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Research Article

Development of a quantitation approach for total human and male DNA based on real time PCR followed by high resolution melting analysis

We developed and validated a total human DNA quantitation technique that simultaneously allows male DNA detection. This assay, called Amel-Y, is a duplex Real Time PCR followed by HRM (high resolution melting) analysis using the intercalating dye SYTO9. Amel-Y duplex produces two amplicons, one for the amelogenin gene (106/112 bp, female/male) and another (84 bp) corresponding to human Y chromosome-specific fragment to detect male DNA. After HRM analysis, two melting peaks differing in 5.3°C–5.5°C are detected if both male and female DNA are present and only one if only female DNA is present. For specificity assessment, the inclusion of high concentrations of bacterial and fungal DNA in the quantitation reactions allowed discarding species cross-reactivity. A set of crime scene evidence from forensic casework has been quantified with commercial kits and compared with Amel-Y duplex. Our method detected male DNA from a concentration of 18 pg/μL and supports autosomal/Y DNA detection ratio up to 200:1. A limitation of the technique is its inability to quantify male and female donnors in a mixed sample. The Amel-Y duplex demonstrated to be an efficient system for quantifying total human DNA being a specific, rapid, sensitive, and cost-effective method suitable for mixed DNA samples and applicable to any field where human DNA quantification is required, such as molecular diagnosis, population genetics, and forensic identification.

Keywords:
DNA quantification / Forensic casework / HRM analysis / Real Time PCR / Y chromosome
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1 Introduction

DNA quantification is a crucial step in STR, SNP, and Indel genotyping affecting the success and quality of the results. A common feature of forensic casework samples is the poor quality and limited quantity of DNA that can be retrieved from evidentiary materials, which affect the STR genotyping success. Commercially available STR amplification kits require a DNA input ranging between 0.5 and 2 ng [1, 2]. Low amounts of DNA can generate stochastic effects (e.g. drop out and drop in, among others) that may hinder the interpretation of a sample's profile. Conversely, high amounts of DNA could generate analytical artifacts (i.e. stutters, spikes, raised baseline, and split peaks, among others) [3]. Moreover, the possibility of identifying male/female relative DNA quantities allows defining best suited experimental strategies to obtain interpretable profiles. Hence, DNA quantification represents a key step in the forensic analytical routine.

Nowadays, DNA quantification methods based on quantitative PCR (qPCR) have replaced traditional ones based on UV spectrophotometry or human-specific hybridization [4]. Quantitation based on PCR demonstrated to be highly sensitive (at the picogram level), human-specific, and able to detect inhibitors. Based on qPCR quantification a variety of analytical decisions can be made, such as re-extraction or purification of the sample (if inhibitors are detected) and precise definition of the DNA input for autosomal and/or Y-STR typing. Alternative systems to commercially available kits have been previously reported although the detection systems employed, such as TaqMan® probes, exceed the complexity of other detection methods, such as intercalating dyes [5, 6]. High resolution melting (HRM) analysis is

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Abbreviations: Amel, amelogenin; **CT**, threshold cycle; **DNA**, deoxyribonucleic acid; **HRM**, high resolution melting; **Indels**, insertion deletion polymorphisms; **m**, reaction slope; **mM**, millimolar; **ng**, nano-gram; **pg**, pico-gram; **pmol**, pico-mol; **q PCR**, quantitative polymerase chain reaction; **SDS**, sodium dodecyl sulfate; **st**, standard concentration point of the calibration curve; **TBL1Y**, Transducin (beta)-like 1, Y-linked gene; **Tm**, melting temperature; **UV**, ultra violet; **μl**, micro-liter; **Y-DNA**, Y-chromosome deoxyribonucleic acid; **Y-STRs**, Y chromosome short tandem repeats

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a post-PCR method enabling the characterization of genetic variations (e.g. SNPs, mutations, and methylations, among others) in PCR amplicons [7–11]. HRM characterizes nucleic acid samples based on DNA strand dissociation (melting) behavior, which depends on the sequence, length, GC content, or complementary-strand interactions [12].

