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qPCR-based mitochondrial DNA quantification: Influence of template DNA fragmentation on accuracy

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ABSTRACT

Real-time PCR (qPCR) is the method of choice for quantification of mitochondrial DNA (mtDNA) by relative comparison of a nuclear to a mitochondrial locus. Quantitative abnormal mtDNA content is indicative of mitochondrial disorders and mostly confines in a tissue-specific manner. Thus handling of degradation-prone bioptic material is inevitable. We established a serial qPCR assay based on increasing amplicon size to measure degradation status of any DNA sample. Using this approach we can exclude erroneous mtDNA quantification due to degraded samples (e.g. long post-excision time, autolytic processus, freeze–thaw cycles) and ensure abnormal DNA content measurements (e.g. depletion) in non-degraded patient material. By preparation of degraded DNA under controlled conditions using sonification and DNaseI digestion we show that erroneous quantification is due to the different preservation qualities of the nuclear and the mitochondrial genome. This disparate degradation of the two genomes results in over- or underestimation of mtDNA copy number in degraded samples. Moreover, as analysis of defined archival tissue would allow to precise the molecular pathomechanism of mitochondrial disorders presenting with abnormal mtDNA content, we compared fresh frozen (FF) with formalin-fixed paraffin-embedded (FFPE) skeletal muscle tissue of the same sample. By extrapolation of measured decay constants for nuclear DNA (λ_{nDNA}) and mtDNA (λ_{mtDNA}) we present an approach to possibly correct measurements in degraded samples in the future.

To our knowledge this is the first time different degradation impact of the two genomes is demonstrated and which evaluates systematically the impact of DNA degradation on quantification of mtDNA copy number.

1. Introduction

Mitochondrial DNA (mtDNA) is the only source of extrachromosomal DNA in animal cells [1]. Human mtDNA is a double-stranded 16,569 bp long, circular molecule containing 13 protein encoding genes (essential subunits of the mitochondrial oxidative phosphorylation system), 22 tRNAs and two rRNA (synthesis of mitochondrial proteins) [2]. Mutations in the mtDNA mutations are either inherited or occur spontaneously in germline or somatic cells [3]. In the absence of detectable mtDNA mutations, abnormal amounts of mtDNA, either depletion or elevation, are indicative of mitochondrial dysfunction. Clinically relevant mtDNA content variations are the result of mutations in nuclear encoded genes involved in (i) mtDNA replication [4], (ii) nucleotide/nucleoside pool balance, (iii) transporters. Thus depletion/overreplication of mtDNA copy number is secondarily induced (e.g. by dysfunctional polymerase γ [5,6]. Mitochondrial depletion syndromes (MDDS) are clinically heterogeneous disorders characterised by a tissue-specific (or multi-systemic) severe quantitative reduction of mtDNA [5,7–11]. Overreplication of mtDNA has been linked to clonal expansion of the mutated mtDNA population in which fast transcription rate may be a compensatory mechanism for inefficient mitochondrial respiratory function [12,13]. Moreover, mitochondrial malfunction due to quantitative changes of mtDNA have been implicated in cancer, neurodegeneration, diabetes, ageing and have shown to be decreased/elevated in several other clinical aspects (e.g. retroviral-drug induced depletions) [14].

As mtDNA depletion/overreplication is indicative of a mitochondrial disorder, quantification of total mtDNA is of crucial importance not only to understand pathological phenotypes and their clinical progression, but also for in-depth knowledge of general changes of mtDNA content. Moreover it can confirm genetic findings functionally in patients with nuclear DNA mutations [5] or serve as diagnostic tool in patients suspicious for a mitochondriopathy. There are numerous quantitative real-time PCR (qPCR) based approaches to determine mtDNA copy number [5,15–17]. Recent studies indicate factors affecting correct mtDNA content determination dependent on DNA isolation procedures [18,19].

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Here we investigate the possible impact of degradation on quantification of mitochondrial DNA for samples from degradation-prone material such as bioptic tissue or highly fragmented DNA from FFPE samples. We present a serial qPCR assay to assess nuclear and mitochondrial DNA degradation individually and simultaneously, indicating that proper normalisation in degraded samples is not only dependent on amplicon size [20], but also on the relative abundance of reference and target. By preparation of degraded DNA under controlled conditions and comparison of FF and FFPE extracted skeletal muscle of the same specimen, we imply quality factors for accurate mitochondrial content determination. These general findings should find application in exclusion of degradation in routinely analysed samples suspicious for abnormal mtDNA content.

