Biased nicking of mitochondrial DNA during extraction can be avoided by choice of isolation method

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Abstract

The integrity of mitochondrial DNA (mtDNA) isolated from solid tissues is critical for analyses such as long-range PCR. We show that a commonly-used DNA isolation procedure preferentially introduces strand breaks into the mtDNA extracted from the skeletal muscle of aged mice, while mtDNA from adult animals is less affected. We present a comparison of mtDNA isolation methods and identify one that avoids this biased loss of muscle mtDNA integrity. Our results highlight the importance of a careful choice of mtDNA isolation method and serve as a resource to researchers planning analysis of mtDNA isolated from solid tissues.

Introduction

Mammalian mitochondrial DNA (mtDNA) is a double-stranded multicopy genome of approximately 16.5 kbp. Because it contains the genetic information encoding for core subunits of the mitochondrial respiratory chain, the integrity of mtDNA is critical to the normal function of the majority of mammalian cells. Alterations in mtDNA, such as mutations or large-scale deletions, can cause mitochondrial disease and are also implicated in a wide range of other pathologies as well as in the normal process of ageing (Szczepanowska and Trifunovic, 2017; Taylor and Turnbull, 2005; Wallace, 2012). For this reason, the accumulation, metabolic consequences and potential repair mechanisms of mutations or various types of mtDNA damage are subject to intense research.

Many of the above research efforts rely on meticulous analysis of mtDNA isolated from solid mammalian tissues. Depending on the requirements dictated by downstream analysis methods, most researchers opt for a DNA isolation method that belongs to one of two main categories: isolation of mtDNA from purified mitochondria, or, if the planned analysis can distinguish mtDNA from a pool of other nucleic acids, the isolation of total genomic DNA (*ie*. nuclear DNA and mtDNA). Both approaches have their benefits and disadvantages. Isolation of mtDNA from purified mitochondria yields highly-enriched mtDNA, but is more labor intense, time consuming, and requires larger amounts of tissue as starting material. Moreover, the tissue homogenization step may require adjustment depending on the type of tissue used. In comparison, isolation of total DNA is faster and more straight forward as it requires little optimization to the type of tissue. It produces sufficient DNA yields from a smaller mass of tissue and is therefore often the method of choice. Many variants of the procedure exist, most of which are easily scaled up for the simultaneous handling of many parallel samples. Tissue lysis is usually achieved with the help of a detergent like SDS in conjunction with proteinase K in an isolation buffer that contains EDTA, conditions that inactivate most cellular endonucleases (Lang and Burger, 2007).

Comparisons of various mtDNA isolation techniques typically only analyze the mtDNA under neutral conditions, which does not yield information on the integrity of the individual strands and thereby prevents comparison of the frequency of single-stranded DNA (ssDNA) breaks. We show that the choice of isolation method has considerable effects on the integrity of the mtDNA isolated from solid mammalian tissues. A widely-used procedure for total DNA preparation yields mtDNA with some ssDNA breaks. Importantly, we find that this isolation method preferentially introduces ssDNA breaks into the mtDNA from the skeletal muscle of aged animals, while mtDNA from the muscle of adult animals is less affected. Finally, we provide a comparison of four different isolation procedures in terms of the integrity, yield and enrichment of the resulting mtDNA. This constitutes an important resource for researchers planning mtDNA analyses that require mtDNA of high integrity.

Materials and Methods

Animal handling and isolation of tissues

Experiments were carried out using surplus wildtype animals from other studies. All mice were maintained at the animal facility at Umeå University under pathogen-free conditions. Mice were housed in an environment with a 12-hour dark/light cycle and *ad libitum* access to food and water. Animal handling and experimental procedure were approved by the ethical committee at Umeå University, complying with the rules and regulations of the Swedish Animal Welfare Agency and with the European Communities

Council Directive of 22 September 2010 (2010/63/EU). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Mice were euthanized by CO_2 inhalation at the following ages: 13 weeks ('adult'), 12 months ('old adult') or 23–29 months ('aged'). The livers and hind leg muscles (gastrocnemius [GAS], tibialis anterior [TA], and mixed thigh muscles) were placed in Eppendorf tubes, quickly frozen in liquid nitrogen and kept at -80°C. As far as possible, samples from an equal number of male and female animals were used in each experiment.

DNA isolation and treatments

In the standard isolation method (Sambrook and Russell, 2001), ca 100 mg of individual mouse tissue was cut into small pieces, incubated at 55°C overnight in SNET buffer (20 mM Tris pH 8.0, 5 mM EDTA pH 8.0, 400 mM NaCl, 1% SDS) containing 400 µg/ml proteinase K (#P6556, Sigma), and total DNA (i.e. nuclear DNA and mtDNA) was isolated by phenol-chloroform extraction and isopropanol precipitation according to standard protocols (Green and Sambrook, 2017).

In the pulverization method, ca 100 mg of frozen tissue was ground to a powder in a mortar containing liquid N_2. The pulverized tissue was incubated in SNET buffer and proteinase K at 55°C for 3 h, followed by phenol-chloroform extraction and isopropanol precipitation as above. Samples were treated with 1.2 µg/µl PureLink RNase A (Invitrogen, #12091-021) at 37°C for 30 min and precipitated again with isopropanol.

In the kit-purification method, total DNA was extracted using the NucleoSpin Tissue kit from Macherey-Nagel (#740952) according to the manufacturer's instructions. Briefly, 100 mg of tissue was cut into small pieces and incubated at 56°C for 3 hours in Buffer T1 with Proteinase K, followed by a 5-minute incubation with RNase A at room temperature. Samples were lysed with 1 volume of Buffer B3 and incubated at 70°C for 10 min. After addition of 1 volume ethanol, the samples were applied to the NucleoSpin Tissue columns and centrifuged for 1 min at 11,000 x *g*. Silica membranes were washed twice with 500 µl Buffer BW with centrifugation for 1 min at 11, 000 x *g*, centrifuged once more to dry the silica membrane and remove residual ethanol, and DNA eluted with 30 µl TE buffer (10 mM Tris pH 8.0; 1 mM EDTA).