Our group previously developed an in-house made approach to quantify DNA by qPCR followed by HRM employing amelogenin primers. Although well-correlated results were obtained, this procedure was not able to efficiently discriminate female from male samples, nor to detect the presence of male DNA in mixed samples, since melting peaks could not be clearly differentiated (106/112 bp, female/male, Δ°C 0.2). To overcome this limitation, the first step was to design Y-specific primers to generate a short amplicon that could be differentiated from the amelogenin amplicon by HRM. Several in silico tools were employed to select finally a pair of primers that allowed amplifying an 84-bp fragment located on the short arm of the Y-chromosome (transducin (beta)-like 1, Y-linked -TBL1Y). These primers, together with amelogenin primers, allowed a specific detection of male DNA in the sample. After HRM analysis, two melting peaks differing in 5.3°C–5.5°C were observed in male samples whereas only one peak in female samples. Ct values are employed to build calibration curve rod (CT vs. DNA concentration). Discrimination, specificity, reproducibility, and resolution were assessed. Additionally, results obtained from crime scene samples were analyzed. Experimental results were compared and correlated with mixed-DNA evidence from routine casework.

We propose this DNA quantification method as a screening tool for samples of forensic interest to improve the quality of genetic profiles. In addition, its use increases experimental efficiency by defining adequate reagent use, saving analysis time, and optimizing the use of quantity limited samples.

2 Materials and methods

2.1 DNA extraction and quantification

DNA samples were extracted by the semi-automated platform Maxwell 16® (Promega Corp., Madison, USA) using the casework sample module according to the manufacturer's protocol.

To define the quantification efficiency of the procedure developed herein, the human-specific Real Time PCR-based commercial kit Plexor HY® (Promega) was used in parallel.

Additionally, species specificity was assessed by nucleic acid quantification of non-human total DNA based on dye mediated fluorescence detection by the Quantus™ Fluorometer (Promega).

2.2 DNA samples

Plexor® HY Male Genomic DNA Standard (Promega) was used to set up the calibration curve for all Plexor quantification assays. For the development of the duplex system, a human male DNA sample was used to build a calibration curve. DNA was extracted from a male human muscle by proteinase K/SDS digestion followed by organic extraction standard protocol [13]. From an initial concentration of 180 ng/μL (quantified by Plexor), successive 1/5 dilutions were prepared. Each dilution was quantified by Plexor. Species-specificity assays were carried out using DNA from several bacteria (*Escherichia coli*; *Pseudomonas aeruginosa; Staphylococcus aureus* and *Proteus mirabilis)* and one yeast species (*Saccharomyces cerevisiae)* as controls. Samples from routine casework were used to test the usefulness of the duplex in real forensic samples (e.g. crime scene swabs and pieces of clothing stained with different biological fluids). DNA extraction was performed according to our Standardized Operating Procedures (overnight proteinase K/SDS digestion followed by DNA purification by the semi-automated platform Maxwell 16® -Promega Corp., according to the manufacturer's protocol).

2.3 Human Y chromosome-specific primer design

Primers for male DNA detection were designed using the online IDT PrimerQuest Tool (https://www.idtdna.com/Primerquest/Home/Index). The predicted amplification product and primer specificity were checked using online UCSC Genome Browser software (http://genome.ucsc.edu/cgi-bin/hgPcr). To obtain broader divergence between melting peaks, amplicons melting temperature (Tm) were predicted using the online Oligo Calculator software (http://www.basic.northwestern.edu/biotools/oligocalc.html). The selected primer sequences (i.e. 5′-GGCACTAGAGGTCTGTACTA-3′ forward and 5′-CAACATAGACGACTCCTTCTC-3′ reverse) complied the following characteristics: single-copy product, short product size (84 bp), Y-chromosome specificity, annealing temperature suitable for amelogenin primers, and melting peaks of 72°C/73°C (depending on the used platform) allowing to differentiate them from the amelogenin melting peaks (77.4°C/78.4°C).

2.4 PCR conditions for the duplex Amel-Y amplification

PCR reaction was carried out in a 25-μL final volume. The reaction mix consisted of 0.3 nM SYTO9 (Invitrogen, USA), 0.05 U/μL GoTaq Polymerase (Promega), 5X Colorless GoTaq Reaction Buffer (Promega), 5 pmol AMEL primers [14], 7.5 pmol Y chromosome-specific primers, 0.15 mM dNTPs, 1.7 mM Mg^{2+}, and 2 μL DNA sample. Amplifications were carried out in a Rotor Gene 6000 (Corbett Life Science, Australia) and in a StepOnePlus (Life Technologies, USA) Real Time PCR equipment. PCR cycling conditions were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 60 s, 59°C for 30 s, 72°C for 30 s. HRM analysis was performed ranging from 69°C to 80°C at 0.2°C/s in Rotor Gene 6000.