2. Materials and methods

2.1. DNA extraction

DNA was extracted from peripheral blood cells with the QIAamp DNA Blood Kit (Qiagen) according to the manufacturer's protocol. FF biopsies were extracted with the QIAamp Tissue Kit (Qiagen) according to the manufacturer's protocol. FF tissue pieces were fixed 24 h at 4 °C in the dark in 1×PBS 3.7% formaldehyde (FA) as reported optimal [21]. Dehydration and Paraffin-embedding steps were performed in an automatic tissue processing device. FFPE tissues were extracted using the QIAamp DNA FFPE extraction kit (Qiagen) according to the manufacturer's protocol or with minor modifications (various incubation times at 90 °C,

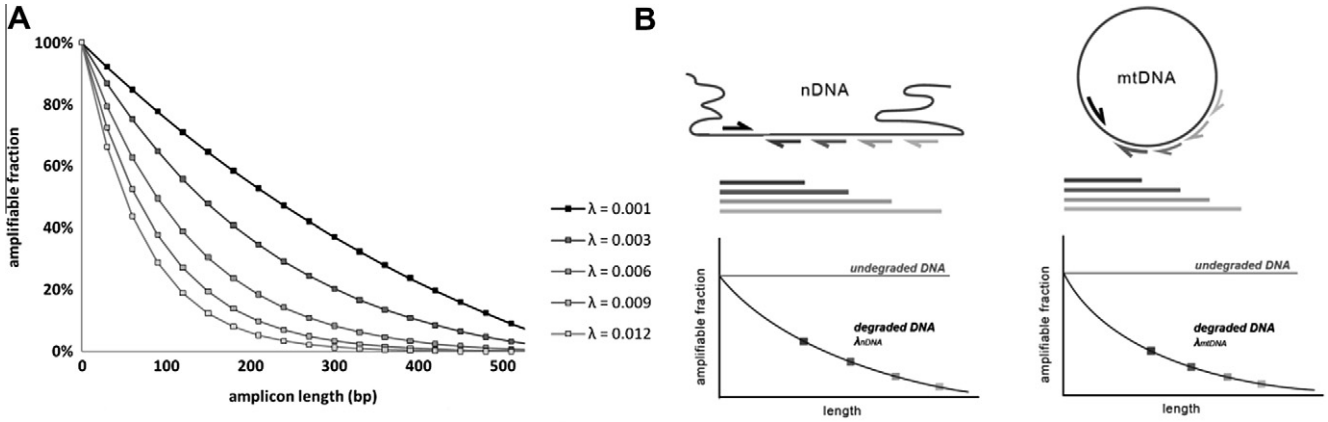

Fig. 1. Serial qPCR assay outline for DNA degradation measurement and subsequent mitochondrial DNA quantification. (A) Computated results for different probabilities of DNA damage (λ) of 0.001–0.012 in relation to amplifiable fraction and increasing amplicon length based on random fragmentation [24]. (B) Increasing amplicons of mitochondrial and nuclear DNA are measured. The derived λ (λ_{mtDNA}, λ_{nDNA}) determine fragmentation status of the two genomes individually and serve as indicators of the quantification error of mtDNA content in degraded samples.

Table 1
Primer sequences.

Name	Fwd primer (5′–3′)	Rev primer (5′–3′)	Nt position	Amplicon (bp)
NCOA3 F	CCTCTGGGCTTTTATTGCGAC		134792–813	
NCOA3 R54		TGACTGACACATTGAACTCTCCAC	134846–822	54
NCOA3 R108		CCCCTCAACACTGCTCTCCTTAC	134900–877	108
NCOA3 R123		CCTTTGATTCTCTGCCCCCT	134915–895	123
NCOA3 R188		CGGTCATCAGAAGAACAGGTAAGT	134980–956	188
NCOA3 R235		CTTTACAACTTGAATCTAGGGGGG	135027–003	235
NCOA3 R341		CGGTGCTTCTCTTGTAACAGTGAC	135133–109	341
NCOA3 R470		TGCTCCTGCTTGACAACATTTC	135262–240	470
B2M F	AAGTTCGCATGTCCTAGCACC		5655–645	
B2M R91		TCACAGCCAAGCATTCTACAAAC	5745–723	91
B2M R213		TGACGCAAAGCACATAAAGTCC	5867–846	213
B2M R295		GCCTTAGTTTCTTCATCTGTGAGAG	5925–949	295
B2M R345		CGATTGCTGCCATTTATCCC	5999–5980	345
B2M R473		CATGCCGCCCAGTTTG	6127–6112	473
mtDNA F	GCCACAGCACTTAAACACATCTCT		322–346	
mtDNA R73		TGAAATCTGGTTAGGCTGGTGTTAG	395–370	73
mtDNA R151		GTATGGGAGTGGGAGGGGA	473–454	151
mtDNA R186		TAGGATGGGCGGGGGT	508–492	186
mtDNA R223		CGGGGTATGGGGTTAGCAG	545–526	223
mtDNA R250		GGGGGTGTCTTTGGGGT	572–555	250
mtDNA R392		TGGAACGGGGATGCTTG	714–697	392
NCOA3 F1*	GAGTTTCCTGGACAAATGAG		126265–285	
NCOA3 R1*		CATTGTTTCATATCTCTGGCG	126399–78	134
B2M F1*	TGCTGTCTCCATGTTTGATGTATCT		6884–6909	
B2M R1*		TCTCTGCTCCCCACCTCTAAGT	6970–6948	86
D-Loop F*	CATCTGGTTCCTACTTCAGGG		16497–16519	
D-Loop R*		CCGTGAGTGGTTAATAGGGTG	35–14	107

