BENCHMARKS

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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Abstract

23 Given that many brain disorders are characterized by mitochondrial dysfunction, there is a growing 24 interest in investigating genetic and epigenetic variation in mitochondrial DNA (mtDNA). One major 25 caveat for such studies is the presence of nuclear-mitochondrial pseudogenes (*NUMTs*), which are 26 regions of the mitochondrial genome that have been inserted into the nuclear genome over evolution 27 and, if not accounted for, can confound genetic studies of mtDNA. Here we show the first systematic 28 study to compare methods for isolating mtDNA from frozen post-mortem human brain tissue, and 29 show that a commercial method from Miltenyi Biotech, which magnetically isolates mitochondria 30 using antibodies raised against TOM22, gives a significant enrichment of mtDNA, and should be 31 considered the method of choice for mtDNA studies in frozen brain tissue.

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Method Summary

38 Here we compare five methods of isolating mitochondrial DNA (mtDNA) to standard phenol-39 chloroform DNA extraction (that isolates nuclear DNA (ncDNA) and mtDNA) to determine the 40 optimal method for enriching mtDNA from frozen post-mortem human brain tissue.

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45 **Main Text**

46 Mitochondria generate ATP, regulate calcium homeostasis [1, 2], mediate apoptosis [3], and produce
47 reactive oxygen species (ROS). Mitochondrial dysfunction has been implicated in a number of
48 diseases, including in the pathogenesis of brain disorders such as Alzheimer's disease [4-6].
49 Mitochondria are unique mammalian organelles in that they contain their own genome; the
50 mitochondrial genome is ~16.6kb of circular DNA (mtDNA) [7], separate to the nuclear genome
51 (ncDNA) and inherited in a maternal, non-Mendelian fashion. The mitochondrial genome comprises
52 37 genes; 13 encode for electron transport chain polypeptides, two for rRNAs and 22 for tRNAs.
53 Because of its role in ROS production, mtDNA has a higher mutation rate (10-17 fold) than ncDNA
54 [8]. Mutations in mtDNA are relatively common, with at least one in 200 healthy humans harboring a
55 potentially pathogenic mtDNA mutation [9]. Indeed more than 300 point mutations in mtDNA are
56 associated with disease risk and pathology in MitoMAP [10]. Interestingly, as each mitochondrion
57 contains 2-10 copies of mtDNA and there are multiple mitochondria in any given cell, somatic
58 mutations result in a mosaic of different mtDNA sequences within a given tissue. This phenomenon is
59 known as mitochondrial heteroplasmy and is linked to various mitochondrial diseases [11]. Such
60 heterogeneity is a potential confounder in studies of mitochondrial diseases, because inter- and intra-
61 individual heteroplasmic variation can confuse the association between a haplogroup and its
62 corresponding phenotype. Therefore, unlike studies of ncDNA variation, it is important to use the
63 specific tissue of interest for etiological research. Another interesting feature of the mitochondria is
64 that over evolution sequences of mtDNA have translocated to the nuclear genome. Traditional
65 mitochondrial genetic research, and more recently studies of mitochondrial epigenetics, can be
66 hampered by the presence of these nuclear-mitochondrial pseudogenes (*NUMTs*) as they share a high
67 homology with their mitochondrial paralogs [12, 13]. Given the interest in studying mtDNA genetic
68 and epigenetic changes in the pathology of brain diseases characterized by mitochondrial dysfunction,
69 it is imperative that *NUMT*s are correctly accounted for [14].

70 The specific isolation of mitochondria prior to downstream processing is vital to fully exclude issues
71 relating to *NUMT* contamination. For this purpose, a number of methods have been developed to

72 specifically isolate mtDNA, although few of these approaches have been specifically optimized for
73 use on post-mortem tissue, a major resource in many epidemiological studies. In fact most studies
74 investigating mtDNA use fresh animal tissue or cell lines. The insult of freezing tissue prior to
75 isolation will potentially alter the effectiveness of these techniques and increase the risk of *NUMT*
76 inclusion in downstream analysis. In this study we compared the effectiveness of five different
77 mitochondrial isolation methods on post-mortem brain tissue using quantitative real-time PCR (qRT-
78 PCR), to determine the optimal method for the specific enrichment of mtDNA, which was
79 subsequently validated by next generation sequencing (NGS). We tested protocols based on A)
80 Percoll gradients, B) linear DNA digestion, C) differential centrifugation, D) rapid differential
81 centrifugation using a commercial kit and E) magnetic isolation of mitochondria using anti-TOM22
82 antibodies.