Isolation of mtDNA from pure mitochondria was modified from (Ekstrand et al., 2004; Frezza et al., 2007; Yasukawa et al., 2005) using ca 200-300 mg of finely-chopped tissue. The liver samples were washed thrice in homogenization buffer (HB; 225 mM mannitol, 75 mM sucrose, 10 mM Hepes-KOH pH 7.8, 10 mM EDTA), while skeletal muscle samples were washed in ice-cold PBS containing 10 mM EDTA, incubated with 0.05% trypsin (w/v) in PBS with 10 mM EDTA for 90 min on ice, centrifuged (200 × *g*, 5 min, 4°C), and the pellet resuspended in HB. Tissues were homogenized by 10 strokes in a hand-held glass/Teflon Potter Elvehjem homogenizer. Nuclei and cell debris were pelleted at 800 × *g* for 10 min at 4°C and the supernatant was further centrifuged at 12,000 × *g* for 10 min at 4°C to pellet mitochondria. The mitochondrial pellet was resuspended in HB buffer containing 100 ng/µl proteinase K and incubated for 15 min on ice. Mitochondria were pelleted (12,000 × *g*, 10 min, 4°C), resuspended in lysis buffer (75 mM NaCl, 50 mM EDTA, 1% SDS, 20 mM Hepes pH 7.8) containing 100 ng/µl proteinase K, and incubated on ice for 30 min. MtDNA was purified by phenol–chloroform extraction followed by ethanol precipitation. For 'ultra-pure' mitochondria such as those in Fig. 4a, the mitochondrial pellet after the first 12,000 × *g* centrifugation was instead resuspended in HB, overlaid on a sucrose gradient consisting of 1 M and 1.5 M sucrose in gradient buffer (10 mM Hepes pH 7.4 and 10 mM EDTA), and

centrifuged at 40,000 × *g* for 1 h at 4°C. The purified mitochondria were diluted in gradient buffer, pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C, and treated with proteinase K, lysed and the DNA isolated as for the pure mitochondria protocol.

All DNA preparations were resuspended in TE and allowed to dissolve o/n. In order to minimize DNA shearing, DNA samples were not vortexed at high speed, and cut or wide-bore tips were used for pipetting whenever feasible. DNA concentrations were determined by Nanodrop. Where indicated, DNA preparations were digested overnight with the SacI restriction endonuclease (Thermo Scientific, #ER1131), followed by ethanol precipitation and resuspension in TE buffer. Treatment with mung bean nuclease (New England Biolabs, #M0250S; at 0.3 U/µg of DNA) was for 15 min at room temperature. T4 DNA ligase treatment (Thermo Scientific, #EL0011; at 3.3 U/µg of DNA) was carried out at 25°C for 6 h, followed by inactivation at 65°C for 10 min in the presence of 25 mM EDTA and 0.2% SDS.

Agarose gel electrophoresis and Southern blotting

Pure mtDNA (500 ng/lane) and total DNA (2 µg/lane) samples were separated on 0.7% agarose gels under either neutral (1 × TAE buffer) or denaturing (30 mM NaOH and 1 mM EDTA) conditions at 1.2 V/cm for 16-18 h at 8°C; 5 ng of GeneRuler 1 kb DNA ladder (Thermo Scientific) was used as a standard for DNA length. The DNA was transferred to a nylon membrane using standard protocols(Sambrook and Russell, 2001) and probed sequentially with selected α-dCTP^{32}-labelled dsDNA probes, stripping extensively with boiling 0.1% SDS between probes. The probes used were *COX1* (nt 5,328–6,872) and D-loop (nt 15,652–15,679) for mouse mtDNA, and 18S (nt 1,245–1,787 of the 18S rRNA on chromosome 6 [gi 374088232]) for nuclear DNA. Blots were exposed to BAS-MS imaging plates (Fujifilm), scanned on a Typhoon 9400 imager (Amersham Biosciences), and the signal was quantified by using ImageJ64 software. Length distributions of individual DNA samples were used to determine the apparent median length of DNA fragments as previously described (Wanrooij *et al.*, 2017). Statistical comparisons were performed by using Welch's unequal variances t-test (for comparisons between two groups) or one-way ANOVA (between three or more groups) in GraphPad Prism software.

MtDNA copy number analysis (enrichment) by qPCR

MtDNA copy number was analyzed in duplicate by quantitative real-time PCR using 1 µl of 1/200-diluted DNA in a 20 µl reaction containing 0.2 µM forward and reverse primers and 10 µl of 2x SyGreen Mix (qPCRBIO #PB20.14-05) in a LightCycler 96 instrument (Roche). Primer pairs targeting cytochrome B (nt 14,682–14,771 of mtDNA; forward GCTTTCCACTTCATCTTACCATTT, reverse TGTTGGGTTGTTTGATCCTG (Ahola-Erkkilä *et al.*, 2010)) and actin (nt 142,904,143–142,904,053 of chromosome 5 [NC_000071]; forward CCACCATGTACCCAGGC, reverse CACCCTCACCAAGCTAAGG) were used with the following qPCR program: 95°C 180 sec, 45 cycles of (95°C 10 sec, 57°C 10 sec, 72°C 20 sec with signal acquisition), melting curve (95°C 5 sec, 65°C 60 sec, heating to 97°C at 0.2°C/sec with continuous signal acquisition). C_q values determined by the LightCycler 96 software (Roche) were used to calculate the copy number of mtDNA relative to nuclear DNA using the Pfaffl method (Pfaffl, 2001) and plotted with GraphPad Prism. Statistical comparisons between multiple groups were performed by one-way ANOVA.

Results

The integrity of skeletal muscle mtDNA isolated by standard methods decreases with age

During the preparation of previous work, we observed that the integrity of mtDNA in total DNA preparations isolated from mouse skeletal muscle, as measured by electrophoretic mobility under denaturing conditions, decreased noticeably with age (Wanrooij *et al.*, 2020). To expand on this observation, we set out to investigate the cause of this phenomenon and whether the integrity of mtDNA isolated from other tissues was similarly affected by the age of the animal.