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When using StepOnePlus, the melting curve stage was 95°C 15 s followed by a 0.3°C continuous ramp from 68 to 80°C.

2.5 Species specificity assays

Species specificity was tested by amplifying human male and female (*Homo sapiens*), *E. coli, P. aeruginosa, S. aureus, P. mirabilis,* and *S. cerevisiae* DNA samples. All samples were previously quantified by Plexor® for human DNA and by the Quantus™ Fluorometer to detect significant amounts of bacterial and fungal DNA.

2.6 Sensitivity assays

To assess the sensitivity, we tested the duplex system in a range of 50 to 0.016 ng/μL by a dilution series of Plexor® HY Male Genomic DNA Standard. Calibration curves were performed in triplicate.

2.7 Accuracy assays

The quantification accuracy of the developed duplex system was analyzed in triplicate of known concentration samples. CT (threshold cycle) standard deviation, calibration curves linearity (R^2), and the reaction efficiency were analyzed by triplicates of the same standard curve points used for the sensitivity assay.

2.8 Crime scene samples DNA quantitation

Sixty-four crime scene DNA samples (i.e. biological stains on cloth and anal, buccal, or vaginal swabs) were quantitated in a Rotor Gene 6000. The DNA used to set up the calibration curve was extracted from a male human muscle, and concentrations were measured with Plexor and ranged from 37.60 to 0.018 ng/μL.

2.9 STR genotyping

PowerPlex® Fusion and Power® Y23 amplification kits (Promega) were used to obtain autosomal and Y chromosome STR profiles respectively, in a Gene Amp® PCR system 9700 (Applied Biosystems, Foster City, USA) following manufacturer´s instructions. Amplification products were separated and detected on an ABI 3500 Genetic Analyzer (Applied Biosystems). Profile analysis was carried out with GeneMapper IDX v1.2 (Applied Biosystems).

Table 1. Species specificity assay of Amel-Y duplex

Species	Total DNA per reaction (ng/μL)	Amel-Y duplex PCR amplification	Amel-Y duplex HRM
H. sapiens (male)	50.0	Amp	A +; Y +
	10.0	Amp	A +; Y +
	0.016	Amp	A +; Y +
H. sapiens (female)	50.0	Amp	A +; Y –
	10.0	Amp	A +; Y –
	0.016	Amp	A +; Y –
E. coli	100.0	N/Amp	A –; Y –
	50.0	N/Amp	A –; Y –
	25.0	N/Amp	A –; Y –
P. aeruginosa	61.2	N/Amp	A –; Y –
	10.2	N/Amp	A –; Y –
S. aureus	68.4	N/Amp	A –; Y –
	11.4	N/Amp	A –; Y –
P. mirabilis	91.0	N/Amp	A –; Y –
	45.5	N/Amp	A –; Y –
	9.1	N/Amp	A –; Y –
S. cerevisiae	105	N/Amp	A –; Y –
	52.5	N/Amp	A –; Y –
	10.5	N/Amp	A –; Y –

Amp = PCR amplification was evidenced; N/Amp = no PCR amplification was evidenced.
A + = Amelogenin melting peak was evidenced; A – = Amelogenin melting peak was not evidenced.
Y + = TBL1Y melting peak was evidenced; Y – = TBL1Y melting peak was not evidenced.

3 Results

In this paper, we present the development and results of a quantitative PCR duplex assay consisting of a fragment of the Amelogenin (Amel) gene and a fragment of the Transducin (beta)-like 1, Y linked (TBL1Y) gene. The system is called: Amel-Y duplex. The main goal of this development was to quantify simultaneously human DNA and detect the presence of male DNA in mixed samples of forensic interest, employing only one intercalating dye. Our development is relevant as in routine casework most mixed samples contain low concentrations of the male component compared to the victim's contribution (over 90%). A second goal was focused on simplicity and cost efficiency. We developed a simple and inexpensive method based on detection with intercalating dyes, replacing more expensive strategies.