Accession: NCOA3 NG_016810.1, B2M NG_012920.1, mtDNA NC_012920.1 [25].
* modified from [15]

for reversion of FA bonding). DNA concentrations were measured using a Nanodrop 1000 spectrometer (Thermo Scientific).

2.2. Artificial DNA degradation

Ultra-sound degradation was performed in 500 µl of DNA (100 ng µl^{-1}), sonicated on a Sonifier 250 (Branson, Danbury) equipped with a 3 mm conical tip on ice. DNaseI (RNAse-free DNase Set, Qiagen) degradation was performed in Mn^{2+} buffer in a final concentration of 50 mM Tris–HCl (pH 7.6) and 10 mM $MnCl_2$ as described [22]. Briefly, 3 µg DNA from blood was DNaseI digested (1/500 from supplied stock solution) in a final volume of 40 µl. Reactions were stopped by direct addition of 360 µl H_2O and 400 µl of phenol:chloroform:isoamyl alcohol (25:4:1) (Fluka), gently vortexed and immediately precipitated.

2.3. Quantitative real-time PCR

Serial qPCR assay was established using a LightCycler480 (Roche Applied Science). qPCR effciencies were determined by serially diluted genomic DNA [23]. FF and FFPE samples have been evaluated for cycling efficiency individually (results not shown). Copy number determinations were obtained using a calculated efficiency of 2. Each reaction contained 10 µl 2× SYBR© Premix Ex Taq (PerfectRealTime™, Takara Bio), 2 µl primers (10 µM) and 8 µl of DNA (0.5–5 ng µl^{-1}) for a final volume of 20 µl. All reactions were performed in duplicates. PCR conditions were: 5 min at 95 °C initial denaturation, followed by 40 cycles of 30 s of denaturation at 95 °C, 15 s of primer annealing at 60 °C and 10 s at 72 °C of extension. The presence of unspecific amplicons was excluded by melting curve analysis and silver-stained polyacrylamide gel electrophoresis. Products were sequenced on an ABI3100 System using BigDye chemistry (Applied Biosystems).

2.4. Analysis of DNA degradation and mtDNA quantification

Parameter of degradation λ was calculated by normalising a short amplicon to increasing amplicons of an untreated sample relative to a treated sample fraction. The random degradation model is adapted as described previously [24]. The parameter λ was determined by fitting exponential regression curves with R^2 values generally >0.9. This allowed to determine λ for nDNA (λ_{nDNA}) and mtDNA (λ_{mtDNA}) individually, reflecting the damage frequency. Low copy numbers C_q > 32 and long amplicons (>350 bp) were excluded in extremely fragmented samples. mtDNA was quantified using two nuclear reference genes (*NCOA3*: NG_016810.1; *B2M*: NG_012920.1) and mitochondrial D-Loop primer or increasing mtDNA amplicons (mtDNA: NC_012920.1 [25]). C_qs were evaluated with the default second derivative method (SDM) of the LightCycler480 (software v.1.5.0.). mtDNA content was calculated using the $\Delta\Delta C_q$ method comparing relative amounts of mitochondrial loci nuclear references *B2M* or *NCOA3* as previously described [26]. Error bars are the average SD of the duplicate $\Delta\Delta C_q$ measurements within the experiment.

3. Results

3.1. Theoretical implications of DNA degradation on mitochondrial quantification

Measurements of different skeletal muscle biopsies from healthy individuals showed great variations in mtDNA content (data not shown). Therefore we addressed the possibility of assessing the quality and degradation state of the DNA by amplification of PCR products with increasing length. Under the assumption, that DNA degrades randomly, the amount of template will decline exponentially with increasing PCR product size [24]. The extent of DNA

