A comparison of mitochondrial DNA isolation methods in frozen post-mortem human brain tissue—applications for studies of mitochondrial genetics in brain disorders

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Given that many brain disorders are characterized by mitochondrial dysfunction, there is a growing interest in investigating genetic and epigenetic variation in mitochondrial DNA (mtDNA). One major caveat for such studies is the presence of nuclear-mitochondrial pseudogenes (NUMTs), which are regions of the mitochondrial genome that have been inserted into the nuclear genome over evolution and, if not accounted for, can confound genetic studies of mtDNA. Here we provide the first systematic comparison of methods for isolating mtDNA from frozen post-mortem human brain tissue. Our data show that a commercial method from Miltenyi Biotec, which magnetically isolates mitochondria using antibodies raised against the mitochondrial import receptor subunit TOM22, gives significant mtDNA enrichment and should be considered the method of choice for mtDNA studies in frozen brain tissue.

Mitochondria generate ATP, regulate calcium homeostasis (1,2), mediate apoptosis (3), and produce reactive oxygen species (ROS). Mitochondrial dysfunction has been implicated in a number of diseases, including brain disorders such as Alzheimer disease (4–6). Compared to other mammalian organelles, mitochondria are unique in that they contain their own genome consisting of ~16.6 kb of circular DNA (mtDNA) (7) that is separate from the nuclear genome (ncDNA) and is inherited in a maternal, non-Mendelian fashion. The mitochondrial genome comprises 37 genes: 13 encode for electron transport chain polypeptides, 2 for rRNAs, and 22 for tRNAs.

Because of its role in ROS production, mtDNA has a higher mutation rate (10–17 fold) than ncDNA (8). Mutations in mtDNA are relatively common, with at least 1 in 200 healthy humans harboring a potentially pathogenic mtDNA mutation (9). Indeed more than 300 point mutations in mtDNA are associated with disease risk and pathology in MitoMAP, a human mitochondrial genome database (10). Interestingly, as each mitochondrion contains 2–10 copies of mtDNA, and there are multiple mitochondria in any given cell, somatic mutations result in a mosaic of different mtDNA sequences within a given tissue. This phenomenon is known as mitochondrial heteroplasmy and is linked to various mitochondrial diseases (11). Such heterogeneity is a potential confounder in studies of mitochondrial diseases, because inter- and intra-individual heteroplasmic variation can confuse the association between a haplogroup and its corresponding phenotype. Therefore, unlike studies of ncDNA variation, it is important to use the specific tissue of interest for etiological research. Another interesting feature of mitochondria is that, through evolution, sequences of mtDNA have translocated to the nuclear genome. Traditional mitochondrial genetic research and, more recently, studies of mitochondrial epigenetics can be hampered by the presence of these nuclear-mitochondrial pseudogenes (NUMTs) as they share a high homology with their mitochondrial paralogs (12,13). Given the interest in studying mtDNA genetic and epigenetic changes in the pathology of brain diseases characterized by mitochondrial dysfunction, it is imperative that NUMTs are correctly accounted for (14).

The specific isolation of mitochondria prior to downstream processing is vital to fully exclude issues relating to NUMT contamination. For this purpose, a number of methods have been developed to specifically isolate mtDNA, although few of these approaches have been specifically optimized for use on post-mortem tissue, a major resource in many epidemiological studies. In fact, most studies of mtDNA use fresh animal tissue or cell lines. Freezing tissue prior to isolation potentially alters the effectiveness of these techniques and increases the risk of NUMT inclusion in downstream analysis.

Here we compare the effectiveness of five different mitochondrial isolation methods on post-mortem brain tissue using quantitative real-time PCR (qRT-PCR), to determine the optimal method for specific enrichment of mtDNA, which was subsequently validated by next-generation sequencing (NGS). We tested protocols based on (A) Percoll gradients, (B) linear DNA digestion, (C)

METHOD SUMMARY

We compare five methods for isolating mitochondrial DNA (mtDNA) to standard phenol-chloroform DNA extraction, which isolates nuclear DNA (ncDNA) and mtDNA, to determine the optimal technique for enriching mtDNA from frozen post-mortem human brain tissue.