Several assays were carried out to determine the most efficient amplification conditions, which have been described in the Materials and Methods section. Specificity, sensitivity, and reproducibility were tested with reference samples, and the efficiency was tested with casework mixed samples. Human specificity was assessed by the amplification of human male and female DNA, as well as in the presence of large amounts of bacterial and fungal DNA (see Table 1). None of the microbial samples showed amplification or characteristic melting peaks, evidencing no cross-reactivity with genetic material of any of the studied species, which could potentially interfere with human DNA amplification.

Accuracy and sensitivity assays were carried out to establish the DNA input range able to produce reliable quantification results. Triplicates of a six-point calibration curve were tested at concentrations ranging from 50 ng/μL to

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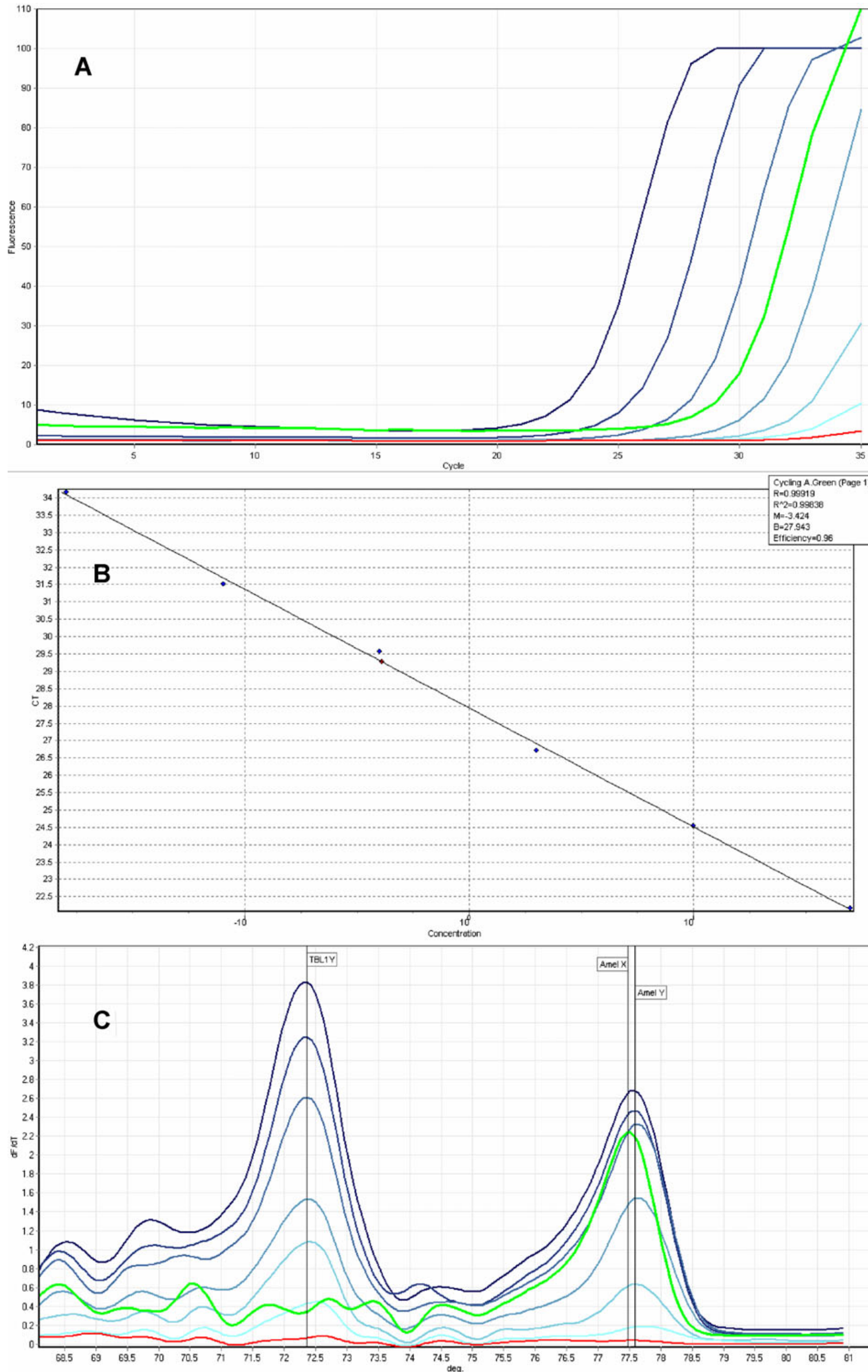

