

Supplementary Material For:

Using Φ X174 DNA as an exogenous reference for measuring mitochondrial DNA copy number

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BioTechniques 47:867-869 (October 2009) doi 10.2144/000113222

Key words: quantitative PCR; mitochondrial DNA copy number; exogenous reference; Φ X174 DNA; DNA extraction

Plasmid DNA and calibrator preparation

Plasmid DNA molecules containing the sequences for the mitochondrial displacement loop (D-loop) region and the nuclear thymidine kinase (*Tk*) gene were constructed using a TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). The plasmid DNA preparations were used as part of the calibrator sample (equal ratio of D-loop and *Tk* plasmid DNA, and Φ X174 DNA) in the quantitative real-time PCR (Q-PCR) experiments and in the $\Delta\Delta C_t$ method validation experiment. PCR amplifications were conducted in an ABI 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using total mouse DNA as the template and primers for the mitochondrial D-loop and the nuclear *Tk* genes (Table S1). The D-loop and *Tk* PCR reactions were performed separately in 30- μ L reactions containing the components of the HotStarTaq Kit (Qiagen, Valencia, CA, USA), 200 μ M each dNTP, and 1 pmole/ μ L each primer. The thermocycling conditions for amplification of the D-loop region consisted of an initial period of denaturation at 95°C for 15 min; then 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min; followed by a final extension step at 72°C for 7 min. The thermocycling conditions for amplification of the *Tk* sequence consisted of an initial period of denaturation at 95°C for 15 min; then 30 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 3 min. Approximately five microliters of the PCR products were loaded on a 1.0% TBE agarose gel, electrophoresed, and visualized after staining with 0.5 μ g/mL ethidium bromide to confirm the PCR product.

Construction of the D-loop and *Tk* plasmid DNA clones was conducted following the manufacturer's protocol for the TA Cloning Kit (Invitrogen). The plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) and the presence of the DNA insert was confirmed by restriction digestion with *Eco*RI and gel electrophoresis. The plasmid DNA preparations containing the insert were sequenced using DYEnamic ET Terminator Cycle Sequencing Chemistry (GE Healthcare Biosciences, Piscataway, NJ, USA) and analyzed on the ABI 377 DNA Sequencer (Applied Biosystems).

Quantification of the plasmid DNA preparations was performed using two separate methods. First, the plasmid DNA preparations were linearized by restriction digestion overnight using the enzyme, *Hind*III. The linearized plasmids were electrophoresed along with a high-mass DNA ladder (Invitrogen) on a 0.8% TAE agarose gel. The gel was stained with Vistra Green Nucleic Acid Stain (GE Healthcare Biosciences) for 10 min and then visualized using a FluorImager (Molecular Dynamics, Sunnyvale, CA, USA). A standard curve was constructed from the molecular weight bands of the high-mass DNA ladder. The unknown plasmid DNA preparations were quantified using the standard curve generated from the DNA ladder. The plasmid DNA quantity was confirmed using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

To generate the calibrator sample, we used plasmid DNA preparations containing the mitochondrial D-loop region, the nuclear *Tk* region, and Φ X174 DNA. An approximately equivalent number

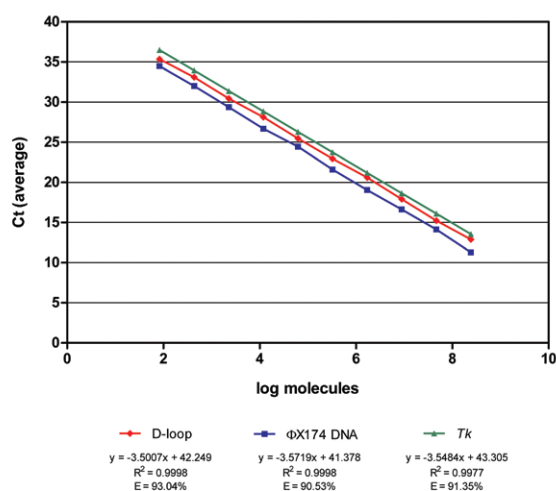
of molecules ($\sim 2 \times 10^9$) of all three were combined with water in a total volume of 50 mL. Aliquots of 20, 30, and 40 μ L were stored as single-use reagents in 0.5-mL microcentrifuge tubes at -20°C.

