Available online at www.sciencedirect.com
ScienceDirect
Forensic Science International: Genetics Supplement Series 1 (2008) 71–73
FSI GENETICS SUPPLEMENT SERIES
www.elsevier.com/locate/FSIGSS

Research article

Quantification of trace amounts of human and non-human mitochondrial DNA (mtDNA) using SYBR Green and real time PCR

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Received 20 August 2007; accepted 9 October 2007

Abstract

There are currently no tests available to quantify total non-human mammalian mtDNA. Standard universal DNA quantification tests are unsuitable due to the large size difference between nuclear and mitochondrial genomes and the ubiquity of human mtDNA. A method has therefore been developed to quantify total mammalian mtDNA and total human mtDNA present in a sample using SYBR Green.

Mammalian primers designed to react with all mammals were designed on the 12S and human specific primers were designed on the cytochrome *b* gene. Each primer set was reacted separately with sample and SYBR Green and detected using RT-PCR. A standard curve was developed using dilutions ranging from 1 billion copies to 100 copies of mtDNA.

Twenty-four human samples were analysed and an average log (copy number) human/universal ratio of 1.00 was obtained. Samples falling below this ratio will contain some non-human mtDNA while samples falling above this ratio contain human mtDNA only.

Twenty-nine mammal samples were also tested. 96.6% of these showed human contamination to some extent. This test is able to quantify mtDNA down to the femtogramme (10E−15g) level.
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Keywords: Quantification; Human mtDNA; Non-human mtDNA; Human contamination

1. Introduction

Quantification of the mitochondrial genome in samples has proven to be a difficult process. Concentrations of mitochondria and mitochondrial DNA (mtDNA) per mitochondrion are assumed to vary by tissue type and estimations of copy number are inaccurate as the literature values vary immensely (Table 1). This wide variation in the literature values makes it impossible to accurately estimate mtDNA copy number, especially in forensic samples where the tissue type may not be known. Mixtures of human mtDNA and non-human mtDNA further complicate this process.

Non-human biological evidence is encountered routinely in forensic casework and is likely to be contaminated with human DNA. It is therefore essential that human contamination, if any, is identified and quantified separately from any non-human DNA present. Standard methods of DNA quantification are none specific and quantify all DNA present. This poses a problem when attempting to quantify mtDNA as the mitochondrial genome, which is more numerous than the nuclear genome, is insignificant when compared by weight. Approximately 386,000 mitochondrial genomes are equivalent to one nuclear genome.

We have developed a test capable of quantifying all mammalian mtDNA and all human mtDNA such that when the human fraction of a sample is subtracted an accurate value for the remaining non-human component of a mixture is obtained. This will aid in determination of proper techniques (sequencing may not be suitable for analyzing a mixture) and in optimization of downstream analyses.

2. Materials and methods

Cytochrome *b* and 12S DNA sequence information was downloaded from the NCBI website and aligned using Clustal W (http://www.ebi.ac.uk/clustalw/). Twenty-nine mammals were used for the cytochrome *b* alignment where the human specific primers were designed. Sixteen mammals were used for the 12S alignment where the universal primers were designed. Universal primers were designed such that they will

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doi:10.1016/j.fsigss.2007.10.103

Table 1
Studies on the number of mitochondria per cell and the number of mitochondrial genomes per mitochondria

Animal	Cell type	mtDNA copy number	Reference
Mouse	L-cell	1100 ± 250	[1]
Human	HeLa	8800	[1]
Human	A2780	500 (average)	[2]
Human	Virtual method	220–1720	[3]
Human	Oocyte	>90,000	[4]
Human	Male germ	2000–3000	[4]

react with all mammalian species. Human specific primers will only react with human samples.

DNA was extracted from blood, tissue, hair, urine or buccal cells using the QIAamp® Micro Kit (Qiagen). Hair samples were allowed to digest in Proteinase K and DTT for up to 48 h.

Standards were prepared for PCR in 50 μL aliquots containing GeneAmp® 10X PCR Gold Buffer (100 mM TRIS–HCl, 15 mM $MgCl_2$, pH 8.0), dNTPs (200 μM), 3.5 units AmpliTaq Gold® (Applied Biosystems), forward and reverse primers (at a final concentration of 0.5 μM), sterile H_2O, and 2.5 μL of DNA. The PCR proceeded for 40 cycles at 95 °C for 45 s, 64 °C for 45 s and 72 °C for 1 min followed by a final extension step of 20 min at 72 °C.

Fragments were visualized on a 2.5% agarose gel with EtBr. Only the expected products were observed. The fragments were then extracted from the gel using the Spin Prep Gel DNA Kit (Novagen®). Extraction proceeded according to the manufacturer's protocol with the addition of a second wash step with solution B. A total of 100 μL of DNA elute was recovered from each column giving a total of 300 μL for each fragment.