83 Method A was a modification of the method by Sims and Anderson (3). 150mg of tissue was
84 dissociated using the gentleMACS dissociator (Miltenyi Biotech:130-093-235) and a mitochondrial
85 extraction kit (Miltenyi Biotech:130-097-340). After removal of the nuclear fraction, the supernatant
86 was spun at 13,000xg for 30 minutes at 4°C to form a crude mitochondrial pellet. The pellet was
87 homogenized in a 12% Percoll solution and added above two layers (26% and 40%) of Percoll
88 solution. Samples were spun at 30,700xg for 5 minutes at 4°C with the lower band containing the
89 enriched mitochondrial fraction. Each mitochondrial fraction was diluted in four volumes of isolation
90 buffer and centrifuged at 16,700xg for 10 minutes at 4°C to form a loose mitochondrial fraction. The
91 supernatant was discarded, and mtDNA extracted using a DNA Mini kit (Qiagen:51304). Method B
92 was based on the method by Zhou *et al* [15] that digests linear DNA but leaves circular DNA intact.
93 20ug genomic DNA (previously extracted using a phenol-chloroform protocol) was treated with 4µl
94 lambda exonuclease (5 U/µl) (New England Biolabs:M0262S) and 12µl $RecJ_f$ (30 U/µl) (New
95 England Biolabs:M0264S) in 400µl 1x lambda exonuclease buffer (New England Biolabs:B0262S) at
96 37^oC for 16 hours. Samples were incubated at 65^oC for 10 minutes to inactivate the enzymes and
97 subsequently purified using a DNA Mini Kit (Qiagen:51304). Method C was based on the method by
98 Clayton and Shadel [16]. 100mg brain tissue was homogenized in 1ml chilled homogenization buffer

99 (0.25M sucrose/10mM EDTA/30mM Tris-HCl, pH 7.5). The homogenate was centrifuged at 1,000xg
100 for 15 minutes at 4°C and supernatant removed. The pellet was re-homogenized in 600µl chilled
101 homogenization buffer and spun at 1,000xg for 10 minutes at 4°C. The supernatant was combined
102 with the supernatant from the previous step and centrifuged at 12,000xg for 30 minutes at 4°C to
103 pellet the mitochondria. MtDNA was extracted using a DNA Mini Kit (Qiagen:51304). Method D
104 was a modification of the method by Clayton and Shadel [16] and purchased as a commercial kit
105 (Promokine:PK-CA577-K280). 100mg of brain tissue was homogenized with the reagents provided,
106 according to the manufacturer's instructions. In Method E 200mg of tissue was dissociated using the
107 gentleMACS dissociator and a mitochondrial extraction kit (Miltenyi Biotech:130-097-340) according
108 to the manufacturer's protocol, with the exception of using an increased quantity of extraction buffer
109 (40µl Solution 1 and 1ml 1x Solution 2). After homogenization the sample was spun at 200xg for 30
110 seconds and passed through a 70µm pre-separation filter (Miltenyi Biotec:130-095-823) and washed
111 with Solution 3. The homogenate was spun at 500xg for 5 minutes at 4°C and the supernatant
112 removed. The supernatant was magnetically labelled with 100µl anti-human TOM22 antibody-
113 microbeads (Miltenyi Biotech:130-094-532) for 1 hour at 4°C under continuous agitation. The eluate
114 was added to a LS column (Miltenyi Biotech:130-042-401) and placed in a MACS separator
115 (Miltenyi Biotech:130-042-302) and washed. Upon removing the column from the magnetic field the
116 mitochondria were pelleted by centrifugation at 13,000xg for 2 minutes at 4°C, washed in 1ml
117 Storage Buffer and centrifuged at 13,000xg for 2 minutes at 4°C. The supernatant was discarded and
118 mtDNA extracted using a DNA Mini Kit (Qiagen:51304). We compared these approaches to DNA we
119 had previously isolated [17] using a phenol-chloroform protocol, which isolates both ncDNA and
120 mtDNA We assessed the purity of each method using qRT-PCR as previously described (6). Briefly,
121 the number of copies of mtDNA relative to ncDNA was determined by dividing the calculated
122 number of copies of mtDNA (MT-CYB assay) by the calculated number of copies of ncDNA (B2M
123 assay).