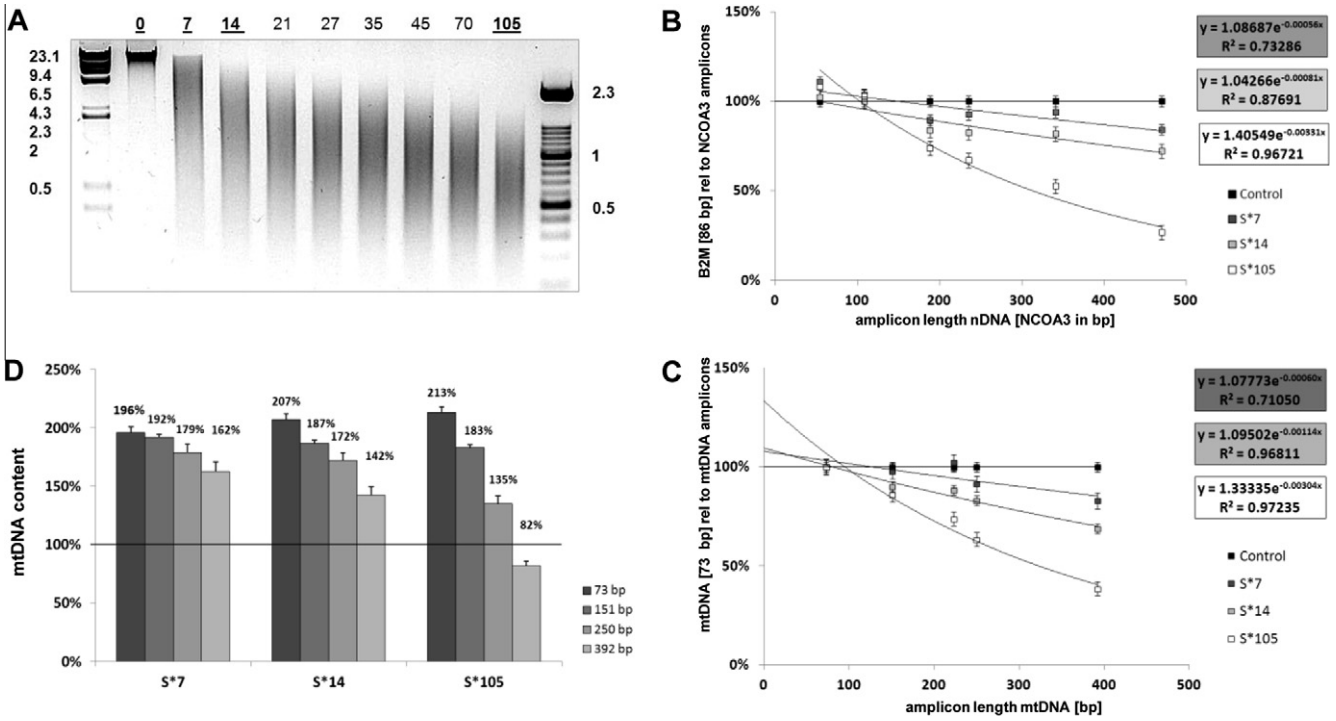

Fig. 2. Serial qPCR analysis of artificially fragmented DNA by sonification. (A) Qualitative assessment of sonicated electrophoresed DNA. (B) Serial qPCR measurement of nuclear gene NCOA3. (C) Serial qPCR measurement of mitochondrial DNA. Plotting of amplicon length against amplification ratio relative to assumed undegraded control yields the constant of decay (λ) to quantify DNA damage. Ratio calculations of different impulses (7, 14, 105) increased λ values for nuclear gene NCOA3 (0.00056, 0.00081, 0.00331) and for mitochondrial DNA (0.00067, 0.00121, 0.00321). (D) Ratio calculations of increasing mtDNA amplicon length (73, 151, 250, 392 bp) normalised to nuclear reference B2M (86 bp) show high overestimation of mtDNA content dependent on degradation state.

damage (λ) is reflected in the decline of the amplifiable fraction (Fig. 1A). To minimise the influence of sequence context, increasing product sizes were generated by shifts of the reverse primer only (Table 1). Single copy reference gene amplicons (Table 1) of the nDNA and mtDNA amplicons (rCRS: 323–714 nt) were designed with increasing length. Under the assumption that mtDNA and nDNA were degrading similarly, the measurement of either would be sufficient to determine degradation state and would not alter quantification. We assumed that in highly degraded sample the damage of the nDNA (λ_{nDNA}) differs from the damage of the mtDNA (λ_{mtDNA}) leading to false quantification outcome. We analysed the possibility of measuring λ_{nDNA} and λ_{mtDNA} to correct each quantification by exponential extrapolation to 0 bp where degradation is assumed to be neglectable (Fig. 1B). If $\lambda_{nDNA} > \lambda_{mtDNA}$ the mtDNA content will be overestimated. If $\lambda_{nDNA} < \lambda_{mtDNA}$ the mtDNA content will be underestimated.