DNA was then quantified using the A_{260} method. Fragment copy/mL was calculated. The human fragment extract was found to be 4.389162562 times more concentrated than the universal fragment extract.

To create standard curves a dilution series was prepared with dilutions from 100 to 1,000,000,000 copies and accounted for the difference in initial concentrations. Samples were analysed using Platinum® SYBR® Green qPCR SuperMix-UDG with Rox (Invitrogen™) on a 72 well Rotor-Gene RG-3000 QPCR machine (Corbett Life Science). The recommended final PCR volume was reduced to 15 μL. The PCRs were prepared containing SuperMix, forward and reverse primers (at a final concentration of 0.5 μM), sterile H_2O, and 1 μL of mtDNA standard or sample. The PCR proceeded for 45 cycles at 95 °C for 45 s, 64 °C for 45 s and 72 °C for 1 min followed by a stepwise from 64–95 °C.

Twenty-four anonymous human samples and 27 animal samples were tested in the same manner as the standards. Animal samples consisted of blood, tissue, hair, urine and buccal samples.

3. Results

Standard curves were created for both human specific and universal fragments (Fig. 1). Testing with human DNA allowed a human/universal ratio to be established and was found to be 1.13 ± 0.07. We recommend a human/universal ratio cut off value of 1.00 due to the increased variation in Ct value observed at lower dilutions. This will ensure that non-human mtDNA is present in the sample.

All animal species reacted showed a human/universal ratio of less than 1. This demonstrates that specificity of the human specific primers as they did not bind with any of the animals tested or a human/universal ratio of greater than 1 would have been observed. Universal primers reacted with all the species tested indicating their versatility. All unrelated human samples showed a human/universal ratio of greater than 1 indicating the human specific primers will produce a product with all human samples. The most dilute

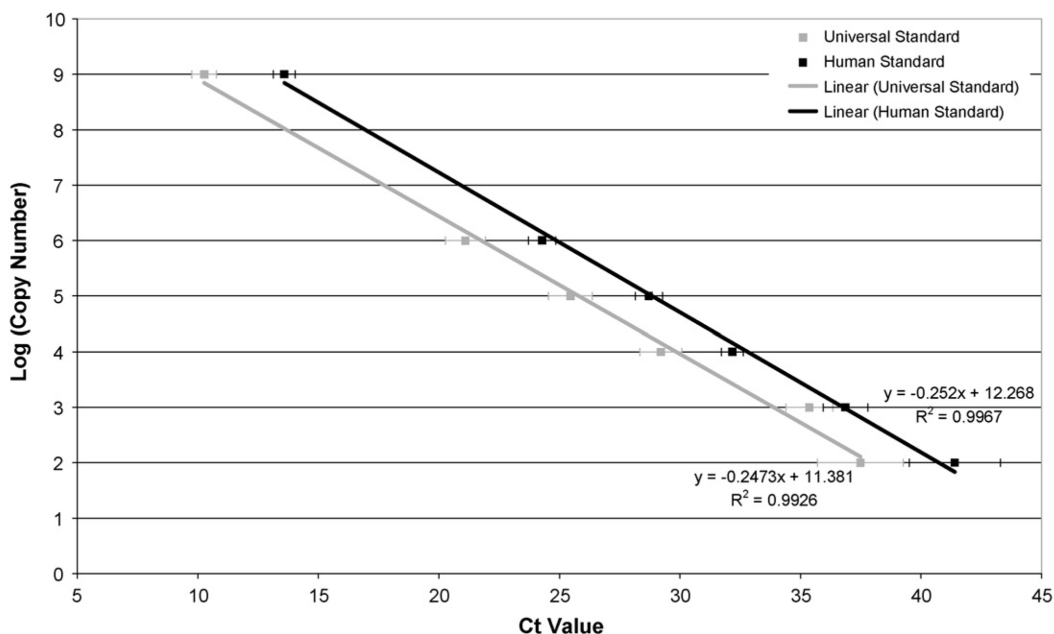

Fig. 1. Standard curves for human and universal fragments. Standard deviation and equations of line are shown.

animal sample analysed showed 12,000 copies per μL (20.4 pg).

4. Conclusions

This method provided for the first time a means to accurately quantify both human and non-human mtDNA within a mixture. This will allow optimization of downstream reactions and will also permit the accurate quantification of mtDNA copies from a wide range of mammalian species regardless of the tissue type.

Acknowledgements

Funding for this project was obtained in part from a Carnegie Research Grant from the Carnegie Trust. We would like to thank the Armed Forces DNA Identification Laboratory (AFDIL) for providing lab space and equipment as well as arranging accommodation. Thanks also to Dr. H. Hird from Central Science Laboratory in York, UK, Linda Horan from the University of Strathclyde and Stuart Becker from the Animal Behavior Group at the University of Liverpool for providing several of the animal samples. We would also like to thank Dr. A. Ricketts for his input and suggestions.

Conflict of interest

None.

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