Figure 1. Sensitivity assay. (A) Amplification curves of genomic male DNA control (Plexor® HY Male Genomic DNA Standard). From left to right, 50-10-2-0.4-0.08-0.016 ng/μL dilutions in blue gradient, female DNA control in green and negative control in red. (B) Calibration curve *rod*. Standards points in blue and female DNA control in red. (C) First derivative of the HRM curve genomic male DNA control .From top to bottom, 50–10–2–0.4–0.08–0.016 ng/μL dilutions in blue gradient, female DNA control in green, and negative control in red. Platform: Rotor Gene 6000.

16 pg/μL of Plexor® HY Male Genomic DNA Standard. The system showed the capacity to detect as low as 16 pg/μL male DNA, and no significant dispersion was evidenced in triplicates (%CV = 1.08) (see Fig. 1). No amplification signals were detected in the negative control samples.

The precision of this qPCR system is reflected by the value of the CT standard deviation for each triplicate of the calibration curve (i.e. SD st1: 0.026, SD st2: 0.026, SD st3: 0.267, SD st4: 0.108, SD st5: 0.110, and SD st6: 0.318). On the other hand, Table 2 shows the parameters associated with the quality of each calibration curve generated with Amel-Y duplex.

Furthermore, we assessed the specificity of the system to the Y chromosome. The system was validated with experimental female/male mixed samples, and whenever a specific Y chromosome melting peak was observed after HRM,

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Table 2. Quality parameters for each Amel-Y duplex calibration curve replicate

Calibration Curve	R^2	Slope (m)	Efficiency (%)
A	0.998	−3.374	98
B	0.997	−3.497	93
C	0.999	−3.420	96

it was possible to obtain a male Y-STR profile by commercially available kits. On the other hand, this real-time quantification approach is aimed mainly to obtain a rapid screening method allowing the detection of low quantities of male DNA in a qualitative way in forensic samples. We tested 64 real casework samples from different sources by the Amel-Y duplex and compared the results with those obtained by two commercial DNA quantification systems, namely Quantus and Plexor (quantification of human autosomal and Y-chromosome DNA). Figure 2 depicts results obtained from routine casework samples (i.e. vaginal swabs or biological stains) quantified by both Plexor and Amel-Y duplex, which concentrations ranged from 0.01 to 21.5 ng/μL and close trend lines were observed between both the methods. We have succeeded in obtaining consistent values similar to those of Plexor quantification, particularly in a concentration range between 0.01 and 10 ng/μL. Regarding Quantus, a higher data dispersion was observed in comparison to both Plexor and Amel-Y duplex. This observation can be explained by the fact that Quantus is a non-specific quantitation system that quantifies total DNA, and quantification varies depending on the presence of non-human DNA in the sample (data not shown).

From a total of 64 evidentiary samples analyzed, 46 exhibited unique male DNA profiles or mixed profiles after amplification with PowerPlex Fusion. In 42 of the 46 samples, complete Y-chromosome haplotypes were obtained, whereas mixed haplotypes were observed in two samples, partial haplotypes were obtained in two samples, and only one sample failed Y-STRs amplification. The latest can be explained by DNA degradation. The autosomal:Y-chromosome DNA ratio ranged from 0.3:1 to 200:1, whereas male DNA quantified by Plexor, ranged from 0.016 to 12.87 ng/μL. Amel-Y duplex detected the Y-amplicon characteristic melting peaks in all of these samples (see Fig. 3).