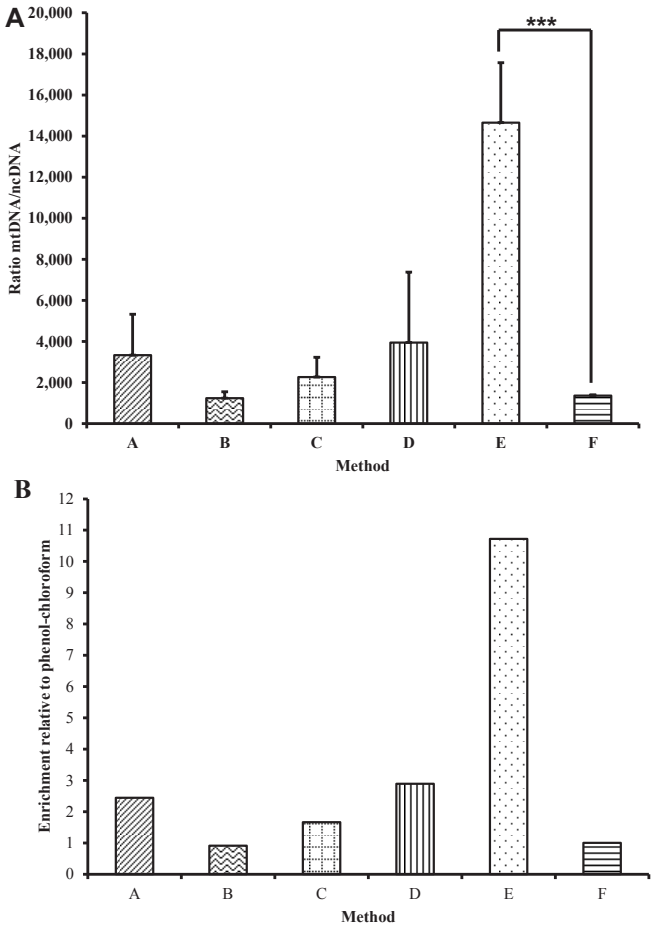

Figure 1. Enrichment of mitochondrial DNA (mtDNA) relative to nuclear genome DNA (ncDNA). In total, five methods [Percoll (A), DNase digestion (B), differential centrifugation (C), rapid differential centrifugation (D), and magnetic microbeads (E)] were compared to a non-enriched standard [phenol-chloroform (F)]. Shown is the ratio of mtDNA/ncDNA (±SEM) (Panel A) and the relative enrichment compared to phenol-chloroform (Panel B). *** denotes $P < 0.005$.

differential centrifugation, (D) rapid differential centrifugation using a commercial kit, and (E) magnetic isolation of mitochondria using anti-TOM22 antibodies.

Method A was a modification of the method described by Sims and Anderson (3). Tissue (150 mg) was dissociated using the gentleMACS dissociator (130–093–235; Miltenyi Biotec, Bergisch Gladbach, Germany) and a mitochondrial extraction kit (130–097–340; Miltenyi Biotec). After removal of the nuclear fraction, the supernatant was spun at 13,000 × *g* for 30 min at 4°C to form a crude mitochondrial pellet. The pellet was homogenized in a 12% Percoll solution and added above 2 layers of Percoll solution (26% and 40%). Samples were spun at 30,700 × *g* for 5 min at 4°C, with the lower band containing the enriched mitochondrial fraction. Each mitochondrial fraction was diluted in 4 volumes of isolation buffer and centrifuged at 16,700 × *g* for 10 min at 4°C to form a loose mitochondrial fraction. The supernatant was discarded, and mtDNA was extracted using a QIAamp DNA Mini kit (51304; Qiagen, Venlo, Netherlands). Method B was based on the method of Zhou et al. (15), which digests linear DNA but leaves circular DNA intact. Twenty micrograms genomic DNA (previously extracted using a phenol-chloroform protocol) was treated with 4 µl lambda exonuclease (5 U/µl) (M0262S; New England Biolabs, Ipswich, Suffolk, UK) and 12 µl $RecJ_f$ (30 U/µl) (M0264S; New England Biolabs) in 400 µl 1× lambda exonuclease reaction buffer (B0262S; New England Biolabs) at 37°C for 16 h. Samples were incubated at 65°C for 10 min to inactivate the enzymes and subsequently purified using a QIAamp DNA Mini kit. Method C was based on the method by Clayton and Shadel (16). Brain tissue (100 mg) was homogenized in 1 mL chilled homogenization buffer (0.25 M sucrose, 10 mM EDTA, 30 mM Tris-HCl, pH 7.5). The homogenate was centrifuged at 1000 × *g* for 15 min at 4°C, and the supernatant was removed. The pellet was re-homogenized in 600 µl chilled homogenization buffer and spun at 1000 ×

Table 1. An overview of starting material for each isolation technique and resulting mitochondrial DNA (mtDNA) and nuclear genome DNA (ncDNA) yields.

