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Mitochondrial DNA as a non-invasive biomarker: Accurate quantification using real time quantitative PCR without co-amplification of pseudogenes and dilution bias

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ABSTRACT

Circulating mitochondrial DNA (MtDNA) is a potential non-invasive biomarker of cellular mitochondrial dysfunction, the latter known to be central to a wide range of human diseases. Changes in MtDNA are usually determined by quantification of MtDNA relative to nuclear DNA (Mt/N) using real time quantitative PCR. We propose that the methodology for measuring Mt/N needs to be improved and we have identified that current methods have at least one of the following three problems: (1) As much of the mitochondrial genome is duplicated in the nuclear genome, many commonly used MtDNA primers co-amplify homologous pseudogenes found in the nuclear genome; (2) use of regions from genes such as β-actin and 18S rRNA which are repetitive and/or highly variable for qPCR of the nuclear genome leads to errors; and (3) the size difference of mitochondrial and nuclear genomes cause a "dilution bias" when template DNA is diluted. We describe a PCR-based method using unique regions in the human mitochondrial genome not duplicated in the nuclear genome; unique single copy region in the nuclear genome and template treatment to remove dilution bias, to accurately quantify MtDNA from human samples.

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1. Introduction

Mitochondria are organelles present in most eukaryotic cells in variable numbers ranging from hundreds to thousands of copies per cell and contain their own extra-chromosomal genome, a 16.6 kb circular molecule of double stranded DNA [1]. An individual mitochondrion can contain more than one mitochondrial genome, the number has been estimated to be between 0 and 11 copies with a mean of 2.0 [2]. The amount of mitochondrial DNA (MtDNA) in a cell could provide a major regulatory point in mitochondrial activity, as the transcription of mitochondrial genes is proportionate to their copy numbers [3,4]. As mitochondria are the major producers of intracellular reactive oxygen species in the cell through free radical generation, a side product of oxidative phosphorylation, it is possible that alteration in MtDNA content could lead to a change in mitochondrial gene transcription and activity and thereby could affect the redox balance of the cell [5].

The role of mitochondrial dysfunction in numerous diseases is well documented with more than 10,000 cited entries in PubMed, NCBI. Studies looking specifically at alterations in MtDNA content in various cell types cover a broad range of human diseases, such as diabetes and its complications [5–10] obesity [11] cancer [12–19], HIV complications [20–24], eye disease [25,26], nasal polyp [27] and others. In addition, links between altered MtDNA content and development [28–30], fertility [31–33], ageing [34], environment [35,36], and exercise [37–39] have been shown. Clearly there is a widespread interest in accurately quantifying human MtDNA in a broad spectrum of human diseases. In order to determine if MtDNA content is a potential biomarker of mitochondrial dysfunction, it is important to validate methods for accurate and reproducible measurement of cellular MtDNA content.

A common method for measuring MtDNA content is to quantify a mitochondrial encoded gene relative to a nuclear encoded gene to determine the mitochondrial genome to nuclear genome ratio (Mt/N) using real time qPCR. However in many cases the methodology being used suffers from at least one of the following problems: Firstly, as much of the mitochondrial genome is duplicated in the nuclear genome [40,41] many MtDNA specific primers co-amplify homologous pseudogenes found in the nuclear genome; secondly, for the quantification of the nuclear genome many studies utilise regions from repetitive and/or highly variable genes

Abbreviations: Mt/N, mitochondrial genome to nuclear genome ratio; MtDNA, mitochondrial DNA.

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such as 18S rRNA which can result in co-amplification errors; thirdly, dilution of genomic template DNA can introduce significant errors in Mt/N values as MtDNA and nuclear DNA do not dilute equally, an effect we have previously described as "dilution bias" and which may be a consequence of the fact that the mitochondrial genome is a circular molecule of 16.5 kb whereas the nuclear genome is composed of linear molecules (chromosomes) of more than 3 million kb in size [5].