3.2. Analysis of artificially fragmented DNA

Artificially degraded DNA was assessed to test sensitivity of our serial qPCR assay. DNA from blood was fragmented by sonification. The effect of fragmentation was qualitatively estimated by electrophoresis (Fig. 2A). By quantification of a short amplicon relative to increasing amplicon length, we obtained the parameter of fragmentation λ by fitting of an exponential function with R^2 generally >0.9. This serial qPCR analysis was performed on differently sonicated fractions for the nuclear single copy gene *NCOA3* (Fig. 2B) and the mtDNA (Fig. 2C) individually. Almost identical λ_{mtDNA} values were obtained when normalised to a short mtDNA amplicon (73 bp) or nuclear reference B2M 86 bp (λ_{mtDNA} 0.00061, 0.00115, 0.00305 and 0.00056, 0.00081, 0.00331, respectively). Based on the normalisation to a short nuclear reference (86 bp), mtDNA content in degraded samples is drastically overestimated. mtDNA content in sonicated samples were for 7 pulses 196%, 14 pulses 207%, and 105 pulses 213%, respectively when comparing a short nuclear reference (86 bp) to a mitochondrial short amplicon (73 bp). Longer mitochondrial amplicons (73–392 bp) resulted in decreased mtDNA amount, which was in good agreement with the degradation status of the differently fragmented fractions (Fig. 2D).

To fragment in another manner, DNA from blood was digested with pancreatic DNaseI for various time intervals. Pancreatic DNaseI is known to fragment DNA quickly and cleaves both strands at approximately the same site when supplemented with Mn^{2+} ions [27] although this cleavage is not entirely sequence unspecific [28]. DNA was digested for various time and directly extracted using phenol:chloroform:isoamylalcohol (PCIAA). Highly fragmented DNA was obtained (Fig. 3A), which showed higher λ_{mtDNA} values than λ_{nDNA} (Fig. 3B,C). Quantification of mtDNA using *B2M* 86 bp as reference and mtDNA target amplicons of various length (73, 151, 186 and 250 bp) resulted in high errors, which worsened with increasing digestion times (3, 5, 7.5 min). With longer mtDNA target amplicons, mtDNA content decreased according to degradation state. These results demonstrate that the use of short amplicons of equal length [20] are not sufficient for accurate mtDNA copy number determination in fragmented samples. This source of error in degraded samples may also be due to the relative abundance of target and reference, which is for the mtDNA/nDNA ratio several orders of magnitude [5,11].

3.3. Analysis of FF & FFPE extracted DNA of the same specimen

To explore the possibility of accurate quantification of mtDNA in archival tissue we compared fresh-frozen (FF) skeletal muscle