We isolated total DNA from the skeletal muscle and the liver of adult (13-week-old) and aged (2-year-old) mice using a standard DNA isolation protocol (Sambrook and Russell, 2001) and carried out gel analysis under two different conditions: neutral conditions where the mtDNA remains in double-stranded form, and denaturing conditions that separate the two strands. For unambiguous interpretation of the results, the DNA separated under neutral conditions was first treated with SacI endonuclease to linearize the mtDNA. The mtDNA was then visualized by Southern blotting using a probe targeting a region of the *COX1* gene. Under neutral conditions, the mtDNA from all samples migrated as expected for a linear molecule of 16.3 kb, demonstrating that it was full-length and that none of the samples contained considerable levels of species with large deletions (Fig. 1a). Under denaturing conditions however, mtDNA from the skeletal muscle of aged animals migrated faster than that from adults (Fig. 1b), in line with our previous findings (Wanrooij *et al.*, 2020). In contrast to the skeletal muscle, liver mtDNA from adult *vs.* aged animals exhibited comparable migration. The difference in migration between mtDNA from adult and aged skeletal muscle could not be attributed to differential handling during isolation. The reproducibility of the findings is further illustrated by the low variation between samples from the same age and tissue (biological replicates) (Fig. 1b). Re-probing of the blots with a probe annealing to the D-loop region of the mtDNA provided equivalent results, showing that the effect was not confined to a specific region of mtDNA (Fig. S1a-b).

To provide a measure of the integrity of the individual strands of mtDNA in the sample preparations, the length distribution of mtDNA in each sample lane of the blot in Fig. 1b was determined relative to size markers run in parallel (Fig. S1c) and expressed as the apparent median length (Fig. 1c). The apparent median length (henceforth referred to as median length) of muscle mtDNA decreased from 4,400 nt in adults to 2,800 nt in aged animals, while adult and aged animal mtDNA from liver were comparable in length (2,900 and 2,700 nt, respectively; Fig. 1c). Notably, the median length of all samples was well below the 16,299 nt expected for an intact, full-length strand of mouse mtDNA. Given that the same preparations migrated primarily as full-length mtDNA under neutral conditions (Fig. 1a), the low median length under denaturing conditions was ascribed to the presence of single-stranded DNA (ssDNA) breaks.

Taken together, these findings demonstrate that mtDNA in total DNA preparations prepared by a standard isolation procedure contain several ssDNA breaks per strand of mtDNA, and that the breaks are more frequently introduced into mtDNA from the muscle of aged animals than that from adult animals.

The standard protocol preferentially introduces nicks into aged animal muscle mtDNA

To find the underlying cause of the more frequent ssDNA breaks introduced into aged animal muscle mtDNA, we further characterized the mtDNA in total DNA preparations from adult *vs.* aged animals. To determine whether the ssDNA breaks were gaps (that lack one or

more nucleotides) or nicks (that merely disrupt continuity of the DNA backbone), we treated the DNA preparations with mung bean nuclease (MBN), a ssDNA-specific endonuclease that digests the DNA backbone opposite gaps but not opposite nicks (Johnson and Laskowski, 1970). After treatment, the DNA was separated on a neutral gel for detection of MBN-inflicted DNA double-strand breaks among the products (inset in Fig. 2a). As controls, we included samples of adult muscle DNA that was either rendered single-stranded by denaturing at 95°C or nicked by treatment with RNase HII that cleaves at the ribonucleotides present in mtDNA (Wanrooij *et al.*, 2020). Non-linearized samples were used to avoid the risk of introducing unspecific nicks during restriction digestion. Under conditions in which MBN digested the ssDNA but did not affect the mobility of the nicked control DNA (Fig. 2a, lanes 13–17), MBN treatment had little effect on the migration of mtDNA from adult or aged mice (Fig. 2a, compare lanes 4–6 with lanes 10–12). Plots of the length distribution of untreated and MBN-treated adult or aged mtDNA were very similar, whereas the distribution of the ssDNA control was markedly affected by MBN treatment (Fig. 2b). Thus, these results indicate that nicks rather than DNA gaps accumulate in aged animal muscle mtDNA during the standard procedure.

Nicks can be inflicted by numerous factors, of which DNA shearing, oxidative damage and various endonucleases appear most relevant to our isolation procedure. Shearing and oxidative damage to DNA often generate "dirty" DNA ends that require further processing before ligation can occur (Bertoncini and Meneghini, 1995; Caldecott, 2008; Ohtsubo *et al.*, 2019), whereas nucleases can leave "clean" DNA ends that are directly ligatable. To determine whether the nicks observed in aged muscle mtDNA contained ligatable ends, we treated the DNA preparations with T4 DNA ligase and separated ligase-treated and untreated samples on a denaturing gel (Fig. 2c). Ligase treatment resulted in an increase in the median length of all samples from both skeletal muscle and liver, but the increase was most prominent for mtDNA from muscle of aged mice (Fig. 2c-d; Fig. S2a-c). These findings suggest that mtDNA isolated from aged animal muscle contains more frequent ligatable nicks than that from adult muscle or from liver.

We hypothesized that nuclease attack during DNA preparation could account for the formation of these ligatable nicks. The higher frequency of nicks in mtDNA from aged animal muscle suggests that these lysates may contain higher nuclease activity than those from adult animals. In support of this idea, even nuclear DNA (nDNA) integrity was lower in aged animal muscle preparations than those from adults (Fig. S1d-e). This nuclease activity is likely to go unnoticed if the quality of DNA preparations was judged only based on neutral gel analysis, as is customary. While the presence of some nicks may not pose a problem for most analyses, their differential introduction into muscle DNA from different ages of animals could lead to experimental bias and is thus problematic. Therefore, we compared different methods for isolation of mtDNA from skeletal muscle with the aim to identify the procedure that best preserves mtDNA integrity.

The integrity of mtDNA isolated by four alternative procedures

We selected four commonly-used methods for isolation of either pure mtDNA or total DNA from murine solid tissues, and evaluated their effect on mtDNA integrity as judged by migration on both neutral and denaturing gels. Additional variables assessed included DNA yield, mtDNA enrichment (the ratio of mtDNA to nDNA), the duration of the procedure, as well as amenability to scale-up to accommodate a larger number of simultaneous samples. The chosen methods had to allow the simultaneous handling of at least six independent

samples and yield a sufficient amount of mtDNA for Southern blot analysis when starting with at most 300 mg of mouse skeletal muscle.