In the current paper we describe improvements to the PCR-based method for quantification of MtDNA. MtDNA copy numbers were determined by identifying and amplifying a short unique region of MtDNA not present as a nuclear pseudogene. Nuclear DNA content was determined by amplification of a segment of unique single copy nuclear gene. In addition, we show the differing effects of template dilution on MtDNA and nuclear DNA and describe a protocol for removing dilution bias.

2. Materials and methods

2.1. Identification of unique regions of the mitochondrial genome and primer/probe design

The duplication of the mitochondrial genome in the nuclear genome was detected using BLAST (http://www.ncbi.nlm.nih.gov) [42]. Unique regions were identified in the human mitochondrial sequence, retrieved from ENSEMBL [43] using FASTA version 3.5.2.7 [44] as follows: The mitochondrial sequence was split into overlapping fragments of length 150 bp with a 50 bp overlap and each fragment was used as a query sequence in a FASTA search against the entire human genome, one chromosome at a time (both strands). The output files were used to extract and order the hits from all the chromosomes to each 150 bp input file. A simple perl script was generated to both sort the data and extract the next best/exact match (mitochondrial to mitochondrial) to any nuclear chromosome (http://www.perl.org/about.html). 'Second best' matches with significant similarity (long overlap and/or significant percent similarity) were identified and excluded from potential 'unique' mitochondrial sequences. Each candidate 'unique' sequence was tested using BLAST to ensure programmatic accuracy. Primers were synthesised at Sigma-Genosys UK and a custom designed taqman probe to the human unique mitochondrial region and the predesigned RNasP taqman probe were used (Applied Biosystems).

2.2. Genomic DNA preparation

Peripheral blood samples obtained under NHS Research Ethics Committee approval (REC 07/H0806/120) were used to prepare genomic DNA using the QIAMP DSP blood DNA kit (Qiagen). The concentration of the DNA was adjusted to10 ng/μl. For shearing the DNA, at least 100 μl of DNA was passed repeatedly through a 1 mm needle for 25 s. For sonication, DNA in a total volume of 100 μl was subjected to sonication for 2–10 min using a Bath Sonicator (Kerry, Pulsa-tron 55) and it was found that 10 min sonication gave the best results. To avoid errors arising from repeated freeze thaw cycles DNA samples were kept at 4 °C for the duration of this study.

2.3. Real time qPCR

MtDNA content was assessed by quantification of a unique mitochondrial fragment relative to a single copy region of the nuclear gene RNaseP or β2 M (Table 1) using a taqman assay. A 62-bp fragment of MtDNA was amplified using the primers: hmito-319F5 and hmito-383R5, and hmitoP5 was used as the hybridization probe, containing the FAM (6-carboxy fluorescein) as a fluorescent reporter dye and NFQ as a quencher dye at the 3′ end. Nuclear content was quantified by targeting an unique region of either RNaseP or the B2 M gene using the ABI-VIC®/TAMRA™ Probes, primer limited (Applied Biosystems).

2.4. Preparation of dilution standards from hmito and RNaseP

Primers for MtDNA (hmitoF3, hmito R3), RNaseP (hRNasePF1, hRNasePR1) used to amplify the respective products from human genomic DNA (Table 1). The PCR product of hmitoF3 and hmitoR3 contains the region amplified by hmitoF5 and hmitoR5 and the PCR product of hRNasePF1 and hRNasePF2 contains the region identified by the commercially available RNaseP assay (Applied Biosystems). Each amplified product was cloned into the vector pGEM-T (Promega), purified and sequenced. Recombinant plasmids were linearised and diluted to known copy numbers calculated as previously described [45,46]. Dilution standards containing a dilution series of 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 copies per μl of each plasmid were prepared in the presence of carrier tRNA (10 μg/ml; Sigma, UK) and used in real time qPCR for copy number quantification. All dilution standards were kept at 4 °C for the duration of the study.