On the other hand, 4/64 samples yielded female profiles, and no male DNA was detected, neither with Plexor nor with Amel-Y. Finally, the remaining 14 samples exhibited unique female profiles, as evidenced by the amelogenin system included in the PowerPlex Fusion kit that showed a single peak at 106 pb. However, in six of these 14 female samples, Plexor detected male DNA, while Amel-Y duplex was unable to detect Y chromosome melting peaks after HRM analysis. Therefore, Amel-Y duplex failed in 9% of the cases in male DNA detection as compared with Plexor HY system. By using the maximal sample volume admitted in the PCR reaction, we obtained a complete Y-chromosome haplotype after PowerPlex Y23 amplification. Interestingly, some peculiar characteristics were observed in the six mentioned samples. First, a high ratio of autosomal to Y-chromosome DNA (higher than 250/1) was observed, and, second, male DNA quantification with Plexor reached values in level of the sixth point of the calibration curve (0.022 ng/μL in average). In the remaining eight samples, Y-DNA concentration was under the detection limit (<0.009 ng/μL) and no Y-STR profile was obtained. The combination of both factors could explain the negative Y-amplification by Amel-Y duplex. Although these observations could constitute a limitation of our development in the forensic field, should be observed that in more than 91% of the cases Amel-Y duplex was successful in detecting male DNA in the sample, and the quantification was concordant to those obtained with Plexor. Furthermore, this development could be applied to quantify any sample for further molecular analysis, such as molecular diagnosis or population genetics.

4 Discussion

We have described the validation of a human DNA quantification system based on RTPCR using an intercalating agent. This dye was selected for its high sensitivity and low cost compared to other detection systems commercially available. The development of a duplex reaction, combining

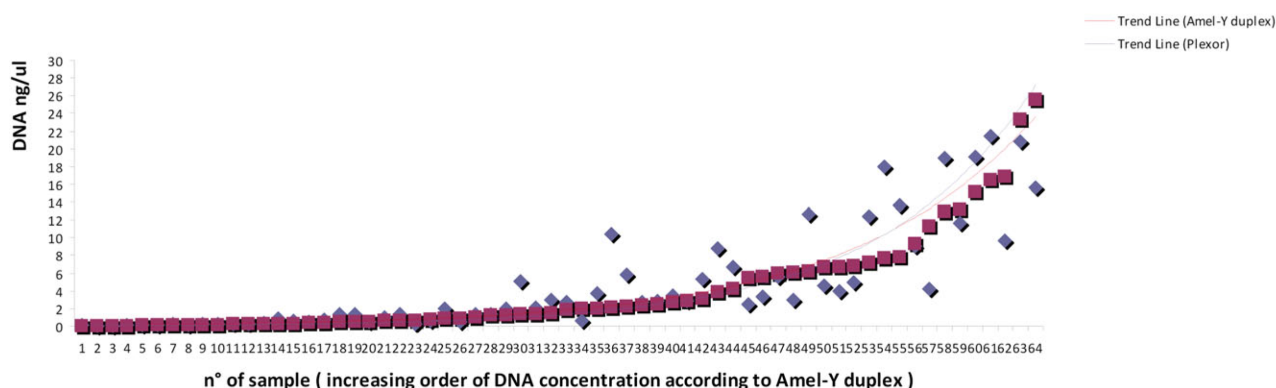

Figure 2. DNA concentration in forensic samples. Trend line of quantification values obtained with Plexor (in blue) and Amel-Y duplex (in red).