In the first chosen procedure, mitochondria were purified from tissue homogenates by differential centrifugation and then lysed in the presence of SDS and proteinase K to allow isolation of pure mtDNA (see Materials & Methods for details). The other three methods were for the isolation of total DNA: the standard procedure applied in the experiments in Fig. 1-2 where finely-chopped tissue is lysed by overnight incubation with SDS and proteinase K (Sambrook and Russell, 2001); a variant of the standard method where frozen tissue is pulverized in a mortar prior to a 3-h cell lysis by SDS and proteinase K; and isolation using the NucleoSpin Tissue kit from Macherey-Nagel. The methods were tested on liver and skeletal muscle. Each individual tissue sample was divided into four parts and DNA isolated by each of the four methods; a total of three samples (biological replicates) was analyzed per method.

We first compared the DNA yield and the enrichment of mtDNA (the ratio of mtDNA to nDNA) isolated by the four methods (Table 1; Fig. 3a). The standard and pulverization methods resulted in highest (total) DNA yields, while the recovery of pure mtDNA was low, only 7.2 and 120 ng DNA per mg of skeletal muscle and liver, respectively (Table 1). When starting with ca 300 mg of skeletal muscle, the total yield is thereby only 2.2 ug of mtDNA. However, the enrichment of mtDNA was high by this method (Fig. 3a). Compared to the standard procedure, the mtDNA from purified mitochondria was enriched *ca* 280 and 570-fold in muscle and liver samples, respectively (Table 1). In terms of yield and enrichment, the pulverization procedure performed similarly to the standard protocol, while the kit-based procedure resulted in an intermediate yield with slight enrichment of mtDNA over nDNA.

Table 1. Comparison of the DNA yield and mtDNA enrichment of the different isolation methods.

		Average yield (ng of DNA / mg of tissue)	Relative yield*	Relative mtDNA enrichment*
Muscle	**Pure mtDNA**	7.2	0.003	280
	Pulverized	3740	1.3	1.00
	Kit	84	0.03	1.7
	Standard	2790	1	1
Liver	**Pure mtDNA**	120	0.01	570
	Pulverized	9200	1.1	0.67
	Kit	230	0.03	4.7
	Standard	8350	1	1

*Relative to the standard procedure. Non-normalized values for mtDNA enrichment (mtDNA/nDNA) are presented in Fig. 3a.

Next, the DNA preparations were separated on denaturing and neutral gels, and mtDNA visualized by Southern blotting. Under neutral conditions, some differences in the degree of background smearing could be observed between samples prepared by different procedures; this was most apparent for liver where pure mtDNA samples appeared more intact than the other preparations (Fig. 3b). As the samples were not treated with restriction endonuclease prior to gel analysis, we could also observe some variation in the fraction of mtDNA molecules in open circle *vs*. linear form. The differences in mtDNA integrity between isolation methods became even more clear when the same preparations were analyzed under denaturing conditions (Fig. 3c). For both muscle and liver, the pure mtDNA samples migrated

noticeably slower than the samples prepared by the standard or kit-based procedures. Accordingly, the median length of mtDNA fragments in the pure mtDNA samples was significantly higher than that of samples prepared by other means (Fig. 3d; distribution plots in Fig. S3a-b). In addition, mtDNA prepared by pulverization of skeletal muscle had a higher median length than that of the kit and standard procedures, while the three total-DNA isolation methods resulted in comparable median lengths of liver mtDNA. We conclude that isolation of DNA from purified mitochondria is the method of choice when mtDNA of the highest integrity is required.

The integrity of mtDNA isolated from purified muscle mitochondria does not vary with age

The comparison in Fig. 3 clearly demonstrated that the isolation procedure involving extraction of mtDNA from purified mitochondria preserved the integrity of mtDNA better than the other approaches tested. We next asked whether the differential introduction of ssDNA nicks into aged animal muscle mtDNA that occurs during the standard procedure (Fig. 1b) is observed with pure mtDNA. We isolated mtDNA from purified muscle mitochondria of adult, old adult (1-year-old) and aged animals, and compared its migration on a denaturing gel. In contrast to the mtDNA prepared by the standard procedure that migrated as a smear over the length of the lane (Fig. 1b), pure mtDNA migrated far slower, indicative of fewer ssDNA breaks (Fig. 4a). Most importantly, the migration of muscle mtDNA from all three ages of animals was comparable and its median length was equally high (Fig. 4a-b; distribution plot in Fig. S4a). These findings show that the mtDNA from the muscle of adult, old-adult and aged animals are of comparable integrity, and that the differential introduction of nicks into aged animal muscle mtDNA observed during the standard preparation procedure can be avoided by the choice of DNA isolation method.

Discussion

The integrity of the utilized DNA preparations is a crucial factor for several types of analyses, such as ones based on long-range PCR (Cui et al., 2013; Lehle et al., 2014). The quality of tissue-derived DNA can be jeopardized by the harsh homogenization methods required for disruption of hard tissues such as skeletal muscle, as well as by attack of nucleases prior to their inactivation by the EDTA and proteinase K in the lysis buffer. We find that although the mtDNA prepared by the most commonly-used procedure for isolation of total DNA from mouse tissues appears intact on a neutral gel (Fig. 1a), the median length of the single-stranded (ss) mtDNA is well below the expected 16,300 nt (Fig. 1b-c), indicative of the presence of ssDNA breaks. Most importantly, the integrity of skeletal muscle mtDNA isolated by this method was differentially affected depending on the age of the animal, an artefact that could lead to biased results in subsequent analyses. These findings prompted us to search for an alternative mtDNA isolation procedure that better preserves the integrity of mtDNA.

While others have optimized mtDNA extraction for high enrichment of mtDNA over nDNA (Kauppila et al., 2018), we here present a comparison of methods for the isolation of high-integrity mtDNA from two mammalian tissues, skeletal muscle and liver. These two tissues pose different types of challenges for the extraction of high-quality DNA. Skeletal muscle is hard and tough, requiring longer incubation with proteinases to disrupt the tissue, and thus providing a longer time window for nucleases to act before their inactivation. If methods involving mechanical disruption are used, the rougher homogenization required to disrupt muscle cells may bring about DNA shearing, which is a problem especially for the high-

molecular weight nuclear genome. Liver, on the other hand, is soft and thus easy to homogenize, but also one of the tissues with the highest endonuclease activity (Barry et al., 1999). We systematically observed lower integrity of mtDNA from liver than from muscle, suggesting that the main threat to mtDNA integrity in all our preparations may be cellular nucleases. This was true even for the procedures involving mechanical homogenization (the pure mtDNA isolation and pulverization methods), showing that shearing caused by mechanical homogenization was not a serious threat to mtDNA quality in our experiments.