Real-time PCR

Primers and TaqMan MGB (minor-groove binder) probes were designed for the mouse mitochondrial D-loop and nuclear *Tk* gene plasmid DNA, and for Φ X174 DNA, using Primer Express v2.0 (Applied Biosystems). Their sequences are given in Table S1. The real-time PCR reaction mixture for a given target sequence contained 1 \times TaqMan Universal PCR Master Mix, 1 μ M each of a forward and reverse primer, 0.25 μ M the corresponding TaqMan probe, and 2 μ L the test sample DNA, in a total of 20 μ L. The DNA and the calibrator samples were prepared and loaded into 96-well plates (Cat. no. AB-0600, Thermo Scientific, Rockford, IL, USA) in duplicate reactions for each amplicon. The ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used to detect and quantify the target sequences. The thermocycling conditions used were: a 2-min hold at 50°C and a 10-min hold at 95°C; followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Sensitivity, linearity, and dynamic range of the real-time PCR

The sensitivity, linearity, and dynamic range were determined using a 5-fold serial dilution series consisting of an approximately equal number of molecules of linearized plasmid DNA containing the mtDNA D-loop region, linearized plasmid DNA containing a portion of the nDNA *Tk* gene, and Φ X174 DNA RF II (relaxed, replicative form). Ten 5-fold dilutions were created ranging from $\sim 2.43 \times 10^8$ to 83 molecules per DNA per reaction. The serial dilutions of the D-loop plasmid DNA, *Tk* plasmid DNA, and Φ X174 DNA were amplified in duplicate by real-time PCR using the gene-specific primers and probes described in Table S1. Standard curves plotting Ct as a function of the logarithm of the starting molecules for the D-loop, *Tk*, and Φ X174 DNA—with their determined slopes, y-intercepts and correlation coefficients—are shown in Figure S1. The amplification of the D-loop plasmid DNA, *Tk* plasmid DNA, and Φ X174



Supplementary Figure 1. Comparison of the standard curves for the mitochondrial D-loop, nuclear *Tk* gene, and ΦX174 DNA. Serially diluted D-loop plasmid, *Tk* plasmid, and ΦX174 DNA (~2.43 × 10⁸–83 molecules) were amplified in duplicate reactions. A linear regression of the average C_t plotted against log(molecules) is shown. C_t, cycle in which target reaches a preset threshold level; D-loop, displacement loop of mtDNA; mtDNA, mitochondrial DNA; *Tk*, nuclear thymidine kinase gene; ΦX174, bacteriophage ΦX174 (relaxed, replicative form).

Supplementary Table 1. List of primers, their sequences, and their experimental use

Designation	Sequence (5'-3')	Experimental use
D-loop-F	TCTTTTATTGGCCTAC	Plasmid cloning
D-loop-R	CACTGAAAATGCTTAGATG	Plasmid cloning
<i>Tk</i> -F	GGTGCCAAGGCTGGGGG	Plasmid cloning
<i>Tk</i> -R	CTTGTAAGTGTAGCTGCCTCGAG	Plasmid cloning
D-loop-F	CCAAAAACACTAAGAACTTGAAGACA	Real-time PCR
D-loop-R	GTCATATTTGGGAAGCTACTAGAATTGATC	Real-time PCR
D-loop probe ^a	AATATTAAGTATCAAAACCTATGTCC	Real-time PCR
<i>Tk</i> -F	GACTGTATTGAGCGGCTTCAGA	Real-time PCR
<i>Tk</i> -R	CATGCTCGGTGTGAGCCATA	Real-time PCR
<i>Tk</i> probe ^a	TTCCCATGCTAAAACCT	Real-time PCR
ΦX174-F	CGCCATTAATAATGTTTTCCGTAA	Real-time PCR
ΦX174-R	CATCCCGTCAACATTCAAACG	Real-time PCR
ΦX174 probe ^b	CGCCTTCCATGATGAGA	Real-time PCR