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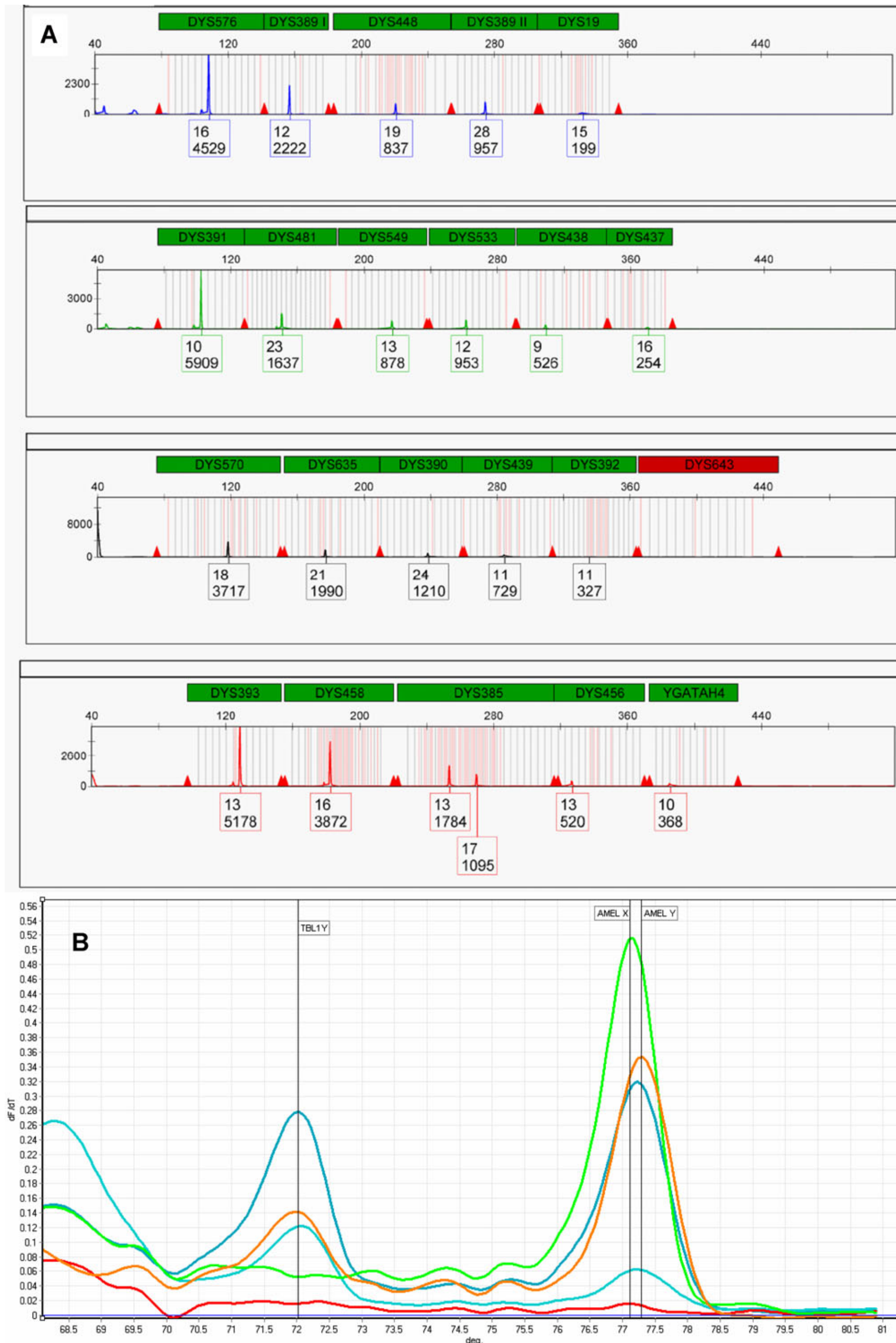

Figure 3. (A) Y-STR profile of an admixture sample (amplified with PowerY23) corresponding to 65 pg/μL of male DNA (quantified by Plexor; autosomal/Y-chromosome DNA ratio 2:1). (B) First derivative of HRM curves showing the lowest male DNA standards (in blue), female DNA control (green), the same crime scene admixture sample from Fig. 3(A) (in orange) corresponding to 65 pg/μL of male DNA (quantified by Plexor) and negative control (in red), Platform: Rotor Gene 6000.

amelogenin and Y-specific primers, allowed to differentiate clearly two melting peaks after HRM analysis, which differed in 5.3–5.5°C (see Fig. 1). It is important to note the specificity of the system for the Y chromosome as it detected male genetic material after HRM. As any sample showed the specific Y chromosome melting peak, it was always possible to obtain a complete and high-quality genetic profile of 23 Y-STR markers, even with a DNA concentration of 20 pg/μL. The species specificity assay was carried out to rule out potential interference of most common microorganisms in a crime scene in the identification of the presence of male genetic material. DNA samples from bacteria and fungi were tested and none of them evidenced Amel-Y melting peaks after HRM analysis, discarding cross-reactivity.

Calibration curve replicates for the developed duplex reaction showed and efficiency of over 90% and good linearity ($R^2 = 0.99$). The reaction slopes (m) obtained showed to be adequate for obtaining a reliable DNA quantification

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since all three slope values were similar to the optimal value ($m = -3.322$) (Rotor Gene 6000^{TM} Operator Manual). Moreover, amelogenin and TBL1Y targets exhibited admissible precision, as indicated by CT standard deviations less than one cycle for all standard curve points. As described by Hudlow et al. [5], the standard deviation in replicates points increases at low DNA concentrations.