Of the four methods tested, the one involving extraction of mtDNA from purified mitochondria yielded mtDNA of the highest integrity, as assessed by the median length of mtDNA fragments under denaturing conditions. This was true for both tissues analyzed. Even this mtDNA is not entirely free of ssDNA nicks, as indicated by a median length below 16,300 nt and by the absence of supercoiled mtDNA forms on the neutral gel (Fig. 3b-d). However, the majority of the pure mtDNA was in open circle conformation, and only a minority contained frequent enough breaks to yield a linear mtDNA molecule (Fig. 3b). Although it results in the highest mtDNA integrity and enrichment out of the methods analyzed in this work, the pure mtDNA procedure has its disadvantages: it has the lowest yield per mg of tissue used, is the most labor-intensive and difficult to scale up for the parallel handling of many samples (Table 2). In practice, the number of samples simultaneously extracted by this method is limited to ca 8 by the capacity of a suitable high-speed centrifuge and by the time it takes to manually homogenize and handle the samples.

Table 2. Summary of the methods assessed for DNA isolation from skeletal muscle.

	MtDNA integrity	MtDNA enrichment	Yield	Hands-on time; overall duration*	Suitability for scale-up
Pure mtDNA	High	High	Low	7 h; 2 d	Low
Pulverized	Medium	None	High	4 h; 2 d	Medium
Kit	Low	Low or none	Medium	1 h; 1 d	High
Standard	Low	None	High	3 h; 3 d	Medium to high

*The estimate of overall duration assumes that the re-dissolving of purified DNA is done overnight for all except the kit-based method, as deemed best for optimal solubility.

Out of the total DNA isolation procedures, the pulverization method resulted in mtDNA of higher integrity than the standard or kit-based methods when skeletal muscle was used, while for liver mtDNA, a similar integrity was observed with all total DNA isolation methods (Fig. 3c-d). The yield of the pulverization protocol is high, and the method is more amenable to scale-up than the one involving mitochondrial purification, although the actual grinding of the frozen tissue can be tedious if lots of samples are to be prepared the same day. Apart from the integrity of muscle mtDNA, the performance of the pulverization method is comparable to the standard procedure that only appreciably surpasses the pulverization method in terms of scalability. Finally, the kit-based approach resulted in mtDNA of similar quality as the standard procedure, but with a lower yield. The main advantage of the kit is therefore not the amount or quality of the product but rather the speed and the high suitability of the procedure for scale-up. The number of samples prepared in parallel is limited only by the capacity of the centrifuge – 24 samples can easily be handled simultaneously.

The main purpose of our comparison was to identify a method that preserves the integrity of skeletal muscle mtDNA isolated from all ages of mice to avoid the artefactual difference in mtDNA integrity seen with the standard procedure. As shown in Fig. 4, mtDNA extraction from purified mitochondria fulfills these criteria by producing mtDNA of equally high integrity from the skeletal muscle of adult, old adult and aged mice. It is therefore the most suitable method for studies that require the highest integrity of mtDNA.

What underlies the higher accumulation of ssDNA nicks in muscle mtDNA during the standard procedure? Based on the fact that pure mtDNA is protected from these nicks, it appears likely that the they are introduced by cytosolic endonucleases that either are more active in aged than in adult muscle, or that have the opportunity to act longer on DNA from aged animal muscle during lysis due to poorer/slower access of buffer components like EDTA and proteinase K to the DNA inside the cells. This could occur if the aged animal muscle tissue is tougher and thus harder to lyse than that of younger animals. While our experiments cannot distinguish between the two options, the higher integrity of mtDNA prepared by the pulverization vs. the standard method demonstrates that the faster access of buffer components to the DNA in skeletal muscle is beneficial for DNA integrity. Future efforts to further optimize DNA isolation procedures will hopefully allow isolation of mtDNA with fewer nicks even from tissues with high nuclease activity such as the liver.

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References

Ahola-Erkkilä, S., Carroll, C.J., Peltola-Mjösund, K., Tulkki, V., Mattila, I., Seppänen-Laakso, T., Oresic, M., Tyynismaa, H., Suomalainen, A., 2010. Ketogenic diet slows down mitochondrial myopathy progression in mice. Human molecular genetics 19, 1974–1984. doi:10.1093/hmg/ddq076

Barry, M.E., Pinto-González, D., Orson, F.M., McKenzie, G.J., Petry, G.R., Barry, M.A., 1999. Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. Hum Gene Ther 10, 2461–2480. doi:10.1089/10430349950016816

Bertoncini, C.R., Meneghini, R., 1995. DNA strand breaks produced by oxidative stress in mammalian cells exhibit 3'-phosphoglycolate termini. Nucleic Acids Research 23, 2995–3002. doi:10.1093/nar/23.15.2995

Caldecott, K.W., 2008. Single-strand break repair and genetic disease. Nat Rev Genet 9, 619–631. doi:10.1038/nrg2380

Cui, H., Li, F., Chen, D., Wang, G., Truong, C.K., Enns, G.M., Graham, B., Milone, M., Landsverk, M.L., Wang, J., Zhang, W., Wong, L.-J.C., 2013. Comprehensive next-generation sequence analyses of the entire mitochondrial genome reveal new insights into the molecular diagnosis of mitochondrial DNA disorders. Genet Med 15, 388–394. doi:10.1038/gim.2012.144

Ekstrand, M., Falkenberg, M., Rantanen, A., Park, C., Gaspari, M., Hultenby, K., Rustin, P., Gustafsson, C., Larsson, N., 2004. Mitochondrial transcription factor A regulates mtDNA copy number in mammals. Hum Mol Genet 13, 935–944.

Frezza, C., Cipolat, S., Scorrano, L., 2007. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. Nat Protoc 2, 287–295. doi:10.1038/nprot.2006.478

Green, M.R., Sambrook, J., 2017. Isolation of High-Molecular-Weight DNA Using Organic Solvents. Cold Spring Harb Protoc 2017, pdb.prot093450–359. doi:10.1101/pdb.prot093450

Johnson, P.H., Laskowski, M., 1970. Mung bean nuclease I. II. Resistance of double stranded deoxyribonucleic acid and susceptibility of regions rich in adenosine and thymidine to enzymatic hydrolysis. J. Biol. Chem. 245, 891–898.