^{a,b}The probe sequences for all three targets contained the reporter dyes FAM (a), or VIC (b) at the 5' end, and a minor-groove binder (MGB) moiety and a nonfluorescent quencher at the 3' end. D-loop, displacement loop of mtDNA; mtDNA, mitochondrial DNA; *Tk*, nuclear thymidine kinase gene; ΦX174, bacteriophage ΦX174 (relaxed, replicative form)

DNA showed strong linearity over a 6-log dynamic detection range, with correlation coefficients (*R*²) equaling 0.9998, 0.9977, and 0.9998, respectively. Although the extreme limits that are possible with this assay were not assessed, the analytical sensitivity limit of the real-time PCR assay was demonstrated to be at least 83 molecules per PCR reaction.

Validation of the ΔΔCt method

The relative quantities of the mitochondrial D-loop, the nuclear *Tk* gene, and ΦX174 DNA in our tissue digest were compared

using the comparative Ct method (ΔΔCt method). The integrated sequence detection system software, SDS software v1.1 (Applied Biosystems), uses the ΔΔCt method to determine the quantity of the target sequence for each DNA extraction. Using the formula 2^{-ΔΔCt}, the quantity of the target gene (normalized to the control reference and relative to a calibrator) was calculated. (1) (<http://docs.appliedbiosystems.com/pebi/docs/04303859.pdf>). For this method to be valid, the efficiency of amplification for each primer and probe set must be approximately equal.

The overall efficiency of PCR for each primer and probe set was calculated from

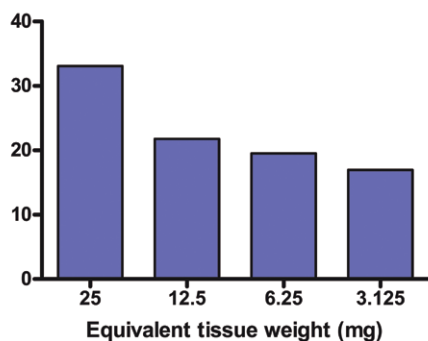
the linear regression of the standard curve plotting Ct versus the logarithm of the starting molecules (Figure S1) and using the following equation: $E = 10^{(-1/slope)}$. The linear regression from the D-loop, *Tk*, and ΦX174 DNA serial dilution plots reveal slopes of -3.50007, -3.5484, and -3.5719, respectively. The corresponding amplification efficiencies (93.04%, 91.35%, and 90.53%) were in the optimal range.

Before the ΔΔCt method for relative quantitation could be used, the similarity of the D-loop, *Tk*, and ΦX174 DNA PCR efficiencies had to be confirmed to be within an acceptable range. To confirm this, the average Ct values generated from equivalent standard curve mass points were used to calculate the ΔCt of each point for each amplicon comparison (i.e., D-loop versus *Tk*, D-loop versus ΦX174 DNA, and *Tk* versus ΦX174 DNA). The ΔCt values were plotted versus the log of molecules (starting amount) to create a semi-log regression line for each comparison (graph not shown). For the amplification efficiencies to be considered equal, the slope of each regression line must be < 0.1. The slope of the line for the ΔCt of D-loop and *Tk* was 0.0477, D-loop and ΦX174 DNA was 0.0711, and *Tk* and ΦX174 DNA was 0.0234. The determined slope for each falls below the maximum slope value of 0.1, thus validating the use of the ΔΔCt calculation for relative quantitation.

