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Development and validation of a human DNA quantification and sex determination approach based on real time PCR followed by high resolution melting analysis

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ABSTRACT

DNA quantitation is one of the most crucial factors affecting the success and quality of DNA typing by PCR. The aim of this work was to develop a DNA quantification assay to be used in routine forensic casework. It should be able to discriminate, simultaneously, the presence of male and female DNA by means of multiplex real time PCR, followed by high resolution melting analysis (HRM), including a fluorescent intercalating dye Syto 9.

The approach is co-amplified fragments of a gene common to both genders, Amelogenin-Amel and a specific sequence of the human Y chromosome (HSYCS) whose melting temperature differs from Amel in 5.3–5.5 °C. Hence, it allows discriminating two peaks after HRM analysis if only male DNA is present in the sample or a single peak if only female template is present. The short length of both amplicons, 106/112 bp for Amel and 84 bp for HSYCS, facilitates quantitation and gender detection in degraded samples that characterize evidentiary material. We achieved the quantification of male, female and experimentally mixed samples with very low DNA quantities (20 pg/μl).

We propose an alternative approach to commercial DNA quantitation kits. Our development showed to be fast, highly sensitive, laborsaving and cost-effective.

1. Introduction

Human DNA quantification in forensic samples is one of the most important factors underlying the efficiency and success of PCR-based genotyping. DNA input between 0.5 and 2.0 ng may result, in case no inhibitors are present in the sample, in complete profile with the available commercial kits.

Since men commit most of the violent crimes, male DNA detection in the evidence is fundamental for determining the best strategy for the investigation [1].

Currently, there are many commercial kits for the quantification of DNA and detection of male DNA simultaneously, shown to be highly sensitive and robust, but their use greatly increases the cost of analysis in forensic casework.

Alternative systems to commercial kits have been previously reported [2,3] although the detection systems employed (such as TaqMan probes) exceeds the complexity of intercalating dyes selected for our development.

Our goal was to develop a human DNA quantization method able to discriminate the presence of male and female human DNA by means of multiplex real time PCR, followed by high resolution melting analysis (HRM), including a fluorescent intercalating dye Syto 9, due to its DNA double strand saturation ability, resulting in higher sensitivity.

Based on previous investigations in our lab, we selected one Amelogenin gene fragment as candidate to quantify human DNA. Presence of male DNA amplifies a 112 bp fragment and female DNA amplifies a 106 bp amplicon. After HRM we observed two melting peaks respectively that differ in 0.2 °C. While this difference in melting behavior allow discriminating the sex identity from DNA, is not optimal to ensure correct resolution of forensic mixed samples. Aiming to boost the discrimination efficiency, we design a new strategy including in the reaction cocktail a second Y-specific primer pair in order to optimize the resolution in sex differentiation.

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2. Materials and methods

2.1. DNA samples

DNA control samples 4997A (female DNA, Promega Corp.), 2800 (male DNA, Promega Corp.) and Plexor HY male DNA Standard (male DNA, Promega Corp.) were used to set up PCR conditions.

Species specificity was tested by amplifying DNAs from rat, cat, dog, horse, monkey and pig.

Samples from routine casework were used to test the usefulness of the duplex in real cases.

2.1.1. Primer design

Optimal primers were designed using Primer Tool software from IDT website (https://www.idtdna.com/Primerquest/Home/Index). The predicted amplification product and primer specificity were checked using UCSC Genome Browser online software (http://genome.ucsc.edu/cgi-bin/hgPcr). In order to obtain broader divergence between melting peaks, amplicons melting temperature (T_m) was predicted using Oligo Calculator software (http://www.basic.northwestern.edu/biotools/oligocalc.html).

The selected primers sequence is: 5′-GGCACTAGAGGTCTG-TACTA-3′ (forward) and 5′-CAACATAGACGACTCCTTCTC-3′ (reverse) and comply the following characteristics: single copy product, short length fragment (84 bp), Y-specific, annealing temperature according with AMEL, melting peak at 72/73 °C (depending upon platform being used) allowing to differentiate it from amelogenin melting peak (77.4/78.4 °C).