Kauppila, J.H.K., Bonekamp, N.A., Mourier, A., Isokallio, M.A., Just, A., Kauppila, T.E.S., Stewart, J.B., Larsson, N.-G., 2018. Base-excision repair deficiency alone or combined with increased oxidative stress does not increase mtDNA point mutations in mice. Nucleic Acids Res 46, 6642–6649. doi:10.1093/nar/gky456

Lang, B.F., Burger, G., 2007. Purification of mitochondrial and plastid DNA. Nat Protoc 2, 652–660. doi:10.1038/nprot.2007.58

Lehle, S., Hildebrand, D.G., Merz, B., Malak, P.N., Becker, M.S., Schmezer, P., Essmann, F., Schulze-Osthoff, K., Rothfuss, O., 2014. LORD-Q: A long-run real-time PCR-based DNA-damage quantification method for nuclear and mitochondrial genome analysis. Nucleic Acids Res 42, e41–e41. doi:10.1093/nar/gkt1349

Ohtsubo, Y., Sakai, K., Nagata, Y., Tsuda, M., 2019. Properties and efficient scrap-and-build repairing of mechanically sheared 3' DNA ends. Commun Biol 2, 409–9. doi:10.1038/s42003-019-0660-7

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29, e45.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning, 3rd ed. CSHL Press, 6.23-6.25.

Szczepanowska, K., Trifunovic, A., 2017. Origins of mtDNA mutations in ageing. Essays Biochem. 61, 325–337. doi:10.1042/EBC20160090

Taylor, R.W., Turnbull, D.M., 2005. Mitochondrial DNA mutations in human disease. Nat Rev Genet 6, 389–402. doi:10.1038/nrg1606

Wallace, D.C., 2012. Mitochondria and cancer. Nat. Rev. Cancer 12, 685–698. doi:10.1038/nrc3365

Wanrooij, P.H., Engqvist, M.K.M., Forslund, J.M.E., Navarrete, C., Nilsson, A.K., Sedman, J., Wanrooij, S., Clausen, A.R., Chabes, A., 2017. Ribonucleotides incorporated by the yeast mitochondrial DNA polymerase are not repaired. Proc. Natl. Acad. Sci. U.S.A. 114, 12466–12471. doi:10.1073/pnas.1713085114

Wanrooij, P.H., Tran, P., Thompson, L.J., Carvalho, G., Sharma, S., Kreisel, K., Navarrete, C., Feldberg, A.-L., Watt, D.L., Nilsson, A.K., Engqvist, M.K.M., Clausen, A.R., Chabes, A., 2020. Elimination of rNMPs from mitochondrial DNA has no effect on its stability. Proceedings of the National Academy of Sciences 117, 14306–14313. doi:10.1073/pnas.1916851117

Yasukawa, T., Yang, M., Jacobs, H., Holt, I., 2005. A bidirectional origin of replication maps to the major noncoding region of human mitochondrial DNA. Mol Cell 18, 651–662. doi:S1097-2765(05)01308-0 [pii] 10.1016/j.molcel.2005.05.002

Figure legends

Figure 1. The standard DNA isolation procedure results in lower integrity of skeletal muscle mtDNA from aged than adult animals. a) DNA isolated from the liver and the tibialis anterior (TA) muscle of 13-week-old (adult) or 2-year-old (aged) animals was linearized with SacI endonuclease and separated on a neutral gel. MtDNA was visualized using a *COX1* probe. Each sample was from a separate mouse. **b)** The same DNA preparations from the liver and the TA muscle of adult or aged animals as in Fig. 1a were separated on a denaturing gel. MtDNA was visualized as above. The signal distribution was quantified and is plotted in Fig. S1c. Dotted lines represent the median length of mtDNA in each group. **c)** The median length of the mtDNA from Fig. 1b was determined based on the signal distribution (Fig. S1c), and age groups were compared using Welch's t-test (n=3). The p-values of statistically significant deviations are indicated. The line indicates the median. The size of the bands in the DNA ladder are indicated in kb. See also Fig. S1.

Figure 2. MtDNA isolated from aged animals by the standard procedure contains ligatable nicks. a) *Inset*: Mung bean nuclease (MBN) cleaves the DNA backbone across ssDNA gaps, but not across nicks. Untreated DNA (lanes 1–6) or MBN-treated DNA (lanes 7–12) from the TA muscle of three adult (lanes 1–3; 7–9) and aged mice (lanes 4–6; 10–12) was separated on a neutral gel and mtDNA visualized by Southern blotting. As controls, samples of adult DNA (lane 17) were rendered single-stranded by boiling (lanes 13–14) or treated with RNase HII to introduce single-strand nicks (lanes 15–16); then treated with mung bean nuclease or left untreated, as indicated. As the DNA was not linearized prior to analysis, it migrates as open circle (*o.c.*) and as linear (*lin.*) species. **b)** DNA size distribution of the untreated (blue) and mung bean nuclease-treated (red) samples from Fig. 2a. Plots for adult and aged groups show the average of the three samples in Fig. 2a. **c)** Untreated (lanes 1–3; 7–9) or ligase-treated (lanes 4–6; 10–12) DNA from adult (lanes 1–6) and aged mice (lanes 7–12) was separated on a denaturing gel and mtDNA visualized by Southern blotting. Dotted lines represent the median of each group. See Fig. S2a for median of individual samples. **d)** The fold change in mtDNA length upon ligase-treatment of the samples in Fig. 2c (TA) or in Fig. S2b (liver); the length of untreated mtDNA was set to 1. The horizontal lines indicate the median. See Fig. S2a, c for the median lengths of untreated and ligase-treated TA and liver samples. The size of bands in the DNA ladder are indicated in kb. See also Fig. S2.

Figure 3. Comparison of mtDNA isolated by different methods. a) The enrichment of mtDNA over nDNA in samples prepared from the skeletal muscle (thigh) and liver of adult mice by four different isolation methods was determined by qPCR. **b)** The same samples as in Fig. 3a were analyzed on a neutral gel and mtDNA visualized by Southern blotting. Each sample corresponds to an individual mouse. *Pulver.*, pulverized; *Std*, standard procedure; *o.c.*, open circle; *lin.*, linear. **c)** The same DNA preparations as in Fig. 3b were separated on a denaturing gel. The signal distribution was quantified and is plotted in Fig. S3a-b. Dotted lines represent the median length of mtDNA in each group. **d)** The median length of the mtDNA from Fig. 3c was determined based on the signal distribution (Fig. S3a-b), and the isolation procedures were compared using one-way ANOVA (n=3). The p-values of statistically significant deviations from the standard procedure are indicated. The line indicates the median. The size of bands in the DNA ladder are indicated in kb. See also Fig. S3.

