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Forensic Science International: Genetics Supplement Series 1 (2008) 86–88

FSI GENETICS SUPPLEMENT SERIES

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Research article

Quadruplex real-time PCR for forensic DNA quantitation

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Received 10 August 2007; accepted 21 August 2007

Abstract

Forensic DNA quantitation is an important initial step preceding PCR amplification of the STR loci even though information concerning the quality of the DNA is not revealed. A quadruplex real-time PCR (qPCR) assay was developed to quantify four DNA targets: (1) the human *RB1* gene in nuclear DNA, (2) the *DAZ* gene present on the human Y chromosome, (3) the *ATPase8* gene present in human mitochondrial DNA and (4) an artificial internal positive control to reveal possible PCR inhibition. Primers labeled with four different fluorophores are used together with a single quencher using the antiprimer quenching-based qPCR method in one reaction, in which the resultant amplicons are less than 127 bp in size. Sensitivity was shown to be less than ten copies for all four targets in the absence of amplification inhibition. The amplification remained sensitive in the presence of an excess of non-human DNA.

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Keywords: Multiplex real-time PCR; PCR inhibition; Forensic DNA quantitation

1. Introduction

The accurate quantitation of extracted forensic DNA is now fairly straightforward with the use of real-time PCR (qPCR). It has become an essential second step after template purification in forensic casework prior to PCR amplification of STR loci or of mitochondrial DNA (mtDNA), so as to optimize or even avoid further sample processing. There have been several reports of forensic qPCR assays [1–7] and commercial kits [8] are now available or becoming available that enable quantification by qPCR of one or more representative targets. Most of these works describe assays which quantify up to three targets.

An equally important issue is the presence of inhibitors that could affect subsequent processing even in the presence of adequate template concentrations. The quantitation step is an ideal moment during which the influence of such inhibitors can be estimated.

In this work, we sought to develop a single-tube qPCR assay which allows the quantitation of three distinct but relevant forensic human DNA targets and gives information on the possible presence of PCR inhibitors. Although template DNA quantitation may not convey information concerning its quality, we endeavoured to amplify targets of less than 130 bp in size, thus giving some information as to the presence of DNA degradation.

2. Materials and methods

2.1. Chemistry and primers

To reduce costs and intellectual property conflicts, we chose a novel real-time chemistry based upon an antiprimer quenching-based qPCR method [9]. The sequence of the antiprimer (AP) is TTCCCTCGGATAGCACT and is 3′-labeled with BHQ2. Primers were designed as suggested [9].

2.1.1. Nuclear autosomal target

The human retinoblastoma gene was selected as a single-copy target, as previously described [1] and the primer sequences are:

F: CCAGAAAATAAATCAGATGGTATGTAACA and R: AGTGCTATCCGAGGGAATGGTTTAG-GAGGGTTGCTTCC and is 5’-labeled with FAM, such that the resultant product size is 95 bp.

2.1.2. Y chromosomal target

The human *DAZ* gene was selected of which there are normally four copies per male haploid genome [10], F:

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doi:10.1016/j.fsigss.2007.08.012