124 Our data showed that Method B (linear DNA digestion) gave the lowest purity (1,242 mtDNA
125 copies/ncDNA copy), and Method E (magnetic-microbeads) the highest purity (14,654 mtDNA

126 copies/ncDNA copy) (**Figure 1A; Table 1**). Of particular interest to the study was the relative
127 enrichment compared to a standard phenol-chloroform extraction (**Figure 1B**). The only method to
128 show no enrichment was Method B (linear DNA digestion). All other techniques showed a positive
129 enrichment of mtDNA compared to phenol-chloroform. Methods A (Percoll), C (differential
130 centrifugation) and D (rapid differential centrifugation) all gave modest positive enrichments of 2.4-,
131 1.7- and 2.9- fold respectively. Although giving one of the lowest yields (3.2μg), the optimal method
132 for enrichment relative to ncDNA was Method E (magnetic-microbeads), which gave a 10.7-fold
133 enrichment, and was the only method to show significantly more copies of mtDNA/ncDNA copy
134 compared to phenol-chloroform extraction ($P=1.88x10^{-3}$). Using this method we saw a significant
135 enrichment of mtDNA/ncDNA compared to Methods A ($P=0.019$), B ($P=6.97x10^{-4}$) and C
136 ($P=8.48x10^{-3}$). To validate our enrichment, two of the biological replicates from Method E were
137 compared to a non-enriched standard from phenol-chloroform extraction using NGS. DNA samples
138 were fragmented by sonication using a Bioruptor (Diagenode:UCD-200) to an average size of
139 ~240bp. Sequencing libraries were prepared using the NEXTflex Rapid DNA-Seq kit (Bioo
140 Scientific) and ligated to pre-indexed adapters (NEXTflex-96 DNA Barcodes; Bioo Scientific).
141 Adapter-ligated DNA was amplified for 10 cycles using Herculase II Fusion DNA Polymerase
142 (Agilent Technologies) and NEXTflex PCR primer mix, then pooled for sequencing on an Illumina
143 HiSeq2500 (100bp paired-end, rapid run mode). Raw reads were quality and adaptor trimmed using
144 TrimGalore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) before being aligned
145 to GRCH37. Only high quality (Phred <20) reads, uniquely mapping to the genome were considered
146 and total read counts were taken. For the non-enriched standard 1.1% of reads mapped to the
147 mitochondrial genome, compared to an average of 18.7% (16.2% and 21.2% respectively) of reads
148 with Method E, demonstrating an average 16.8 fold enrichment.

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150 Despite Method E providing a greater enrichment than Method C (differential centrifugation) in
151 mouse liver [18] and a similar enrichment to Percoll in an osteosarcoma cell line [19], higher levels of
152 mitochondrial enrichment have previously been reported [20]. However, this method, like Method B

153 in our study, relies on the circular nature of the intact mitochondrial genome, which, whilst present in
154 cell lines and blood, may be more degraded in frozen, archived brain. In the context of genomic
155 studies of mtDNA, where the exclusion of *NUMTs* is imperative, the relative enrichment of mtDNA is
156 of far greater importance than the yield. Thus, although we saw a lower yield with magnetic-
157 microbeads (Method E) compared to the majority of methods tested, we observed the greatest purity
158 with this method. The reasons for the observed greater enrichment of mtDNA/ncDNA relative to
159 Percoll (Method A) in our study compared to the analysis by Hornig-Do and colleagues potentially
160 may include i) our use of qRT-PCR, which is more sensitive than western blot, ii) our use of frozen
161 samples, rather than fresh samples and iii) the use of brain, rather than a cell line, as brain has high
162 levels of mitochondria. To our knowledge our study represents the first to systematically compare and
163 contrast methods for isolating mtDNA from small quantities of frozen, post-mortem human brain. Our
164 findings suggest that magnetic-microbeads provide a significant enrichment of mtDNA compared to
165 any other method tested. This may be due to a number of reasons, for example the automated
166 homogenization of tissue in this protocol could provide a more consistent and gentle approach than
167 other techniques and the use of magnetically labelled antibodies provides a specific capture of intact
168 mitochondria, which may also contain less degraded mtDNA. We recommend that given the current
169 interest in studying the mitochondrial genome in human brain, that the magnetic-microbead method
170 from Miltenyi Biotech is used prior to DNA extraction to minimize the inclusion of NUMTs in
171 downstream analyses.