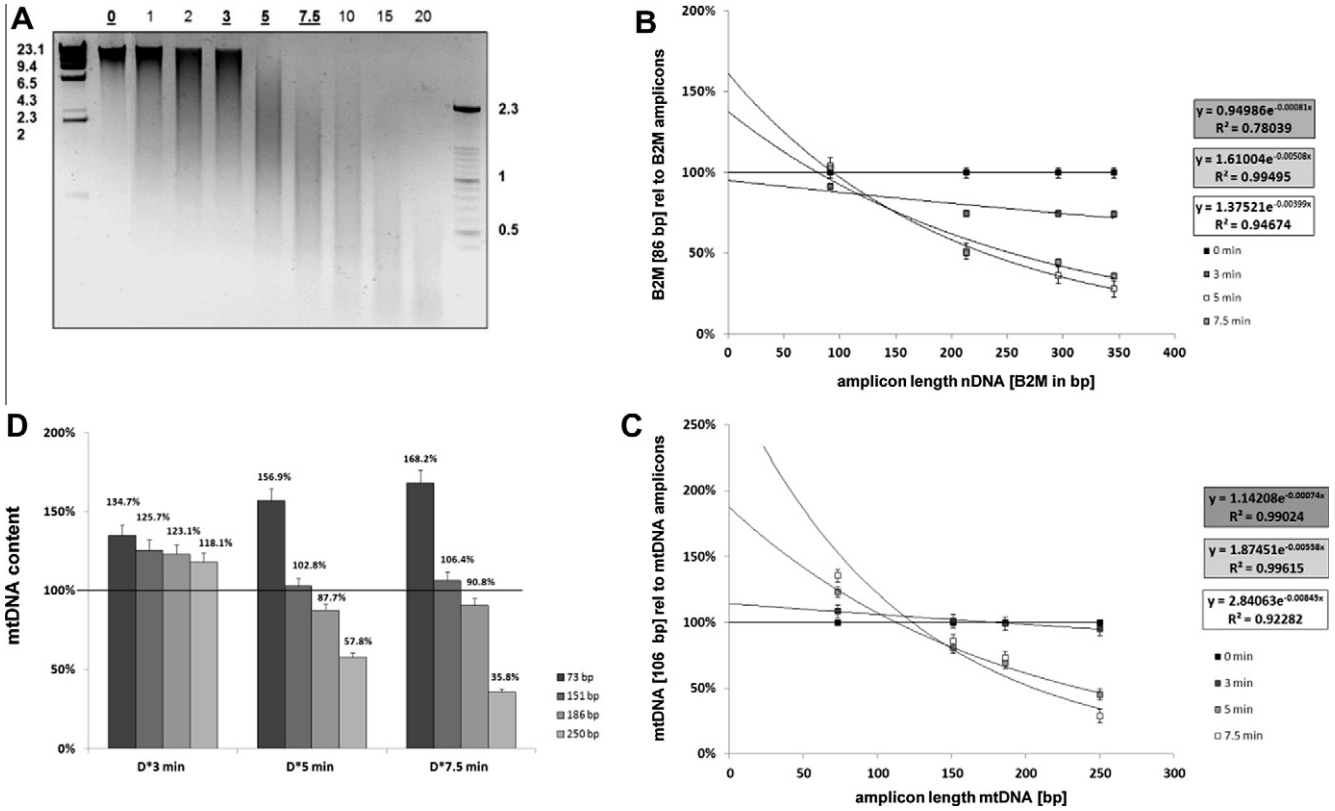

Fig. 3. Serial qPCR analysis of artificially fragmented DNA by DNAseI digestion. (A) Qualitative assessment of electrophoresed DNAseI digested DNA (0–20 min incubation). (B) Quantification of mitochondrial DNA content of a DNA sample systematically fragmented by DNaseI. Various incubation times (3, 5 and 7.5 min) normalised to 0 min of the same sample lead to increasing overestimation of mitochondrial DNA. This false estimation is reflected in the differing degradation state of nDNA (B) and mtDNA (C). (D) Ratio calculations of increasing mtDNA amplicon length (73, 151, 250, 392 bp) normalised to nuclear reference B2M (86 bp) show high over-or underestimation of mtDNA content dependent on degradation state and did not depend on equally short amplicon size alone.

to FFPE skeletal muscle of the same sample to estimate the extent of damage by fixation and embedding.

A FF skeletal muscle sample was extracted twice freshly whereas the remaining piece was directly fixed in 1×PBS 3.7% formaldehyde overnight. After fixation the sample was FFPE embedded by routine procedures. The FFPE sample was extracted as described previously and subjected to three different incubation times at 90 °C (60, 75, 90 min) to evaluate fragmentation and DNA yield. 90 °C incubation reverses the formaldehyde bonding. Quantification of mtDNA content from the FF control extraction to its equivalent FFPE embedded counterparts at three incubation times lead to underestimation of mtDNA content when normalised to a nuclear reference (B2M 86 bp) relative to increasing mtDNA amplicons (Fig. 4D). Amplification efficiencies were the same for the FF and FFPE sample (results not shown). Fitting regression curves of increasing amplicon length of mtDNA normalised to B2M 86 bp, the initial mtDNA content was approached by extrapolation to an assumed amplicon of infinite shortness. Extrapolation of the three FFPE samples results (Fig. 4D) to 0 (intersection *y*-axis to 0 bp) to an approximation of 99.6% ($\lambda_{mtDNA} = 0.00287$, $R^2 = 0.98$) for FFPE 60 min, 109.6% ($\lambda_{mtDNA} = 0.00284$, $R^2 = 0.97$) for FFPE 75 min and 92.6% ($\lambda_{mtDNA} = 0.00324$, $R^2 = 0.92$) for FFPE 90 min of mtDNA content relative to its FF counterpart.

4. Discussion

For a comprehensive molecular diagnosis of mitochondrial disorders, accurate tissue-specific quantitative analysis of mitochondrial DNA is extremely important. mtDNA copy number (of healthy individuals) are also influenced by (i) extraction procedures [18,19], (ii) quality of DNA, (iii) physical status, (iv) age [13], (v) tissue specificity, (vi) biopsy location, (vii) autolytic processes, (viii) storage, (ix) assay stability and (x) degradation (Table 2).

As MDDS are highly tissue-specific [11] and preparation of tissues is degradation-prone, fragmentation cannot be prevented in the analysis of mtDNA content. Degradation may occur in every step of preparation such as: biopsy, autolytic processes, freezing, thawing, extraction, long time storage, etc. Thus it is important that an assay for exclusion of degradation is independent of the different protocols of tissue preparation/asservation in use.