Preliminary studies on DNA recovery

Studies using a silica-based DNA purification kit

Genomic DNA recovery from animal tissue. Total genomic DNA recovery efficiency was assessed for the silica-based column DNeasy Blood and Tissue Kit (Qiagen). Four 25-mg pieces of liver from an adult B6C3F₁ mouse were digested fully overnight in a rotating incubator with 220, 440, 880, and 1760 μL digestion solution [1× ATL lysis buffer (Qiagen) and proteinase K enzyme (600 mAU/mL solution; Qiagen)]. This gave a 2-fold dilution series of the liver digest (referred to in the following experiments as 25-mg, 12.5-mg, 6.25-mg, and 3.125-mg tissue weights). These tissue weights are within the range suggested by the manufacturer. The samples were heated at 95°C for 15 min to heat-inactivate the proteinase K. Doubling volumes of AL buffer were added to the corresponding digests and incubated at 70°C for 10 min. Four hundred microliters of each digest was removed and 200 μL 100% EtOH was added to each sample,



Supplementary Figure 2. Genomic DNA recovery from animal tissue using a silica-based DNA purification kit. Input tissue weight affects the efficiency of DNA recovery. 25-, 12.5-, 6.25-, and 3.125-mg tissue samples yielded 1.32, 1.74, 3.12, and 5.42 µg DNA per mg tissue, respectively.

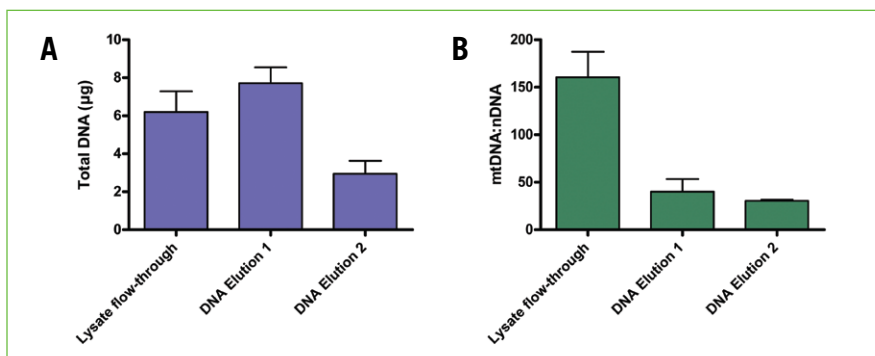
then loaded onto the column. The following steps in the DNA isolation were carried out according to manufacturer’s protocol and the DNA was eluted with 200 µL of the supplied elution buffer. DNA concentrations were measured for each sample using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

The efficiency of DNA recovery from liver using the DNeasy Blood and Tissue Kit is shown in Figure S2. It would be expected (if extraction efficiency was 100% and input tissue weight had no effect) that DNA yield would reduce by approximately half for each half-reduction of tissue weight, and that the DNA yield per mg of tissue would remain constant. However, it appears the input tissue weight markedly impacted the efficiency of DNA recovery: 25 mg tissue yielded 1.32 µg DNA per mg tissue, and 3.125 mg tissue yielded 5.42 µg DNA per mg tissue. Arbitrarily setting the 3.125 mg tissue sample to 100% DNA extraction efficiency, the 6.25, 12.5, and 25 mg tissue sample DNA extraction efficiencies can be calculated to be 57.62%, 32.15%, and 24.41%, respectively.

Relative mtDNA:nDNA recovery from animal cells. To assess mtDNA:nDNA recovery using the silica-based column DNeasy Blood and Tissue Kit (Qiagen), genomic DNA was extracted from three mouse 3T3 cell pellets (2 × 10⁶ cells/pellet) following the manufacturer’s protocol for cultured cells including treatment with RNase A. DNA was eluted twice with 100 µL supplied elution buffer. In addition, the cell lysate flow-through was collected for DNA precipitation with 100% EtOH. DNA concentrations of the lysate flow-through and DNA elutions 1 and 2 were measured using a NanoDrop ND-1000 spectrophotometer (Figure

