Quantification of Human and Non-Human Mitochondrial DNA (mtDNA) Using SYBR Green

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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.

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	> 90000	[4]
Human	male germ	2000 - 3000	[4]

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 (Figure 1), is insignificant when compared by weight (Figure 1). 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.

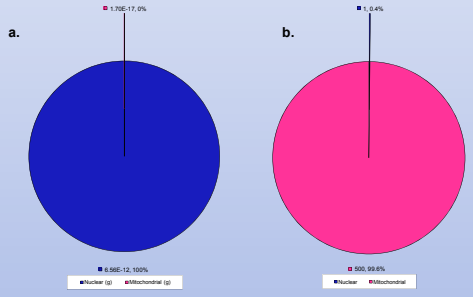

Figure 1: a) The nuclear genome compared to the mitochondrial genome by weight. b) The nuclear genome compared to the mitochondrial genome by copy number per cell. 500 is used as an average value but this number can vary from several hundred copies to several thousand copies per cell.

MATERIALS AND METHODS:

Sequence Information and Primer Design

Cytochrome *b* and 12S sequence information were downloaded from the NCBI website and aligned using ClustalW (http://www.ebi.ac.uk/clustalw/). Twenty nine mammals were used for the cyt*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 react with all mammalian species. Human specific primers will only react with human samples.

DNA Extraction

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 hours.

Creating Standards

Samples 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 ºC for 45 seconds, 64 ºC for 45 seconds and 72 ºC for 1 minute followed by a final extension step of 20 minutes at 72 ºC.

Fragments were visualized on a 2.5 % agarose gel with EtBr (Figure 2a). 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.

The success of the gel extraction was tested by separating the products on a 2.5 % agarose gel (Figure 2b). Two bands were clearly visible indicating a successful extraction. 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.

$$[(A \times 5.0 \times 10^{-7}\ g/mL \times dilution) / (mol\ weight\ per\ copy)] \times 6.02 \times 10^{23} = Fragment\ copy/mL$$

Where A is the absorbance at 260 nm, 5.0×10^{-7} g/mL is constant for dsDNA. Fragment copy is equal to one double stranded copy of the target sequence.

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 (Table 2). Samples were analyzed 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 ºC for 45 seconds, 64 ºC for 45 seconds and 72 ºC for 1 minute followed by a stepwise from 64-95 ºC.

All 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 ºC for 45 seconds, 64 ºC for 45 seconds and 72 ºC for 1 minute followed by a stepwise from 64-95 ºC.

Table 2: Dilution series (showing the adjustment of 4.4 times more universal fragments in the initial dilution) used for standard curve showing copy number per µL, weight of copies and equivalent weight of mitochondrial genome.

Dilution	Copy number		Weight of fragments (g)		Equivalent mitochondrial genome weight (g)	
	Universal	Human	Universal	Human	Universal	Human
1	2.463×10^{11}	1.081×10^{12}	5.00×10^{-08}	2.25×10^{-07}	4.19×10^{-06}	1.84×10^{-05}
1×10^{-03}	1.084×10^{09}	1.081×10^{09}	2.20×10^{-10}	2.25×10^{-10}	1.84×10^{-08}	1.84×10^{-08}
1×10^{-06}	1.084×10^{06}	1.081×10^{06}	2.20×10^{-13}	2.25×10^{-13}	1.84×10^{-11}	1.84×10^{-11}
1×10^{-07}	1.084×10^{05}	1.081×10^{05}	2.20×10^{-14}	2.25×10^{-14}	1.84×10^{-12}	1.84×10^{-12}
1×10^{-08}	1.084×10^{04}	1.081×10^{04}	2.20×10^{-15}	2.25×10^{-15}	1.84×10^{-13}	1.84×10^{-13}
1×10^{-09}	1.084×10^{03}	1.081×10^{03}	2.20×10^{-16}	2.25×10^{-16}	1.84×10^{-14}	1.84×10^{-14}
1×10^{-10}	1.084×10^{02}	1.081×10^{02}	2.20×10^{-17}	2.25×10^{-17}	1.84×10^{-15}	1.84×10^{-15}

Weight is based on the calculation: (Copy #)(Fragment size bp (617.5g/mol)/(6.02×10^{23}bp/mol)).
Genomic weight is based on the human mitochondrial genome with a total weight of 1.70×10^{-11}g multiplied by the number of fragment copies.

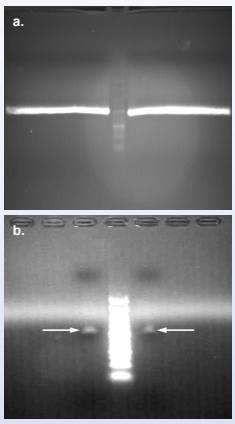

Figure 2a: Human (left) and universal (right) fragments separated with a 100 bp ladder on a 2.5 % agarose gel. No superfluous fragments were observed. 2b: Confirmation of a successful gel extraction for human (left) and universal (right) fragments separated with a 100 bp ladder on a 2.5 % agarose gel.

Sample Testing

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.

RESULTS:

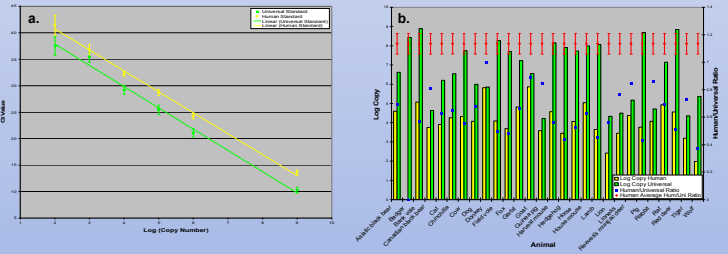

Figure 3a: Standard curve for human and universal fragments showing standard deviation. ■ - human standard (y = -3.9551x + 48.618, R2 = 0.9967) ; ■ - universal standard (y = -4.0135x + 45.875, R2 = 0.9926). 3b: Animal samples, their human and universal copy number, human/universal ratio and the average human/universal ratio obtained with human test samples. Animal samples with a human/universal ratio below the average obtained from the human test samples contain non-human mtDNA. ■ - human/universal ratio for the animal samples and; ♦ - average human/universal ratio for human test samples showing standard deviation.

CONCLUSIONS:

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 (Figure 3a). 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 (Figure 3b). Human specific primers are specific for human mtDNA and 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.

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.

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