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174 **Author Contributions**

175 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
176 experiments. C.T. and S.A-S provided tissue from the London Neurodegenerative Disease Brain
177 Bank. M.D. and K.L. analyzed the data and drafted the manuscript. All authors approved of the final
178 manuscript prior to submission.

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184 ## Competing Interests

185 The authors declare that they have no conflicts of interest in regard to this work.

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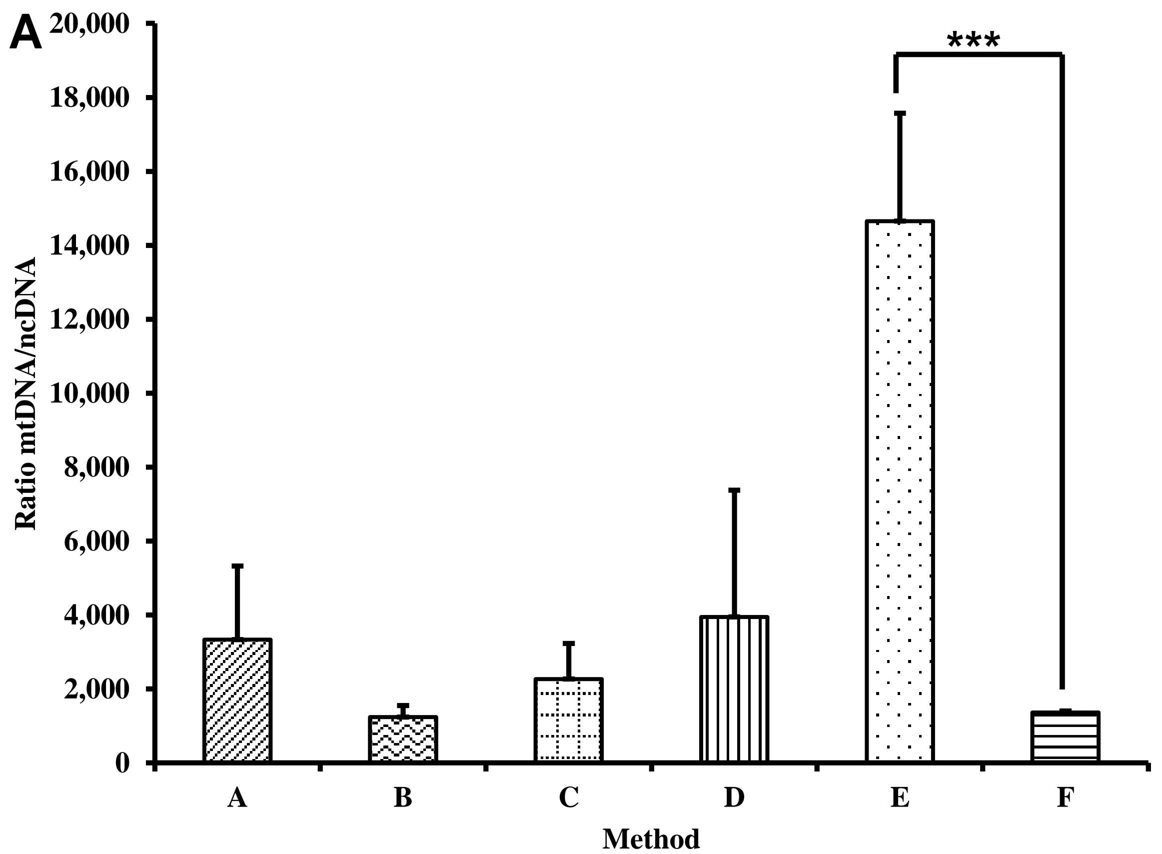
Table 1. An overview of starting material for each isolation technique and resulting yield.