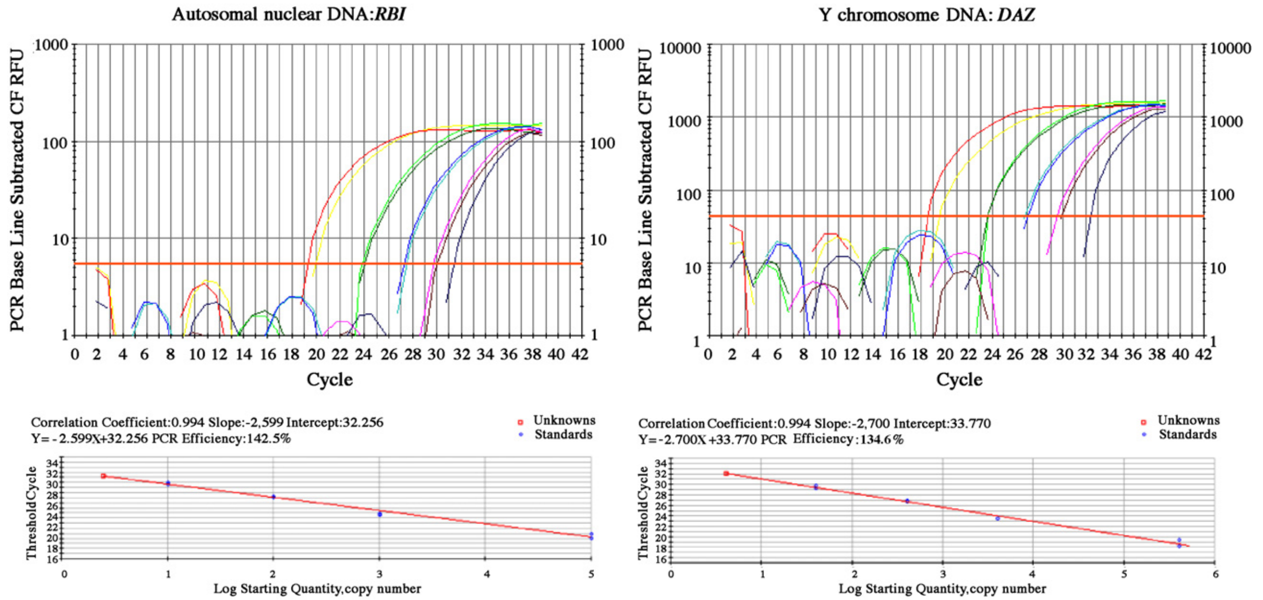

Fig. 1. Amplification curves (duplicates) and serial dilution plots for *RB1* and *DAZ* gene targets.

AGTGCTATCCGAGGGAAGAATCCTGTAACTCAGCA-CGTTC and is 5'-labeled with Cal Fluor Red 610 and R: TTACTATGGAAAGTGTAGATACAATTCC, such that the resultant product size is 126 bp.

2.1.3. Mitochondrial DNA target

The human *ATPase8* gene was used as described previously [4], F: AATATTAAACACAAACTACCACCTACCT and R: AGTGCTATCCGAGGGAATGGTTCTCAGGGTTTGTTA-TAATTT 5'-labeled with HEX, 96 bp amplicon.

2.1.4. Internal positive control (IPC)

This synthetic 77 nt oligonucleotide has the following sequence [7]: AAGCGTGATATTGCTCTTTCGTATAGTTAC-CATGGCAATGCTTAGAACAATACTAATGTTGTAATCTG-TCGCTATGT and is detected with these primers: F: AGTGC-TATCCGAGGGAAAGCGTGATATTGCTCTTTCGTATAG which is 5'-labeled with Cy5 and R: ATAGCGACAGATTA-CAACATTAGTATTG.

2.2. qPCR

Total reaction volume was 25–40 μl. Final concentrations were 400 μM dNTPs, 5 mM Mg^{2+}, 1 μM AP, 400 nM mtDNA primers, 200 nM remaining primers, 0.1 U/μl DNA polymerase. Thermocycles comprised 15 s at 95 °C, 30 s at 60 °C, 45 s at 50 °C during which fluorescence was measured. Multiplex signal collection was carried out using a Bio-Rad iCycler instrument.