Supplementary Table 2. DNA recovery from mouse tissues using an organic solvent extraction

Tissue	Equivalent tissue weight	DNA extracted µg (µg/mg)	Extraction efficiency (%)
Muscle	3.125 mg	1.80 (0.58)	100
	6.25 mg	2.61 (0.42)	72.64
	12.5 mg	4.82 (0.39)	66.99
	25 mg	7.14 (0.29)	49.61
Brain	3.125 mg	2.36 (0.76)	100
	6.25 mg	4.6 (0.74)	97.37
	12.5 mg	9.42 (0.75)	99.55
	25 mg	23.28 (0.93)	123.08
Heart	3.125 mg	5.17 (1.65)	100
	6.25 mg	9.86 (1.58)	95.46
	12.5 mg	16.82 (1.35)	81.39
	25 mg	34.81 (1.39)	84.22
Liver	3.125 mg	3.28 (1.05)	100
	6.25 mg	10.80 (1.73)	164.46
	12.5 mg	45.85 (3.67)	348.96
	25 mg	62.53 (2.50)	237.95



Supplementary Figure 3. Genomic DNA and relative mtDNA:nDNA recovery from animal cells using a silica-based DNA purification kit. (A) Total DNA measured in the lysate flow-through, DNA elution 1, and DNA elution 2 samples from three 3T3 cell pellets. (B) Q-PCR comparing relative mtDNA:nDNA recovered in the lysate flow-through, DNA elution 1, and DNA elution 2 demonstrates the disproportional loss of mtDNA in the discarded lysate flow-through.

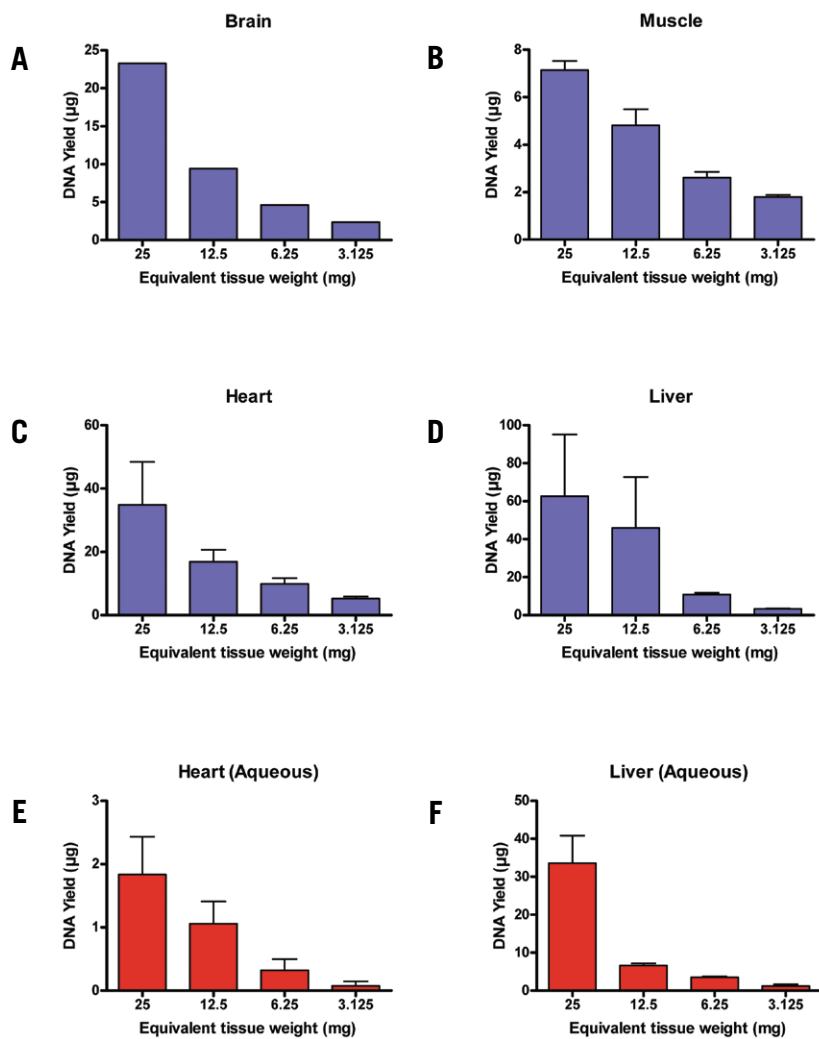
S3A). A substantial quantity of DNA was lost in the lysate flow-through (~80% of that found in Elution 1). In addition, the coefficient of variation (CV) for the measured DNA concentrations of the lysate flow-through, DNA elution 1, and DNA elution 2 were 30.60%, 18.80%, and 39.99%, respectively.

