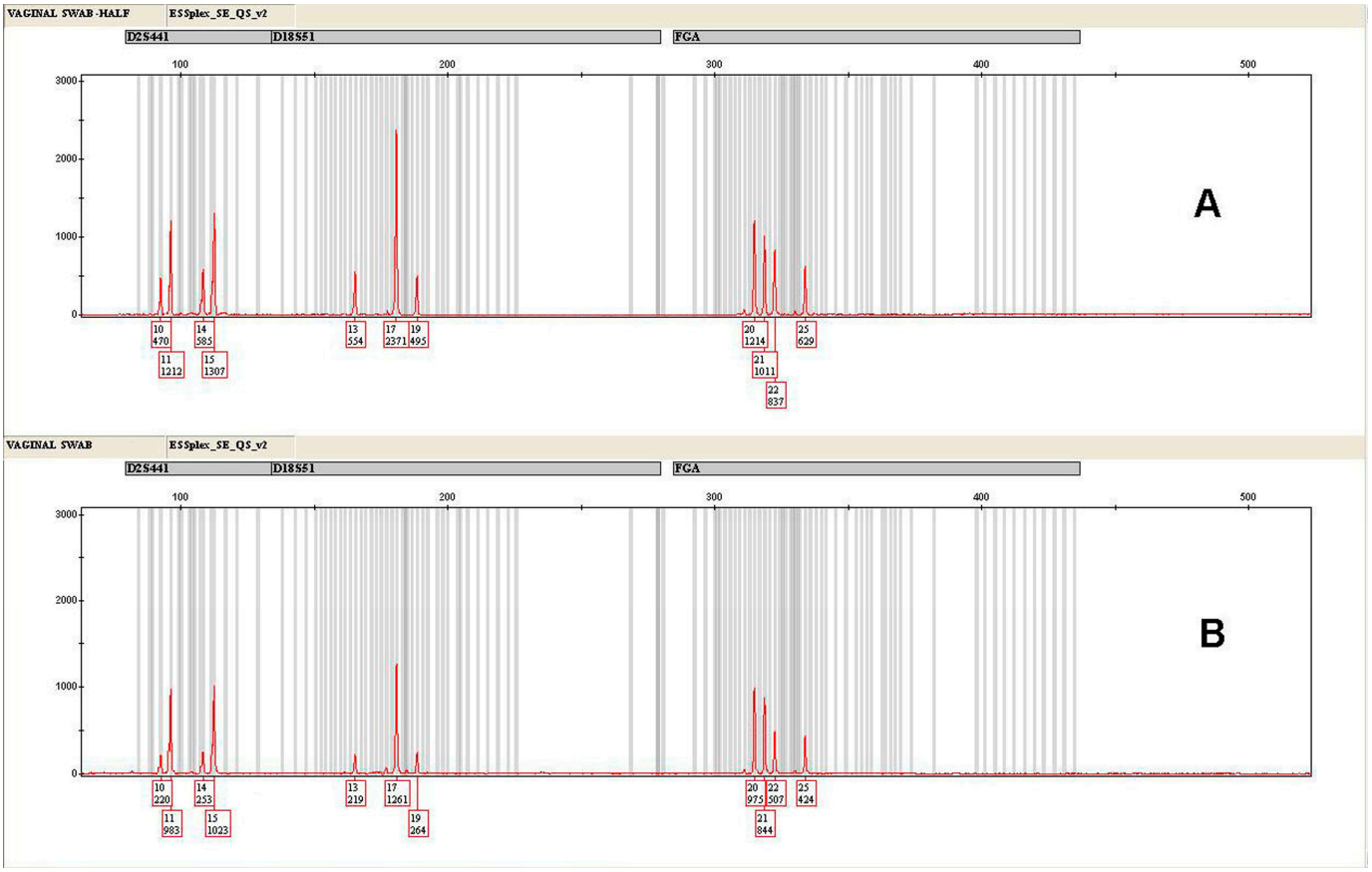

Figure 7. Electropherograms of 0.5 ng DNA extracted from a vaginal swab and amplified using half (**A**) and regular (**B**) PCR volumes. Example of concordance for the yellow channel.

4. Conclusions

In previous years, several commercial multiplexes have been developed that allow the simultaneous amplification of European- and USA-database core loci. They are widely used either in forensic analysis or in paternity tests because of their ease of use and high discriminatory power.

The Investigator ESSplex SE QS (Qiagen) is one of the new-generation PCR kits; it utilizes fast-cycling technology and allows simultaneous amplification of the 16 STR markers plus the Amelogenin locus in just 1 h.

Forensic laboratories conduct hundreds of DNA tests annually and it has become common to use reduced PCR reaction volumes. Because of this, we focused on verifying the performance of the Investigator ESSplex QS using half of the standard prescribed reaction volumes.

The present study confirms that the Instigator ESSplex SE QS kit is a robust PCR multiplex capable of delivering reliable results across a wide variety of forensic samples. It enables the recovery of information, even when dealing with challenging samples. Additionally, the presence of the two quality sensors proves useful in providing information about PCR efficiency, the presence of PCR inhibitors, and DNA degradation status. We observed that by reducing the total reaction volumes while maintaining all unaltered component ratios, it is possible to obtain reliable DNA profiles from both control and real casework samples, comparable to those obtained using regular reaction volumes.

Although both reaction volumes require the same optimal DNA quantity, reduced volumes produce peaks with higher signal intensities.

Furthermore, the use of half reaction volumes had no impact on the detection of quality sensors, included as internal PCR controls.

Finally, the use of half volumes for PCR amplification allows the analysis of a larger number of samples than the standard protocol, thereby reducing laboratory costs while maintaining the quality level of the analysis.

Author Contributions: A.B. (Anna Barbaro) carried out project design, sample analysis, results interpretation, manuscript preparation, and revision. A.B. (Aldo Barbaro) supervised and approved the project design. G.F. and A.L.M. collected samples, drafted and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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

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