Method	A	B	C	D	E	F
Overview	Percoll	DNAse digestion	Differential centrifugation	Rapid differential centrifugation	Magnetic microbeads (anti-TOM22)	Phenol-chloroform
Quantity of starting Material	150mg (tissue)	20µg (DNA)	100mg (tissue)	100mg (tissue)	200mg (tissue)	100mg (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 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 copies of ncDNA (±SEM)	155 (85)	8,768 (3,731)	20,746 (11,256)	720 (449)	402 (86)	4,011 (585)
Average ratio mtDNA/ncDNA (±SEM)	3,337 (1,988)	1,242 (309)	2,270 (960)	3,949 (3,424)	14,654 (2,922)	1,367 (35)
Fold Enrichment 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

In brief 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. We compared the yield and enrichment to a non-enriched standard (phenol-chloroform) using an unpaired two-tailed t-test.



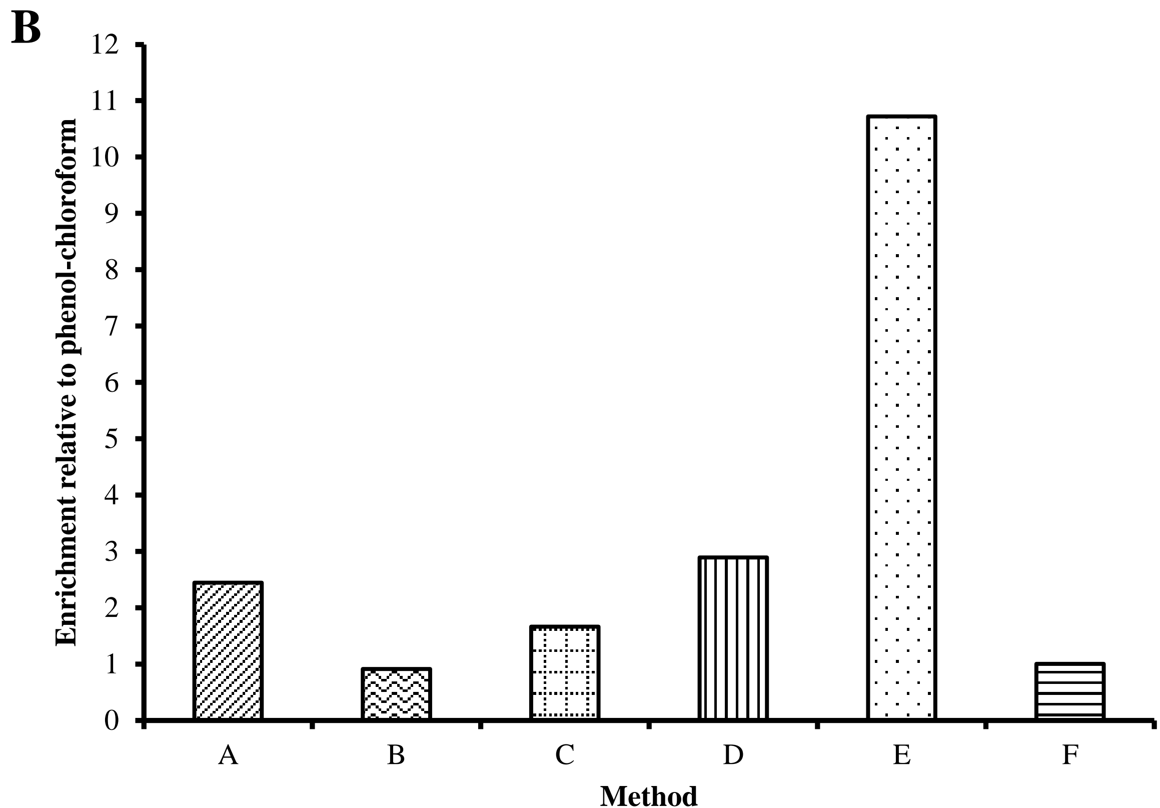


Figure 1: Enrichment of mtDNA relative to ncDNA. In total five methods (Percoll (A), DNase digestion (B), differential centrifugation (C), rapid differential centrifugation (D) and magnetic microbeads (E) were compared to a non-enriched standard (phenol-chloroform (F)). Shown is the ratio of mtDNA/ncDNA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