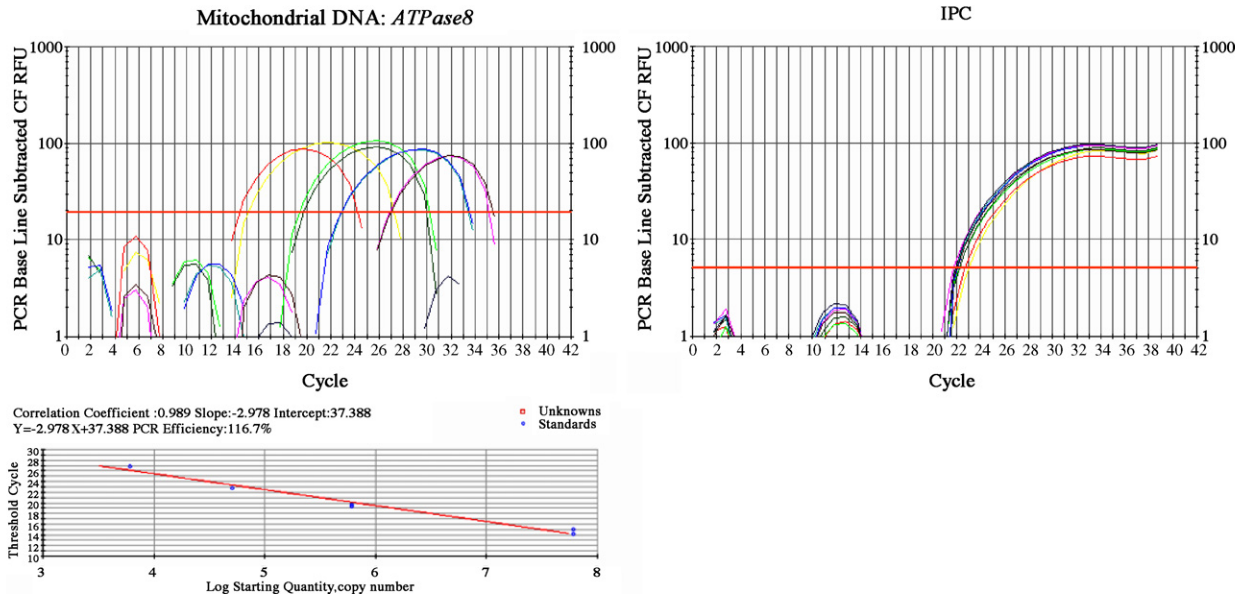

Fig. 2. Amplification curves (duplicates) and serial dilution plots for *ATPase8* gene, and for fixed IPC target.

2.3. Standards

Genomic DNA target standards were made by serially diluting genomic DNA previously quantified using a Nanodrop 3300 fluorospectrometer and PicoGreen. Mitochondrial DNA standards were obtained by serially diluting human DNA containing a known quantity of mitchondrial copies. IPC copy number standards were made by serially diluting a known mass of molecule previously determined by the oligonucleotide manufacturer.

3. Results and discussion

The above data were generated from quadruplex qPCR reactions of serially diluted human genomic and mtDNA. The reactions also contained 1.14×10^5 copies/μl of IPC. The sensitivity under these conditions is 10 copies per 40 μl qPCR reaction (Figs. 1 and 2).

The calculated amplification efficiencies for the human DNA targets are all >100%, most likely reflecting the inherent difficulties of amplifying four targets simultaneously when present at higher copy numbers; this was not observed when carrying out singleplex amplifications of each target separately. The amplification curves observed when amplifying a target alone or as part of a multiplex were mostly superimposable. Despite extensive optimization efforts we were not able to improve upon these results and we found that the universal multiplex conditions previously described [11] referring to 5′ nuclease probes did not apply in this case.

Amplification of mtDNA under multiplex conditions inevitably gave amplification curves in which the signals decreased after reaching a maximum, as shown above, a phenomenon not observed in singleplex reactions. Agarose gel electrophoresis of the multiplex qPCR reactions did not show any additional nonspecific products. As yet we have no explanation for these findings and they could represent the 'hook effect' [12] which has no effect on the quantitation.

Amplification of the fixed IPC under multiplex conditions varied by a maximum of one C_t regardless of the varying copy numbers of the other three targets, as shown above. This shows that, under these conditions, IPC signal generation is independent of that of the other three targets and that the IPC is functioning as anticipated. Preliminary experiments (data not shown) demonstrated that when the IPC is spiked into samples known to have PCR inhibitors, its amplification profile reflects this fact in terms of the shape of the amplification curve and in terms of the increased C_t.

Future work will be directed to cloning the three genomic targets so as to permit setting up the standard curves more easily and reliably using plasmids.

Conflict of interest

None.

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