3. Results

3.1. Duplication of the human mitochondrial genome in the nuclear genome

Using blastn, we compared the mitochondrial genome with the nuclear genome and found that more than 97% of the

Table 1
Primers/probes used in the study.

Gene accession no.	Primer/probe	Oligonucleotide sequence	Product size (bp)
Human mitochondrial genome NC_012920	hmito F3	CACTTTCCACACAGACATCA	129
	hmito R3	TGGTTAGGCTGGTGTTAGGG	
	hmito F5	CTTCTGGCCACAGCACTTAAAC	64
	hmMito R5	GCTGGTGTTAGGGTTCTTTGTTTT	
	hmito P5	FAM-ATCTCTGCCAAACCCC	
Human RNaseP AF479321	hRNaseP F1	CCCCGTTCTCTGGGAACTC	175
	hRNaseP R1	TGTATGAGACCACTCTTTCCCATA	
Human β2M Accession number M17987	hB2M F1	TGTTCCTGCTGGGTAGCTCT	187
	hB2M R1	CCTCCATGATGCTGCTTACA	
	hB2M F2	GCTGGGTAGCTCTAAACAATGTATTCA	93
	hB2M R2	CCATGTACTAACAAATGTCTAAAATGGT	
	hB2M P2	VIC-CAGCAGCCTATTCTGC	

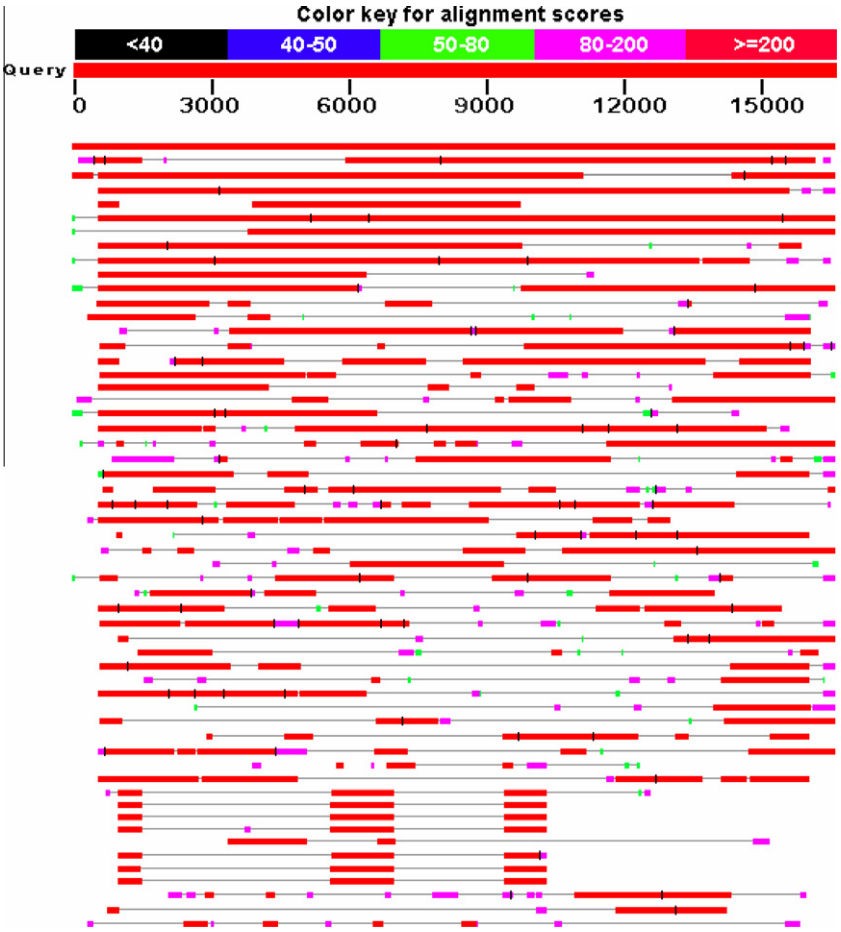

Fig. 1a. Duplication of the mitochondrial genome in the nuclear genome. The thick red line (query) representing the mitochondrial genome sequence accession number NC-001807, was blasted against the reference sequence of the human genome using blastn, the first 50 best matching sequences are shown, the top red line being an exact match to the mitochondrial genome, whereas the remaining 49 lines are regions of the nuclear genome showing a high degree of identity. The colour key for alignment scores is given at the top of the figure with red being the highest alignment score. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