Q-PCR results indicate that a significant and disproportional loss of mtDNA is evident in the lysate flow-through (Figure S3B). The mtDNA:nDNA ratios for the lysate flow-through, DNA elution 1, and DNA elution 2 samples were measured using primers and TaqMan MGB probes for the mouse mitochondrial D-loop and nuclear *Tk* gene. In addition, the variation of mtDNA:nDNA ratio in the lysate flow-through and DNA elution 1 was 28.84% and 57.69%, respectively. The variance of

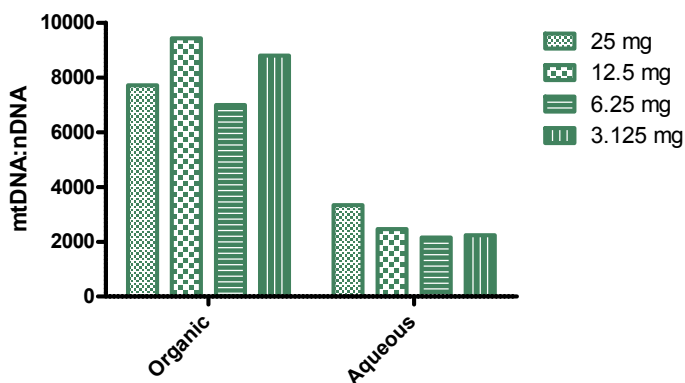
the mtDNA:nDNA ratio in the DNA elution 1 samples appear to result from the variable loss of mtDNA in the lysate flow-through.

Studies using organic solvent extraction

Genomic DNA recovery from animal tissues. Genomic DNA recovery and mtDNA:nDNA ratio were assessed for the organic solvent TRI Reagent DNA extraction (guanidinium thiocyanate-phenol-chloroform extraction) (Sigma-Aldrich, St. Louis, MO, USA). Four 25-mg skeletal muscle, heart, brain and liver samples from an adult B6C3F₁ mouse were digested overnight with doubling volumes of digestion solution. As explained above, this gave digests with the equivalent of 25-mg, 12.5-mg, 6.25-mg, and 3.125-mg



Supplementary Figure 4. Genomic DNA recovery from animal tissues using organic solvent extraction. Total genomic DNA was extracted from mouse (A) brain, (B) muscle, (C) heart, and (D) liver. DNA extraction yield and efficiency varies with the type of tissue used. DNA extracted from the aqueous phase from (E) heart and (F) liver indicate loss of DNA in the discarded phase. DNA was undetectable in the aqueous phase from the brain and muscle tissues (data not shown).