Moreover, FFPE tissue is an invaluable source for genetic retrospective studies and allows to analyse the tissue-specific pathomechanisms in morphologically defined biopsies (e.g. using laser-assisted micro-dissection). As tissue processing is not standardised and several protocols are applied (e.g. biopsy, fixation time, temperature), the exclusion of false-quantification due to degradation had to be assumed. All DNA samples suffer to some extent the damage introduced through endogenous endonuclease activity, spontaneous depurination, strand breaks, oxidative damage, molecular crosslinking [24]. In FFPE extracted samples this damage is aggravated by chemical fixing agents, leading to highly fragmented nucleic acids [21,24] with average fragment lengths of 200–400 bp [29]. Moreover, inhibitory substances may be present, which are necessary components for the extraction of nucleic acids from FFPE tissue (e.g. Xylene [30], Formalin [31]).

To systematically approach the possibility of accurate mtDNA copy number quantification, we analysed DNA of artificially fragmented freshly extracted samples or highly fragmented FFPE sam-

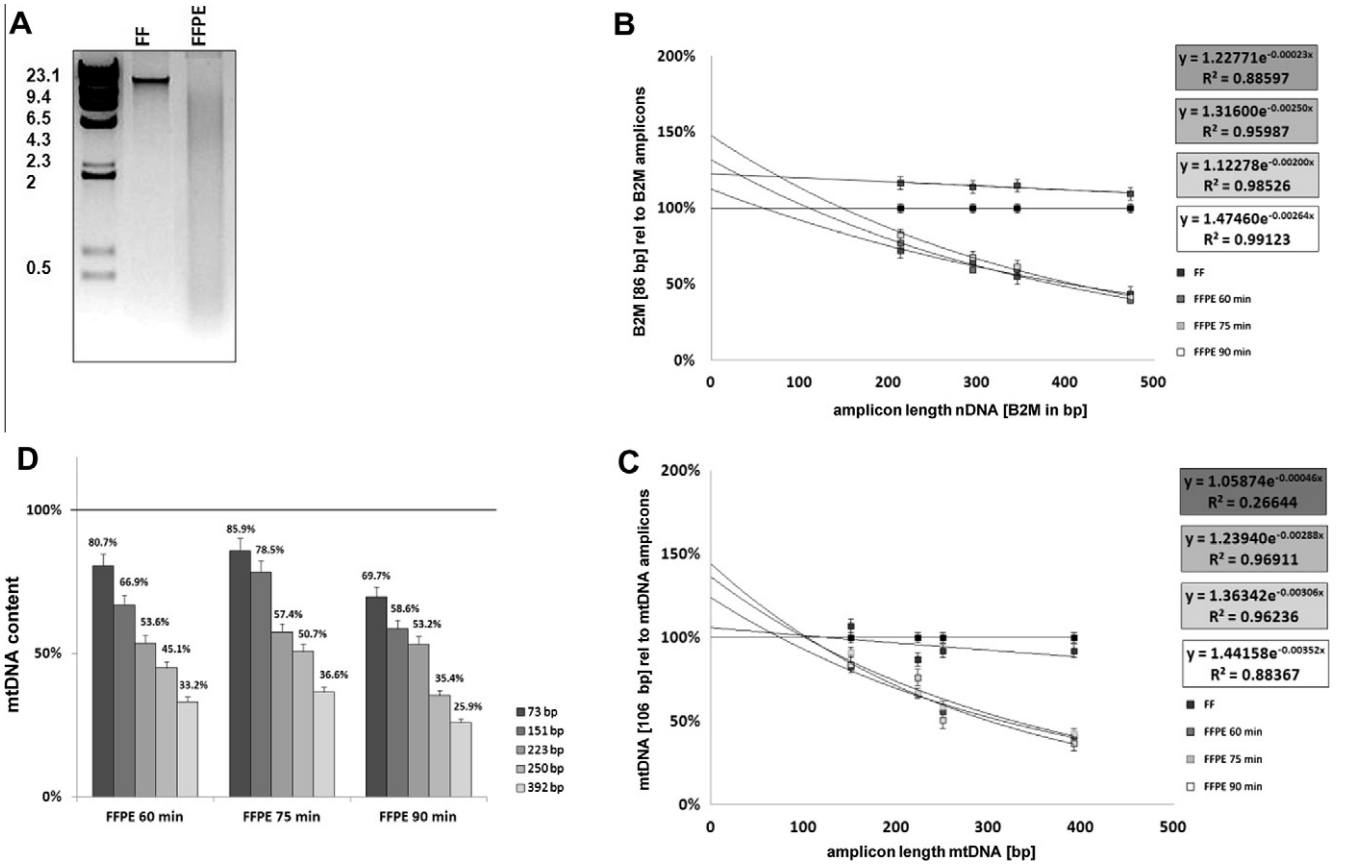

Fig. 4. Serial qPCR analysis of FF and FFPE skeletal muscle. (A) Qualitative assessment of electrophoresed DNA of FF and FFPE extractions of same skeletal muscle specimen. Serial qPCR measurement of nuclear gene B2M (B) and mtDNA (C) with corresponding fragmentation derived λ_{nDNA} and λ_{mtDNA}. FFPE extracted DNA was normalised to FF extracted sample. (D) Ratio calculations of increasing mtDNA amplicon length relative to B2M (86 bp) of FFPE extracted samples at various heat extraction time points normalised to their FF counterpart.