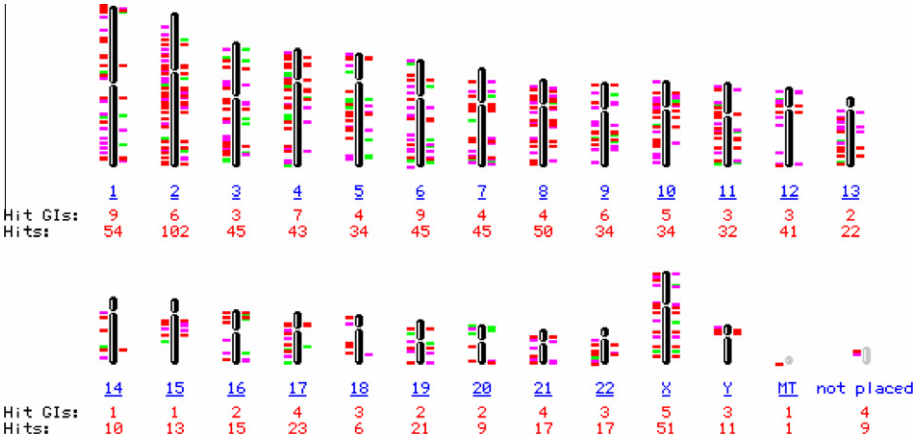

Fig. 1b. Duplication of the mitochondrial genome in the nuclear genome. Mitochondrial pseudogenes in the nuclear genome are shown as bars against the relevant human chromosome, the extent of the homology is shown as a colour code indicated in (1A), and the number of hits is shown in red numbers below the chromosome number shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MtDNA sequence shows homologies with regions in the nuclear genome (Fig. 1A). More than 200 regions of the nuclear genome contained significant matches to the mitochondrial genome, and these matches were scattered throughout the nuclear genome (Fig. 1B).

3.2. Identification of unique regions of the mitochondrial and nuclear genome

The entire mitochondrial genome sequence was split into fragments of 150 bp long overlapping by 50 bp and used to identify a

Table 2
Accuracy of the MtDNA and RNaseP assay and the effect of serial 10-fold dilutions using untreated template DNA.

Dilution factor of template DNA	Mito			RnaseP			Mt/N
	Copy numbers	Mean copy number (SD)	Copy number corrected for dilutions	Copy numbers	Mean Copy number (SD)	Copy number corrected for dilutions	
Neat	46,781 42,582 44,560 43,130 43,328	44,076 (1676)	44,076	1009 909 860 883 997	931 (67.5)	931	47.3
1 in 10	1784 1885 2131 2083 1979	1972 (141)	19,720	58 61 54 61 61	59 (3.08)	590	33.4
1 in 100	100 99 98 87 101	97 (5.7)	9700	4.54 4.54 3.89 7.59 7.59	5.6 (1.8)	560	17.2
1 in 1000	6.8 7.1 5.2 6.0 6.9	6.4 (0.7)	6400	0.23 0.15 0.09 0.44 0.10	0.2 (0.14)	200	32
1 in 10,000	2.0 1.8 1.6 1.7 1.9	1.8 (0.15)	18,000	0 0 0.164 0.07 0.07	0.1 (0.06)	1000	18

Values show numbers (standard deviation).10 ng of neat DNA was used followed by 10-fold dilutions. $n = 5$ for each dilution factor.

unique region of the human mitochondrial sequence between positions 241 and 390 which was the 'least similar' to any human nuclear genomic region (Table 1). Primers were designed to RNaseP and β2 M genes and the primers and amplicons were tested using blast to ensure that they were present only once in the nuclear genome and did not show similarity to any other regions in the genome.