Supplementary Figure 5. Relative mtDNA:nDNA recovery from animal tissue using organic solvent extraction. Q-PCR comparing relative mtDNA:nDNA recovered in the organic and aqueous phase from brain tissue. Variable mtDNA:nDNA ratios are evident in the organic/interphase extraction. Minor loss of mtDNA occurs in the discarded aqueous phase.

tissue weights. The skeletal muscle, liver, and heart extractions were performed twice on separate days. After overnight digestion in a rotating incubator at 55°C, the sample was heated at 95°C for 15 min to heat-inactivate the proteinase K. DNA extraction from the tissue digests was performed using 150 µL tissue digest and 450 µL TRI Reagent (Sigma-Aldrich). After phase separation, the aqueous phase was removed and placed in a new 1.5-mL tube. Genomic DNA was extracted from both the organic/interphase and aqueous phase following the manufacturer's protocol with the addition of a 0.1M sodium citrate/10% EtOH wash and overnight storage in 75% EtOH.

DNA concentrations were measured for the aqueous- and organic-phase DNA extractions for each tissue using a NanoDrop ND-1000 spectrophotometer. The efficiencies of DNA recovery from the mouse skeletal muscle, brain, heart, and liver tissue [using TRI Reagent (Sigma-Aldrich) organic solvent extraction on tissue digests] are shown in Figure S4, A–D and Table S2. As explained for the silica-based extractions above, if extraction efficiency was 100% and input tissue weight had no effect, the yield of DNA should be reduced by approximately half for each half-reduction of tissue weight, and the DNA yield per mg of tissue would remain constant. As shown, the DNA extraction yield and efficiency varies with the type of tissue used. In our experiment, the brain and heart yielded relatively consistent quantities of DNA regardless of input tissue weight (Figure S4, A and C; Table S2). DNA was below the limit of detection in the aqueous phase for the brain tissue. In the case of heart tissue, small quantities of DNA were extracted from the aqueous phase, indicating some loss (<2 µg for 25 mg tissue) (Figure S4E). Skeletal muscle tissue extraction efficiency appears to be moderately influenced by input tissue weight. Arbitrarily setting the 3.125 mg tissue to 100% DNA extraction efficiency, a 25-mg skeletal muscle sample yields 7.14 µg DNA per mg tissue, corresponding to ~50% efficiency (Figure S4B and Table S2). DNA was below the limit of detection in the aqueous phase for the skeletal muscle tissue. In contrast with the brain, heart, and skeletal muscle, when DNA was extracted from the liver, the efficiency of extraction increased with increasing tissue weight (Figure S4D and Table S2). This also is in direct contrast to that observed with extraction of DNA from liver tissue using the silica-based column extraction kit. Not only did the DNA extraction from liver tissue behave differently from

the other tissues examined, but also, there was considerable DNA loss in the aqueous phase (Figure S4F). For 25 mg liver tissue, >30 μg DNA was found in the aqueous phase (~10% loss), compared with <2 μg for the equivalent amount of heart tissue, and non-detectable DNA in the aqueous phase from skeletal muscle and brain.

Relative mtDNA:nDNA recovery from animal tissue. The mtDNA:nDNA ratios for the brain organic- and aqueous-phase DNA extractions were measured using primers and TaqMan MGB probes for the mouse mitochondrial D-loop and nuclear *Tk* gene. It would be expected (if extraction efficiency for both mtDNA and nDNA were 100% and input tissue weight had no effect) that the mtDNA:nDNA ratio should remain constant for each half-reduction of tissue weight. Q-PCR results indicate an inconsistent yield of mtDNA and nDNA extracted in the organic/interphase portion with varying tissue weights (Figure S5). Although the CV of the mtDNA:nDNA ratio between all four extractions was only 13.2%, comparing the mtDNA:nDNA ratio for the 12.5-mg and 6.125-mg tissue samples demonstrates a 30% difference between the two extractions. This variation in the mtDNA:nDNA extracted may compromise accurate quantitation of mtDNA. Q-PCR on the aqueous phase indicated that a small amount of mtDNA and nDNA were amplified, even though the DNA levels were below the detection limits of the NanoDrop device. Compared with the silica-based column kit, where significant and disproportional loss of mtDNA occurred in the discarded lysate flow-through, the organic solvent extraction shows a relatively minor loss of mtDNA in the discarded aqueous phase.

References

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