Figure 4. Muscle mtDNA isolated from purified mitochondria shows equally high integrity in all age groups.
a) MtDNA isolated from purified mitochondria of adult, 1-year-old (old adult) or aged animal GAS muscle was separated on a denaturing gel and mtDNA visualized in Fig. 1. **b)** The median length of the mtDNA from Fig. 4a was determined based on the signal distribution (Fig. S4a).

Figure 1.

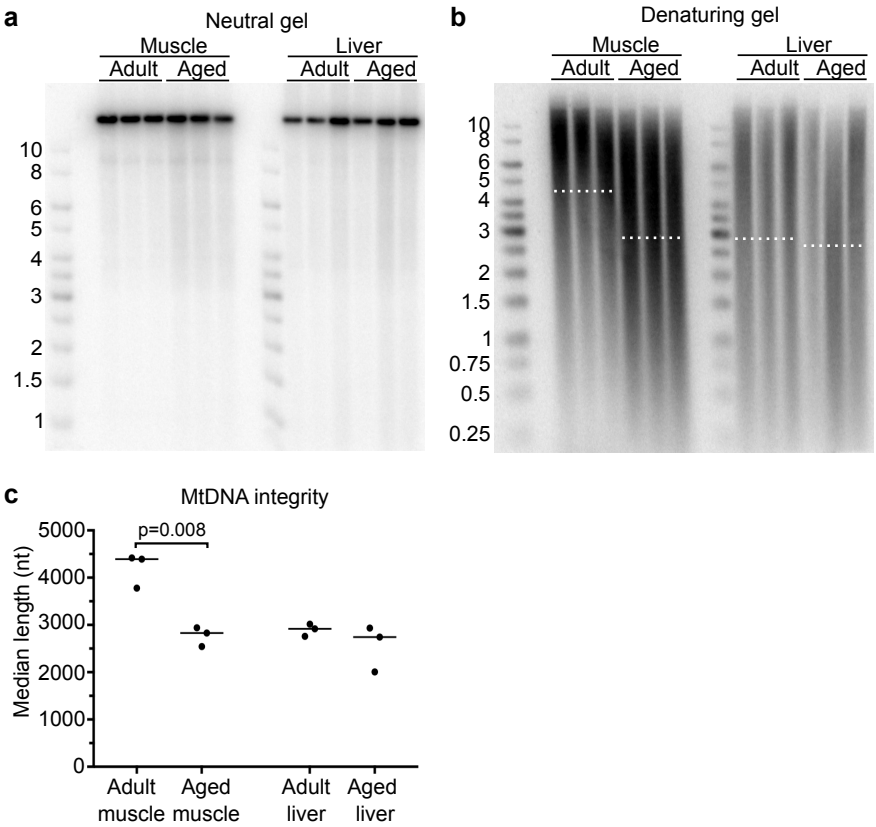

a
Neutral gel
Muscle
Liver
Adult
Aged
Adult
Aged
10
8
6
5
4
3
2
1.5
1
b
Denaturing gel
Muscle
Liver
Adult
Aged
Adult
Aged
10
8
6
5
4
3
2
1.5
1
0.75
0.5
0.25
c
MtDNA integrity
p=0.008
Median length (nt)
5000
4000
3000
2000
1000
0
Adult muscle
Aged muscle
Adult liver
Aged liver

Figure 2.

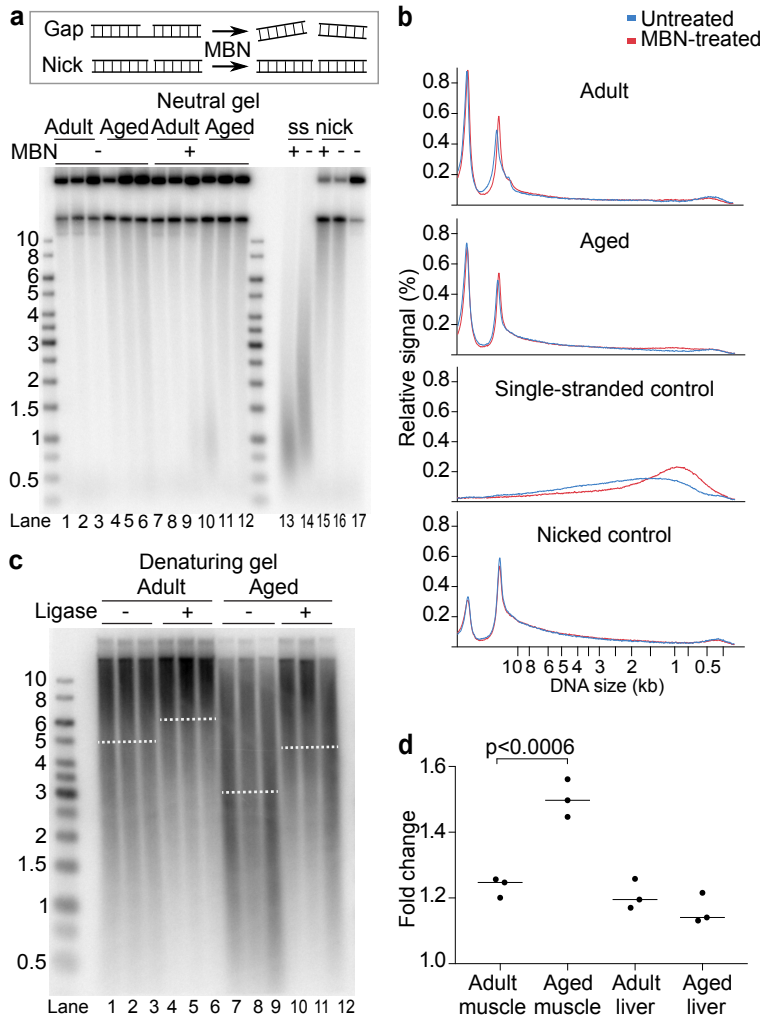

a
Gap
Nick
MBN
Neutral gel
Adult Aged Adult Aged
ss nick
MBN - +
+ - + - -
10
8
6
5
4
3
2
1.5
1
0.5
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
b
Untreated
MBN-treated
Adult
Aged
Single-stranded control
Nicked control
Relative signal (%)
0.8
0.6
0.4
0.2
10 8 6 5 4 3 2 1 0.5
DNA size (kb)
c
Denaturing gel
Adult Aged
Ligase - + - +
10
8
6
5
4
3
2
1.5
1
0.5
Lane 1 2 3 4 5 6 7 8 9 10 11 12
d
p<0.0006
Fold change
1.6
1.4
1.2
1.0
Adult muscle
Aged muscle
Adult liver
Aged liver

Figure 3.