3.3. Dilution of template DNA can lead to large experimental errors: Dilution Bias

We examined the accuracy and sensitivity of the hmito MtDNA and RNaseP taqman assays as well as the effect of diluting the template DNA. When replicates from the same sample were compared to each other at a single dilution, both MtDNA and RNasP values were almost identical showing that the real time qPCR assays show good reproducibility (Table 2). However values were markedly different when different dilutions of the same template DNA were compared to each other. Ten-fold dilution of template DNA did not result in the expected 10-fold dilution of MtDNA or RNAseP copies. Instead, MtDNA copy numbers of the starting material varied by more than 10-fold and RnaseP copy numbers varied by more than 5-fold depending on which dilution of the template was used for real time qPCR. The consequence of this was that Mt/N values for the exact same sample differed by as much as >3fold from the same template DNA. Clearly such large differences in quantification from the same sample at different dilutions results in unacceptable errors in the quantification of Mt/N.

This result suggests that mitochondrial DNA and nuclear DNA do not dilute correctly and have termed this effect as "dilution bias", which may be a consequence of (a) the high molecular weight of nuclear genomic DNA resulting in viscosity and (b) the different sizes of the nuclear and mitochondrial genome, the latter being much smaller and circular compared to the nuclear linear chromosomes.

3.4. Ultrasonic fragmentation of template DNA to remove dilution bias

We previously described the use of manual shearing of template DNA by passage through a syringe prior to PCR to remove dilution bias [5]. However, manual shearing of DNA is not practical when large numbers of samples are being processed and furthermore could introduce variability and/or contamination. Therefore we set out to determine if ultrasonic fragmentation, known to fragment DNA [47], could be utilised to pre-treat template DNA in order to remove dilution bias and improve accuracy of Mt/N determination. Several variables were examined including sample volume, DNA concentration and ultrasonic time as well as the presence of DNA carriers such as tRNA to reduce dilution bias (data not shown). A comparison of the effect of pre-treatment of template DNA by shearing and by sonication is shown in Fig. 2. Serial 10-fold dilution of template DNA should result in 10-fold decrease in RnaseP and MtDNA copy numbers as determined by real time qPCR. However in untreated DNA neither the RNaseP nor the MtDNA copy number show accurate 10-fold reduction (Fig. 2A and B). Treatment with shearing improves the values for MtDNA but the RnaseP values remain underestimated in the diluted samples (Fig. 2A and B) resulting in Mt/N values being underestimated when diluted samples are used. The best results are obtained when the template DNA is sonicated, with both RNaseP and MtDNA copy showing the expected 10-fold reduction in values at 10-fold dilutions. This results in the Mt/N ratio being the most accurate for the sonicated DNA (Fig. 2C) regardless of the dilution of template DNA used.

Sonication significantly improved accuracy allowing the dilution of template DNA to 1000-fold without the loss of accuracy, however at 10,000-fold dilution the accuracy was compromised (data not shown). Therefore, it is possible to accurately measure Mt/N values in as little as 1 pg of DNA template using this method. For comparison we also undertook real time qPCR using the SYBR