Method	A	B	C	D	E	F
Overview	**Percoll gradient**	**DNAse digestion**	**Differential centrifugation**	**Rapid differential centrifugation**	**Magnetic microbeads (anti-TOM22)**	**Phenol-chloroform**
Quantity of starting material	150 mg (tissue)	20 µg (DNA)	100 mg (tissue)	100 mg (tissue)	200 mg (tissue)	100 mg (tissue)
Number of samples	4	6	4	3	5	5
Average concentration (ng/µl) DNA collected (±SEM)	162.4 (11.6)	40.3 (10.1)	23.7 (8.0)	257 (138.4)	32.4 (7.0)	N/A
Average yield (µg) DNA collected (±SEM)	8.1 (0.58)	2.0 (0.50)	4.7 (1.60)	13.7 (6.35)	3.2 (0.70)	N/A
Average number of copies of mtDNA (±SEM)	41,530 (15,468)	12,092,501 (7,742,804)	28,886,594 (12,405,939)	403,381 (194,557)	5,321,960 (1,246,724)	459,264 (751,075)
Average number of copies of ncDNA (±SEM)	155 (85)	8768 (3731)	20,746 (11,256)	720 (449)	402 (86)	4011 (585)
Average rmtDNA/ncDNA ratio (±SEM)	3,337 (1988)	1,242 (309)	2,270 (960)	3,949 (3424)	14,654 (2922)	1367 (35)
Fold-enrichment of mtDNA/ncDNA relative to phenol-chloroform (*P*-value)	2.44 (0.297)	0.91 (0.725)	1.66 (0.320)	2.89 (0.342)	10.72 (1.88×10^{-3})	N/A

We compared five methods of isolating mtDNA in post-mortem human brain tissue: (A) discontinuous Percoll gradient, (B) DNase digestion of linear DNA, (C) differential centrifugation, (D) rapid (commercial) mitochondrial isolation via differential centrifugation, (E) magnetic labelling and pull-down of mitochondria using an antibody to TOM22. Yield and enrichment for each method were compared to a non-enriched standard (phenol-chloroform) using an unpaired two-tailed *t*-test.

g for 10 min at 4°C. The supernatant was combined with the supernatant from the previous step and centrifuged at 12,000 × *g* for 30 min at 4°C to pellet the mitochondria. mtDNA was extracted using a QIAamp DNA Mini kit. Method D was a modification of the method by Clayton and Shadel (16) and was performed using a commercial kit (PK-CA577-K280; Promokine, Heidelberg, Germany). Brain tissue (100 mg) was homogenized with the reagents provided, according to the manufacturer's instructions. For Method E, 200 mg of tissue was dissociated using the gentleMACS dissociator (Miltenyi Biotec) and a mitochondrial extraction kit (Miltenyi Biotec) according to the manufacturer's protocol, with the exception of using an increased quantity of extraction buffer (40 µl Solution 1 and 1 mL 1 × Solution 2). After homogenization, the sample was spun at 200 × *g* for 30 s, passed through a 70 µm pre-separation filter (130–095–823; Miltenyi Biotec), and washed with Solution 3. The homogenate was spun at 500 × *g* for 5 min at 4°C, and the supernatant was removed. The supernatant was magnetically labeled with 100 µl anti-human TOM22 antibody–microbeads (130–094–532; Miltenyi Biotec) for 1 h at 4°C under continuous agitation. The eluate was added to an LS column (130–042–401; Miltenyi Biotec), placed in a MACS separator (130–042–302; Miltenyi Biotec), and washed. Upon removing the column from the magnetic field, the mitochondria were pelleted by centrifugation at 13,000 × *g* for 2 min at 4°C, washed in 1 mL Storage Buffer, and centrifuged at 13,000 × *g* for 2 min at 4°C. The supernatant was discarded, and mtDNA was extracted using a QIAamp DNA Mini Kit. We compared DNA isolated with these approaches to DNA we had previously isolated (17) using a phenol-chloroform protocol, which isolates both ncDNA and mtDNA. We assessed the purity of each method using qRT-PCR as previously described (6). Briefly, mitochondrial and nuclear copy numbers were determined using qPCR assays for the mitochondrial gene *MT-CYB* and the nuclear gene *hB2M*. The number of copies of mtDNA relative to ncDNA was then determined by dividing the calculated number of copies of these two genes.