2.1.2. Multiplex PCR conditions

PCR reaction was carried out in 25 μl final volume. The reaction mix contained: Syto9 (Invitrogen, USA) 0,3 nM; Go Taq polymerase

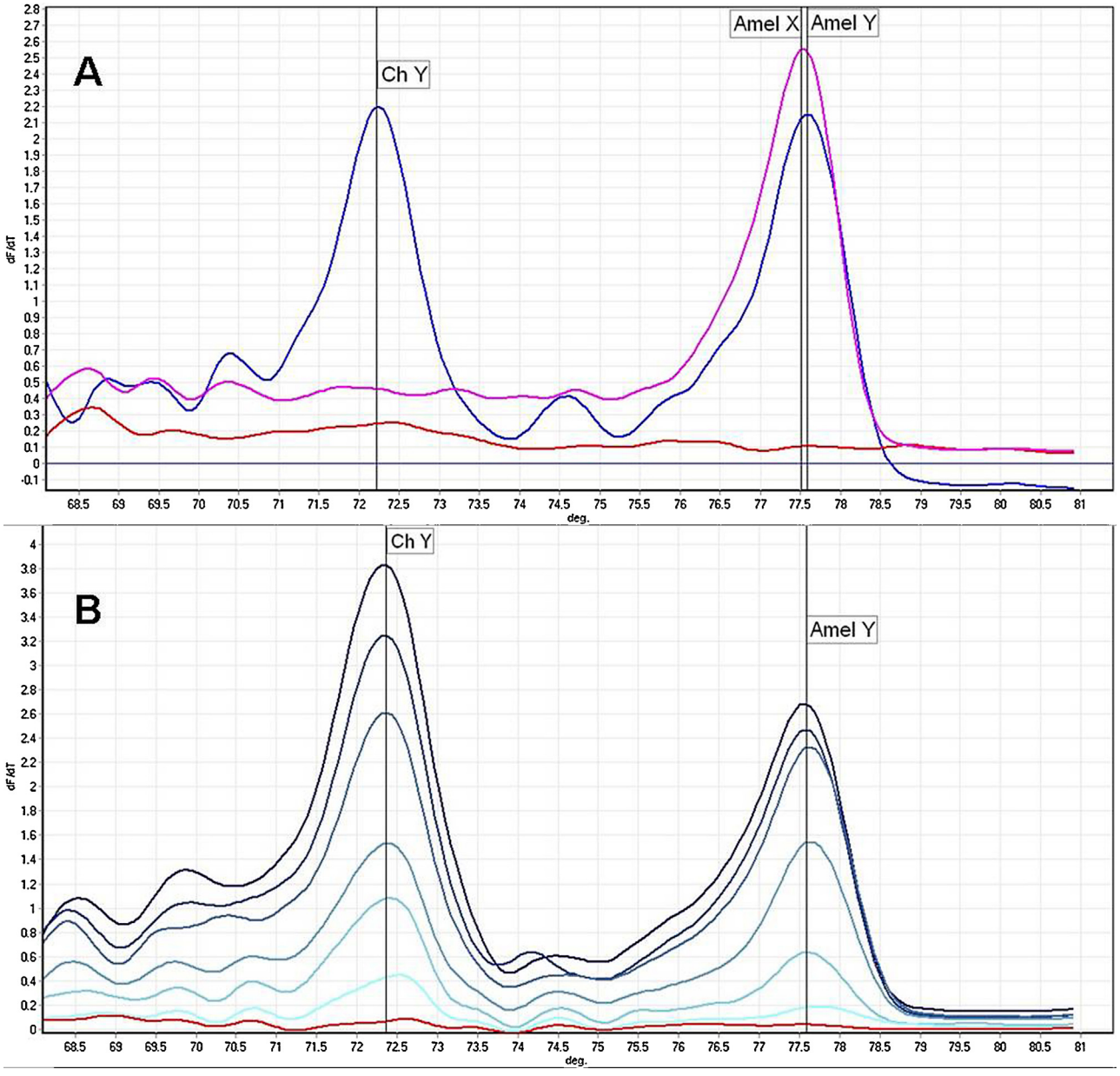

Fig. 1. (a) First derivate from HRM analysis plot of control DNAs: 2800 male (in blue) and 4997A female (in pink); negative control (in red). Platform: Rotor Gene 6000. (b) Sensitivity assay: first derivate from HRM analysis plot of genomic male DNA control (Plexor® HY ADN male Standard). From top to bottom 50–10–2–0.4–0.08–0.016 ng/μl dilutions; Negative control (in red). Platform: Rotor Gene 6000.

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(Promega, USA) 0.05 U/μl; 5× colorless GoTaq reaction buffer (Integrated DNA Technologies) 1×; AMEL primers 5 pmol; Y-ch specific primers 7.5 pmol; dNTPs 0.15 mM; Mg 1.7 mM; and 2 μl sample DNA.

Amplifications were performed in a Rotor Gene 6000 (Corbett Life Science, Australia) and in a StepOne Plus (Life Technologies, USA) Real Time PCR equipment. PCR cycling conditions were: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C 60 s, 59 °C 30 s, 72 °C 30 s. HRM analysis was performed ranging from 69 °C to 80 °C at 0.2 μC/s for Rotor Gene 6000. When using StepOne Plus, the melting curve stage was: 95 °C 15 s followed by a 0.3% continuous ramp from 68 °C to 80 °C.

3. Results

3.1. Chromosome specificity assay

DNA samples were amplified by RT PCR reaction containing both primers (AMEL and HSYCS) according to the amplification conditions detailed previously. After HRM analysis two characteristic melting peaks for male DNA samples and a single peak for female DNA were observed (Fig. 1a). These peaks could be clearly differentiated by 5.3–5.5 °C allowing discriminating the sex of the DNA samples.

3.2. Species specificity assay

DNA samples from several mammalian species were tested and none of them evidenced the specific HSYCS melting peak after HRM analysis, exhibiting no cross-reactivity. Even more, no spurious cross-reactivity to human autosomes or to the X-chromosome was detected.