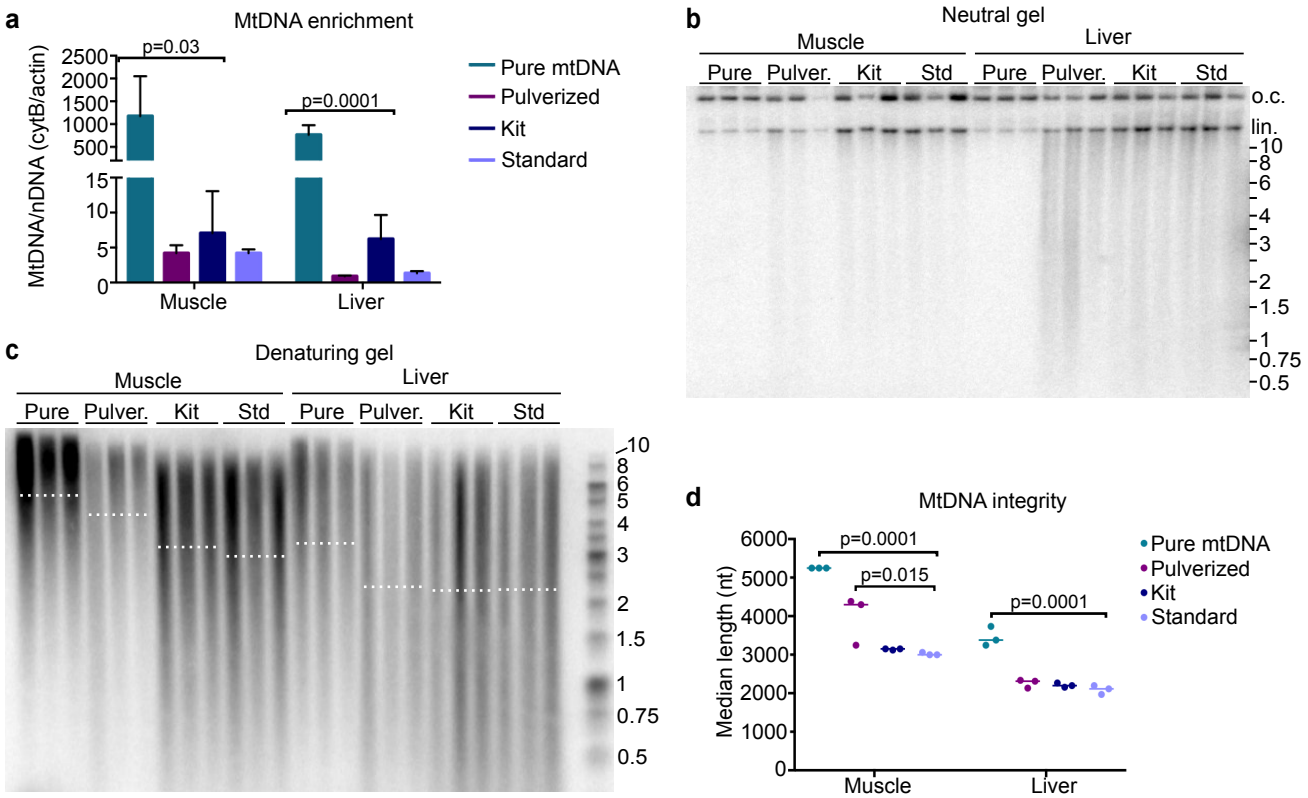
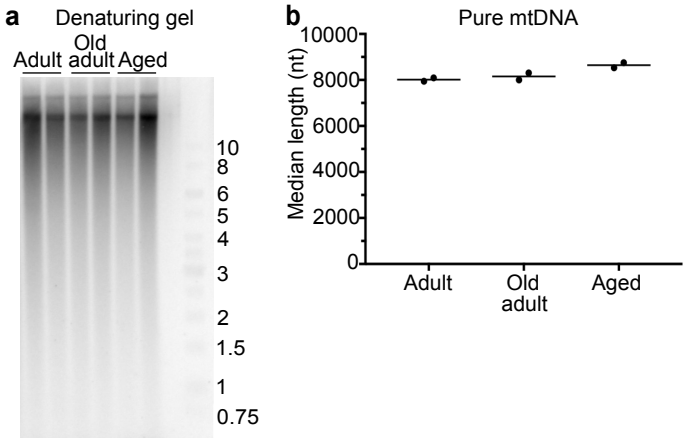

a
MtDNA enrichment
MtDNA/nDNA (cytB/actin)
p=0.03
p=0.0001
2500
2000
1500
1000
500
15
10
5
0
Muscle
Liver
Pure mtDNA
Pulverized
Kit
Standard
b
Neutral gel
Muscle
Liver
Pure
Pulver.
Kit
Std
Pure
Pulver.
Kit
Std
o.c.
lin.
10
8
6
4
3
2
1.5
1
0.75
0.5
c
Denaturing gel
Muscle
Liver
Pure
Pulver.
Kit
Std
Pure
Pulver.
Kit
Std
10
8
6
5
4
3
2
1.5
1
0.75
0.5
d
MtDNA integrity
Median length (nt)
6000
5000
4000
3000
2000
1000
0
p=0.0001
p=0.015
p=0.0001
Muscle
Liver
Pure mtDNA
Pulverized
Kit
Standard

Figure 4.


a
Denaturing gel
Adult
Old adult
Aged
10
8
6
5
4
3
2
1.5
1
0.75
b
Pure mtDNA
Median length (nt)
10000
8000
6000
4000
2000
0
Adult
Old adult
Aged