Our data showed that Method B (linear DNA digestion) gave the lowest purity (1242 mtDNA copies/ncDNA copy), and Method E (magnetic microbeads) produced the highest purity (14,654 mtDNA copies/ncDNA copy) (Figure 1A and Table 1). Of particular interest to the study was the relative enrichment compared with a standard phenol-chloroform extraction (Figure 1B). The only method to show no enrichment was Method B (linear DNA digestion). All other techniques showed a positive enrichment of mtDNA compared with phenol-chloroform. Method A (Percoll), Method C (differential centrifugation), and Method D (rapid differential centrifugation) all gave modest positive enrichments of 2.4-, 1.7-, and 2.9-fold, respectively. Although giving one of the lowest yields of DNA (3.2 µg), the optimal method for enrichment relative to ncDNA was Method E (magnetic microbeads), which produced a 10.7-fold enrichment and was the only method to show significantly more copies of mtDNA/ncDNA copy compared with phenol-chloroform extraction ($P = 1.88 \times 10^{-3}$). Using this method, we saw a significant enrichment of mtDNA/ncDNA compared with Method A ($P = 0.019$), Method B ($P = 6.97 \times 10^{-4}$), and Method C ($P = 8.48 \times 10^{-3}$). To validate our enrichment findings, two of the biological replicates from Method E were compared with a non-enriched standard from phenol-chloroform extraction using NGS. DNA samples were fragmented by sonication using a Bioruptor (UCD-200; Diagenode, Seraing, Liege, Belgium) to an average size of ~240 bp. Sequencing libraries were prepared using the NEXTflex Rapid DNA-Seq kit (Bioo Scientific, Austin, TX) and ligated to pre-indexed adapters (NEXTflex-96 DNA Barcodes). Adapter-ligated DNA was amplified for 10 cycles using Herculase II Fusion DNA Polymerase (Agilent Technol-

ogies, Santa Clara, CA) and NEXTflex PCR primer mix, then pooled for sequencing on an Illumina (San Diego, CA HiSeq2500 (100 bp paired-end, rapid run mode). Raw reads were quality and adaptor trimmed using TrimGalore! (www.bioinformatics.babraham.ac.uk/projects/trim_galore/) before being aligned to human genome assembly GRCH37. Only high quality reads (Phred < 20), uniquely mapping to the genome were considered, and total read counts were taken. For the non-enriched standard, 1.1% of reads mapped to the mitochondrial genome, compared with an average of 18.7% (16.2% and 21.2% respectively) of reads with DNA purified using Method E, demonstrating an average 16.8-fold enrichment.

Although Method E provided a greater enrichment than Method C (differential centrifugation) in mouse liver (18) and a similar enrichment to Method A (Percoll) in an osteosarcoma cell line (19), higher levels of mitochondrial enrichment have previously been reported (20). However, this study, like Method B in our study, relies on the circular nature of the intact mitochondrial genome, which, while present in cell lines and blood, may be more degraded in frozen, archived brain. In the context of genomic studies of mtDNA, where the exclusion of NUMTs is imperative, the relative enrichment of mtDNA is of far greater importance than the yield. Thus, although we saw a lower yield with magnetic microbeads (Method E) compared with the majority of methods tested, we observed the greatest purity with this method. The reasons for the observed greater enrichment of mtDNA/ncDNA relative to Percoll (Method A) in our study compared with the analysis by Hornig-Do and colleagues (19) potentially may include (*i*) our use of qRT-PCR, which is more sensitive than Western blot, (*ii*) our use of frozen samples rather than fresh samples, and (*iii*) the use of brain tissue rather than a cell line, as brain has high levels of mitochondria. To our knowledge, this is the first study to systematically compare and contrast methods for isolating mtDNA from small quantities of frozen, post-mortem human brain. Our findings suggest that magnetic microbeads provide a significant enrichment of mtDNA compared with the other methods we tested. This may be due to a number of reasons—for example, the automated homogenization of tissue in this protocol could provide a more consistent and gentle approach than other techniques, and the use of magnetically labeled antibodies provides specific capture of intact mitochondria, which may also contain mtDNA that is less degraded. Given the current interest in studying the mitochondrial genome in human brain, we recommend that the magnetic microbead method from Miltenyi Biotec is used prior to DNA extraction to minimize the inclusion of NUMTs in downstream analyses.

Author contributions

M.D. J.M., and K.L. conceived the idea for the study. M.D., J.B., R.C., M.J., and A.R.J. performed the experiments. C.T. and S.A.-S. provided tissue from the London Neurodegenerative Disease Brain Bank. M.D. and K.L. analyzed the data and drafted the manuscript. All authors approved of the final manuscript prior to submission.

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Competing interests

The authors declare no competing interests.

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