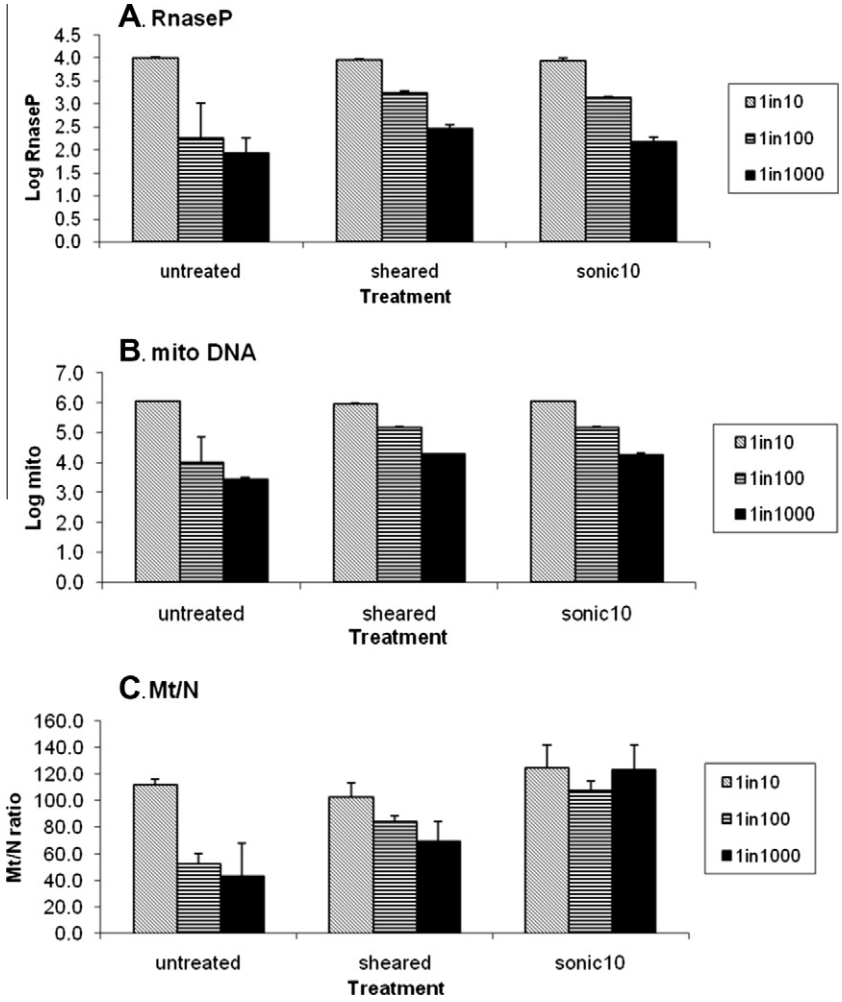

Fig. 2. The effect of pretreatment of template DNA on quantification of mito, Rnase P and Mt/N values. Genomic DNA prepared from peripheral blood was adjusted to 10 ng per μl and was used (A) untreated as control, (B) sheared prior to use by passing repeatedly through a 1 mm needle for 25 s (C) sonicated for 10 min using a Bath Sonicator (Kerry, Pulsa-tron 55). Following treatment the template DNA was used to determine MtDNA and RNase P copy numbers using real time qPCR with the taqman probe method in the presence of dilution standards for each condition ($n = 6$). 1 in 10 refers to neat DNA i.e. 10 ng, 1 in 100 is expected to be 1 ng and 1 in 1000 is expected to be 0.1 ng.

green incorporation assay as opposed to the taqman assay using both RNaseP and β2 M as the nuclear genes and found that both methods give good reproducible and comparable results compared to untreated DNA (Data not shown).

4. Discussion

In the last decade with the wider availability of real time qPCR, there has been a substantial increase in publications reporting changes in MtDNA content in human cells from tissues and circulating cells, and changes in MtDNA have been reported for a wide range of human diseases from cancer to diabetes as well as in development, ageing and exercise [5–37]. Altered MtDNA content or copy number, has demonstrated in circulating blood cells [5,20,21] in cell free serum [48,49], saliva [12] and sperm [31,32]. The results of these and many other studies suggest that MtDNA could be an attractive and non invasive biomarker for predictive and diagnostic purposes. As such it is important to improve the accuracy and reproducibility of methods for measuring MtDNA. We have identified significant problems associated with the accuracy of measurement of MtDNA with current methodology. There is widespread use of primers designed to amplify regions of MtDNA which are highly duplicated in the nuclear genome in the form of pseudogenes as well as nuclear regions which are duplicated or repetitive (Appendixes A and B). Furthermore, the inability to dilute template DNA accurately due to the differing genome sizes of the nuclear and mitochondrial genomes, described here as dilution bias, could result in unacceptable errors in Mt/N values. Furthermore, the method used for template DNA isolation before MtDNA quantification can influence results, as it has been shown that solvent based methods using phenol extraction may give different results to column based methods [50–52].