3.3. Sensitivity assay

Since our most important goal was to design a DNA quantitation system, dilutions of male genomic standard DNA was analyzed. Male standard DNA was serially diluted in order to obtain six standard points curve in a concentration range from 50 ng/μl to 16 pg/μl (fig. 1b). To test reproducibility all samples were amplified by triplicate. All replicates samples analyzed showed the two specific melting peaks, PCR efficiency (greater than 90%), satisfactory quality standard curve (correlation coefficient), high precision of quantification values and no amplification in no-template control samples. The quantification was sensible at 20 pg/μl.

3.4. Mixture samples assay

In order to test the sensibility of the duplex to detect male DNA in presence of high female DNA quantities, experimentally mixed samples were tested. Male and female DNA was combined in different ratios in order to simulate sexual crime samples. The system was able to detect human male DNA in presence of high female DNA quantities, in the ratio of 125:1 female/male DNA, obtained a full Y-STRs profile by means of Power Y23 commercial kit amplification.

4. Conclusion

DNA quantification plays a key role in all areas where DNA analysis is required. In addition, quantification of human DNA in forensic samples is crucial for defining the input DNA needed to obtain interpretable STR profiles.

We have developed a duplex Real Time PCR technique that allows human DNA quantification and simultaneously male DNA detection. Additionally, the duplex PCR system showed to be highly sensitive to detect male DNA in mixed samples with high female DNA quantities.

The results correlate well with many commercial kits available in the market.

The system showed to be fast, sensitive, specific, cost effective and suitable for routine casework.

Conflict of interest

The authors report no declarations of interest.

Acknowledgments

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