Table 2
Mitochondrial DNA content determination factors.

	Individual	Technical	Methodical
Sampling	Individual variations [34]	Biopsy location	
	Tissue specificity	storage & storage time [35]	*Serial qPCR*
	Physical status	Autolytic processus	*Serial qPCR*
	Age-matched [11]	(e.g. Post mortem)	
	Affectedness		Sequencing
	(heteroplasmy, deletions)		Long range PCR [36]
Extraction	Dna quality	Protocol (column-based, pciaa) [18,19]	qPCR, electrophoresis
	Incubation time		*Serial qPCR*, electrophoresis
Controls			
Inclusion	Age-matched	Degraded	*Serial qPCR*
Exclusion	Several loci	Inhibitory substances	Standard curves [23], sequencing
	Internal calibrator	Polymorphism in binding sites [37]	
Content determination		Short amplicons [20]	*Serial qPCR*
		Assay variations	
		Absolute quantification [38]	
	qPCR inhibitors		
	Efficiency calculations [23]		
	Single copy reference gene (s)		

ples. By artificial degradation of fresh DNA from blood either by sonification or DNaseI digestion and subsequent analysis of variedly fragmented samples, we observed an increasing error in mtDNA copy number determination with higher fragmentation relative to the untreated starting material of the same specimen.

Development of a serial qPCR assay to individually address fragmentation of nuclear and mitochondrial DNA and subsequent application to fragmented samples, showed differing preservation qualities of the two genomes.

Comparison of the individual decay constants of nDNA and mtDNA, results in three possible scenarios in which the decay constant of the mtDNA (λ_{mtDNA}) is either steeper, flatter or equal to the decay constant of the nDNA (λ_{nDNA}). If $\lambda_{mtDNA} > \lambda_{nDNA}$ the mtDNA copy number would be underestimated (overreplication would remain undetected). If the $\lambda_{mtDNA} < \lambda_{nDNA}$ the mtDNA copy number would be overestimated (a degraded sample being depleted remains undetected). If the $\lambda_{mtDNA} = \lambda_{nDNA}$ the mtDNA copy numbers would theoretically be unaffected by fragmentation.

Our efforts to develop a mathematical correction model by the application of λ_{mtDNA} and λ_{nDNA} to individual measurement (assuming exponential decay) failed to meet the desired precision. We attributed the failing precision for mathematical correction to several problems: (i) assumption of random degradation [24], (ii) not enough data points for accurate mathematical description of λ, (iii) loss of small fragments due to silica-based extraction columns introduces a bias of abundancy, (iv) sequence-dependent degradation, (v) observation of increasing estimation of short amplicons in little fragmented relative to untreated samples. Little fragmented DNA has more free binding sites to be accessed by primers within the DNA matrix influencing qPCR efficiency at early cycles. This leads to an overestimation, which remains undetected in standard curve measurement, which only reflect PCR efficiency of amplicons generated on amplicons. This will lead to quantification errors in highly degraded samples [32]. The initial sequence context of the DNA in early cycles thus is not represented and inhibitory effects of unequal melting remain undetected. Moreover, there is a lack of experience in sequence-dependent degradation (see Supplementary). Table 2 summarises possible sources of errors in quantification of mtDNA and includes strategies for monitoring quality of analysed samples, demonstrating the potential of serial qPCR in exclusion of degradation-induced false quantification.

We have shown that serial qPCR accurately measures even at low degradation states (Fig. 2A). Moreover, we show that a high (mtDNA) to low (nDNA) abundance of target and reference is highly error prone, indicating that short amplicons are not sufficient to accurately quantify in highly fragmented samples due to individual degradation qualities of the two genomes. This may also be reflected in expression studies, where target and reference differ in several magnitudes. Dependency on short amplicons for reliable measurements on degraded RNA [20] and false-positive copy number calls in multiplexed qPCR assays in degraded samples [33] have been reported.

To our knowledge this is the first report on systematic evaluation of the impact of DNA degradation on quantification of mtDNA copy number. In good laboratory practice we have routinely implied these general findings for bioptic material received for mtDNA quantification. In conclusion, we describe a serial qPCR assay to analyse fragmentation status of nuclear and mitochondrial DNA independently and demonstrate erroneous quantification of mitochondrial DNA copy number in highly fragmented samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.121.

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