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Amelogenin as a target for real time PCR quantitation of forensic templates

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Abstract. PCR is the ubiquitous method of forensic DNA analysis, but prior to amplification, two other processes are crucial to obtaining a satisfactory result: template DNA extraction and template quantitation. Here we will presume the extraction process has been performed, and concentrate on the quantitation step: a focus of recent advances. Real time quantitative PCR (RT-QPCR) is an advantageous alternative to probe hybridisation or fluorescent dye association, which are (respectively) laborious and less accurate procedures. We reviewed the available commercial methods of RT-QPCR and concluded that for our requirements, a more attractive solution was the in-house development and validation of an ultra-rapid, small batch size solution. Our solution is real time detection using the Roche LightCycler 2.0 and the amplification of a 106/112 bp amelogenin amplicon. Melt-curve analysis and back extrapolation to the starting template-dependant crossing point generates results from 32 samples in ~30 min (post PCR assembly) and this approach has advantages in that a positive quantitation result implies that in the SGM Plus™ amplification that follows, at the very least, an amelogenin product should be generated. The use of the amelogenin target also provides an indication of the possibility of PCR product travelling from the separate PCR product room backwards into the clean PCR set-up environment, something that the use of telomerase or β-globin amplicons cannot provide. We have validated the use of our LightCycler-amelogenin based quantitation system and have seen significant improvements in the reliability of quantitation measurements in our forensic laboratories. © 2006 Published by Elsevier B.V.

Keywords: Real time PCR; Amelogenin; LightCycler

1. Introduction

Accurate quantitation of template DNA concentration in a forensic extract is crucial to maximising the potential of gaining a full STR profile. Modern multiplex analyses are optimised over a fairly tight template range, and deviating above or below these limits could compromise the profile obtained.

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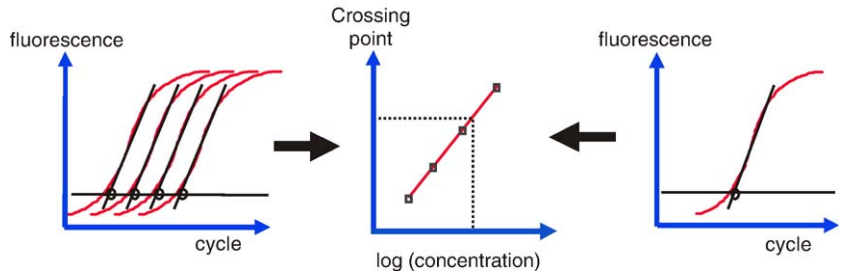

Fig. 1. The left hand graph depicts the accumulation of fluorescence at four (decreasing) concentrations. The right hand depicts the result from an unknown sample. The centre depicts reading the unknown crossing point from the standard curve of the knowns.

Real time PCR has the advantages of being human specific, rapid and accurate. We investigated commercially available real time PCR kits and surmised that the targets used were not necessarily as informative as they might be. Additionally, plate based formats demanded that 96 samples were quantified at the same time, meaning that either queuing of samples, or uneconomical lab procedures had to be tolerated periodically.

The amelogenin gene is well known to the forensic community as giving an indication of the sex of the donor of a specific sample, although this is not infallible [1]. We chose to target the amelogenin gene as the real time PCR target of amplification onboard the Roche LightCycler 2.0 instrument, which is significantly faster than plate-based formats and can be used to process small sample numbers economically.

2. Methods

Amplification primers flank the 6 bp deletion in the amelogenin gene that is used to discriminate gender of the sample [2]. These primers are compatible with the SGM Plus™ annealing temperature of 59 °C.

Forward primer

$^{5'}$CCCTGGGCTCTGTAAAGAATAGTG$^{3'}$

Reverse primer

$^{5'}$ATCAGAGCTTAAACTGGGAAGCTG$^{3'}$

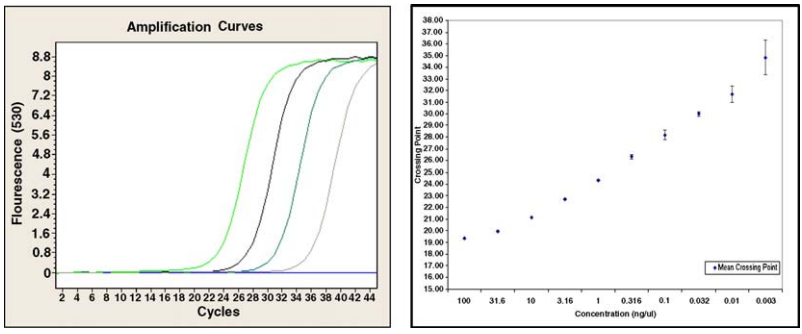

Fig. 2. Actual data from the devised system over four separate ‘standard’ values of 10, 1, 0.1 and 0.01 ng/μl. The graph to the right indicates the results obtained over a more extensive range (100–0.003 ng/μl). Error bars increase towards the lower end of the concentration range as might be anticipated.

Table 1
Representative results of normal and low level DNA content samples

Sample number	Sample type	Quantiblot conc. (ng/μl)	LightCycler conc. (ng/μl)
F12019	Saliva swab	0.4	0.3
F12092	Cigarette end	0.0	0.00457
F12098	Touch (glove)	0.0	0.00197
F12101	Hair	0.0	0.0387

Samples which are rated as zero-quant by hybridisation techniques return very low but detectable levels of DNA employing real time PCR.

Reduced volume (5 μl) PCRs were assembled in the capillary of the LightCycler. Each amplification contained only 1.25 μl of template extract and 3.75 μl of Q-PCR MasterMix (LightCycler® FastStart DNA Master SYBR Green I Kit, Roche Applied Science). Capillaries were transferred to the LightCycler and were cycled through fully 40 rounds of PCR, whilst monitoring the accumulation of fluorescence.

Comparison of the accumulated fluorescence with the results obtained from a standard curve (10 ng/μl, 1 ng/μl, 0.1 ng/μl, 0.01 ng/μl) enables back extrapolation of each line to the 'crossing point' and accurate quantitation of the amount of DNA present in the unknown samples (Fig. 1). The crossing point of the unknown can be read from the graph of crossing points from the standard curve, giving an indication of the concentration of extracted DNA present in the sample.

The system devised demonstrated that it is possible to generate results from diverse sample types and that the real time PCR system would reliably give quantitation results where zero quantitation values had been obtained from the Quantiblot (probe hybridisation) kit (Fig. 2).

3. Results and discussion

Targeting the amelogenin gene in order to quantify extracted DNA offers several advantages over other more exotic PCR targets. The amelogenin target is amplified under the same conditions as the SGM Plus reaction will be undertaken, and so gives a strong indication that if the quatitation gives a positive result, the diagnostic SGM Plus reaction should (despite being carried out on a very different thermal cycler) be expected to generate an amelogenin profile at the very least. The use of a reduced volume does of course mean that for very low level templates (around 1 cell copy per μl) there is the chance that the 1.25 μl of extract sampled will be devoid of any amelogenin target. A zero quantitation must therefore be concentrated and the quantitation repeated before the result can be relied upon as being truly negative.

Despite this, we find that the LightCycler quantitation method is typically more sensitive than classical probe hybridisation systems such as Quantitiblot (ABI). Table 1 demonstrates a few such examples.

References

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[2] K.M. Sullivan, et al., BioTechniques 15 (1993) 637–641.