The concept of the mitochondrial genome being duplicated in the nuclear genome has been known for many years. Parfait et al. undertook a systematic study using mitochondrial primers and testing them for co-amplification in a mitochondrial null cell line and identified primer pairs that did not show any co-amplification with nuclear DNA [40]. Subsequently, more than 1105 regions in the nuclear genome with strong sequence identity to MtDNA were shown to carry 286 Mt pseudogenes [41] with many of the nuclear pseudogenes showing polymorphisms [53]. We have confirmed here that more than 95% of the mitochondrial genome is duplicated in the nuclear genome at numerous locations. In the current paper we describe primers designed to a unique region of MtDNA which will not co-amplify any nuclear pseudogenes.

Many studies using real time qPCR have tended to use primers for estimation of nuclear genome content from control genes previously used for mRNA quantification, such as β-actin and

18SrRNA. The rationale for using these genes for mRNA quantification is that they are assumed to have "housekeeping" functions and are assumed to be expressed at equal levels in all cells. However when quantifying copy numbers from genomic DNA these genes are not the best choice as many of the regions being amplified are duplicated in the genome and are not single copy (Appendix A). For example, there are several β-actin pseudogenes in the nuclear genome some of which show high levels of sequence identity to the active gene. Similarly the use of the highly repetitive and variable 18SrRNA sequences present in thousands of copies in the nuclear genome should be avoided for nuclear DNA quantification. In the current paper we provide primers for RNaseP and β2M which are both unique single copy genes which could be used for nuclear genome quantification.

We propose that for Mt/N determination template DNA should be fragmented prior to PCR to avoid dilution errors. We have shown that Mt DNA and nuclear DNA show different dilution patterns which could be a consequence of the relatively small circular genome of MtDNA (~50 kb) compared to nuclear DNA, which consists of chromosomes, linear molecules of large molecular weight. We have shown that dilution of genomic DNA can lead to large inaccuracies in quantification of both mitochondrial and nuclear genomes and could have serious implications for the Mt/N determination. As many studies are reporting changes as small as ~2-fold in MtDNA content, it is important to ensure that these changes are not a consequence of the dilution effect.

The method we describe here is applicable to quantification of Mt DNA from any cells of human origin including tissues and blood cells. Furthermore pre-treatment of template DNA prior to dilution is also applicable where Mt DNA has been proposed as a circulating biomarker in serum [48,49]. The dilution effect we have described could also be applicable to other situations where real time qPCR is used with genomic DNA, for example in copy number variation research [53,54]. If changes in MtDNA copy numbers contribute to oxidative stress related diseases then Mt DNA could be a potential non-invasive biomarker of mitochondrial dysfunction and as such accurate and reproducible quantification of Mt/N is essential. As such the steps we propose in the current paper should help to move towards this aim.

Appendix A

"Analysis of primers used in various studies for the estimation of the nuclear genome used for Mt/N determination"

We used the primers described in various published studies as the nuclear gene to see if they correspond to a single region in the nuclear genome. Many of the nuclear genes used were duplicated in several places in the nuclear genome, the results are shown at: http://www.kcl.ac.uk/ip/philcunningham/afshan/afhan2.html.

Appendix B

"Analysis of primers used for the quantification of mitochondrial DNA: detection of nuclear pseudogenes"

We used the primers described in various published studies to amplify Mt DNA to see if the primers used corresponded to nuclear pseudogenes. In most cases primers used corresponded to nuclear pseudogenes which could be erroneously co-amplified. The results are shown at: http://www.kcl.ac.uk/ip/philcunningham/afshan/z3.html.

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