DNA samples from routine forensic casework (e.g. sex assaults) extracted from evidences such as vaginal swabs or clothes were tested with Plexor, Quantus, and the homemade quantification system, and concordant results were obtained by both methods (Plexor and Amel-Y duplex). Therefore, the quantification results obtained by Amel-Y duplex are close to those obtained by Plexor system, especially in low concentration DNA samples, showing that our Real Time PCR approach is efficient and can complement systems such as Plexor. For higher concentration samples more dispersion was evidenced. However, Poetsch et al. [15] described that the comparability of quantification results between different kits does not seem to be possible due a greater variance of results found by analyzing serial dilutions of standard DNA.

Six out of 64 samples analyzed by Amel-Y duplex failed in detecting Y chromosome melting peaks, however, Y-chromosome profiles were obtained. Amel-Y duplex is only reliable when positive male detection is obtained but we must emphasize the importance of using an alternative male detection method in samples where no male DNA identification was observed by Amel-Y duplex. While the latest represents a limitation of our development, it is important to note that in these six samples two factors could make male DNA detection difficult, namely low Y-DNA concentration and low proportion compared to autosomal DNA. While the above mentioned fact is a limitation in the forensic field, it is not for research (i.e. molecular diagnosis, population genetics) where the sample of interest is from a single source.

Furthermore, Poetsch et al. [15] reported the comparison among four human DNA quantification kits (Plexor, Quantifiler, Quantiplex and PowerQuant) and they described that full or partial profiles could be found in samples where their quantification value was under the threshold line. Therefore, failures in reliability are found even in commercial kits where complexity and costs exceeds those compared home-made techniques such as Amel-Y duplex.

It is not possible to determine the exact amount of female or male human DNA in an admixed sample. The Amel-Y duplex was developed for determining total human DNA concentration and simultaneously detecting male DNA (in a qualitative manner) by high resolution melting analysis. The importance of Amel-Y duplex performance in admixed samples is its ability to detect human male DNA, evidenced by 72°C specific Y chromosome melting peak, making use of only one dye detection channel. Therefore, employing only one intercalating dye (syto9) in the system quantifies total human DNA and detects male genetic material in case it is present.

As described in Fig. 3B, a melting peak at 72°C is observed in an admixture crime scene sample where the female component is in major proportion than the male one, evidenced by the imbalance among the melting peaks heights corresponding to Amelogenin and Tbl1y amplicons. Thus, Amel -Y duplex allows male DNA detection in admixture samples, evidenced by TBL1Y characteristic melting peak.

A semi-quantification of the male genetic component could be attempted from the melting curves plot analysis by comparing Y melting peak heights obtained for each point of the calibration curve with Y melting peak heights obtained for the sample under analysis.

The DNA quantification system described herein is routinely used in our laboratory. It has proved to be useful in samples of different origins as crime scene swabs, pieces of clothing, muscle, bones, saliva, blood, and semen as well as in samples used for population genetics studies and molecular diagnosis. While there are differences in the quantification compared to commercial systems such as Plexor, Amel-Y duplex has provided quantification results consistent with the quality and resolution of the genetic profiles obtained afterward. Additionally, the developed assay has been tested on another platform (i.e. StepOnePlus), and results matched those obtained with Rotor Gene 6000, exhibiting analytical platform flexibility.

4.1 Conclusion

The possibility to simultaneously quantify and determine the genetic constitution to samples of forensic interest is an extremely useful tool as it improves work efficiency, reduces analytical time, and allows adopting a suitable strategy for STR markers amplification. The Amel-Y duplex approach proved to be useful in samples from different sources and quantified DNA successfully allowing obtaining full and high-quality genetic profiles. Male DNA detection after HRM was possible even at low concentrations and in mixed samples, where female DNA is present in a higher proportion than male DNA. The system showed to be fast, sensitive, specific, cost-effective, and suitable for routine casework, population genetics, and molecular diagnosis.

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The authors have declared no conflict of interest.

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