Benchmarks

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

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Quantitative real-time PCR has become a popular method to analyze and quantify changes in the copy number of mitochondrial DNA (mtDNA), and nuclear DNA (nDNA) is often used as an endogenous reference for mtDNA abundance. In our experience, using nDNA as a reference is problematic, due to differences in the extraction efficiency of nDNA and mtDNA and variation in the ploidy of experimental samples. Here, we report that the ratio of mtDNA to nDNA varies in repeated DNA extractions but that Φ X174 DNA, added before DNA extraction, is extracted with a similar efficiency to mtDNA, making it a suitable alternative reference for quantifying mtDNA copy number.

Changes in mitochondrial DNA (mtDNA) copy number have been related to mitochondrial disease and other pathologies, such as those associated with the use of nucleoside reverse transcriptase inhibitors (1–3). Quantitation of mtDNA is often accomplished by quantitative real-time PCR (Q-PCR), using nuclear DNA (nDNA) as an endogenous reference for normalization (4–7). Our experience, with both nonorganic and organic solvent-based systems, indicates that consistent, quantitative recovery of nDNA and mtDNA for Q-PCR is problematic, possibly because the two DNA species differ greatly in molecular weight, base composition, and quantity. Furthermore, many of the tissues of interest for analysis are polyploid (e.g., liver, muscle), and age, pathological conditions (such as those that produce oxidative damage), and chemical exposure can result in changes in ploidy (8–10). If the tissues of interest have had a change in their nuclear ploidy related to the experimental variable, this could compromise the accuracy of the mtDNA copy number estimate.

In preliminary studies, we found that the choice of genomic DNA extraction method can influence mtDNA recovery and poten-

tially impact Q-PCR results. For example, with a silica-based column DNA purification kit, we have observed the disproportional loss of mtDNA relative to nDNA in the discarded lysate flow-through (see Supplementary Material). DNA loss was not as great using an organic solvent DNA extraction. In fact, while our manuscript was in review, an advanced online publication by Guo et al. (11) described problems with DNA recovery similar to what we had observed. Comparing organic solvent extraction and two silica-based column DNA purification kits, they found that the extraction method had a major influence on the yield of DNA and whether or not it was possible to detect experimentally induced differences in mtDNA copy number.

Here we report that, even when a relatively efficient organic DNA extraction method is used, the ratio of mtDNA to nDNA varies in repeated extractions, thus compromising accurate mtDNA copy number estimates when nDNA is used as the endogenous control reference. We have developed a method for mtDNA quantitation by Q-PCR that uses Φ X174 DNA as an exogenous reference. Like mtDNA, Φ X174 DNA (replicative form) is a relatively

small (~5.3 kb), double-stranded, circular DNA molecule, and likely to be recovered with a similar efficiency to mtDNA by DNA extraction.

The following experiment serves to illustrate that Φ X174 DNA is a more suitable reference molecule than nDNA for quantifying mtDNA copy-number. A small piece (~15 mg) of skeletal muscle was excised from the hind leg of an adult B6C3F₁ mouse, transferred to a 1.5-mL microcentrifuge tube, and weighed. A freshly made digestion solution was then added at a ratio of 350 μ L per 15 mg of tissue; the digestion solution consisted of 1 $\times$ ATL lysis buffer (Qiagen, Valencia, CA, USA), proteinase K enzyme (600 mAU/mL solution; Qiagen), and 1 μ g/mL Φ X174 (RF II) DNA (New England BioLabs, Ipswich, MA), prepared at a ratio of 36:6:5 (v:v:v). 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. Four (total) DNA extractions from the single tissue digest were performed concurrently using 150 μ L tissue digest and 450 μ L TRI Reagent LS (Sigma-Aldrich, St. Louis, MO, USA). Further steps in the DNA extraction were as described by the manufacturer. All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the National Center for Toxicological Research (Jefferson, AR, USA).

A calibrator sample was run on every plate used for Q-PCR analysis. This allowed all the samples to be expressed as an *n*-fold difference relative to the calibrator. The calibrator, which is fully described in the Supplementary Material, consisted of an equal number of molecules of three DNA types: plasmid DNA containing the mtDNA displacement loop (D-loop) region, plasmid DNA containing a portion of the nDNA thymidine kinase (*Tk*) gene, and Φ X174 DNA. Q-PCR was conducted with an ABI 7000 (Applied Biosystems, Foster City, CA, USA) in duplicate reactions using primers and TaqMan MGB probes designed for the mouse mtDNA D-loop, *Tk* nDNA, and for Φ X174 DNA (see Supplementary Material). The PCR amplification efficiency for each target sequence was determined to be within the acceptable range as described previously (<http://docs.appliedbiosystems.com/pebi/docs/04303859.pdf>) (12) (see Supplementary Material). Relative DNA amount was estimated using SDS software v1.1 (Applied Biosystems) and the comparative Ct method ($\Delta\Delta$ Ct method) (12).

Table 1 shows the relative mtDNA copy number for the four extractions using the D-loop as the mtDNA target and nDNA

Figure 1. Q-PCR results for D-loop and ΦX174 DNA normalized to the nuclear *Tk* gene. Shown are the relative quantities of D-loop and ΦX174 DNA in each extraction when normalized to *Tk* and relative to the calibrator sample. Each sample was run in duplicate reactions and averaged. mtDNA, mitochondrial DNA; D-loop, displacement loop of mtDNA; *Tk*, nuclear thymidine kinase gene; ΦX174, bacteriophage ΦX174 (relaxed, replicative form).

(*Tk*) as the reference. The Q-PCR analysis estimated up to a 9-fold difference in the mtDNA copy number between the four extractions, with a coefficient of variation (CV) of almost 75%.

To determine the source of the variation in the mtDNA copy number, the averaged Ct value of the D-loop and *Tk* amplifications for each DNA extraction were compared. The CV for the *Tk* amplification using the four extractions was only 4.6%, whereas the CV for the D-loop was 77% (Table 1). This indicates that mtDNA does not extract at the same efficiency as nDNA and its quantity varies in replicate DNA extractions, whereas the extraction efficiency for nDNA remains relatively constant.

Table 1 also shows the relative quantity of ΦX174 DNA for each DNA extraction

using nDNA (*Tk*) as the reference. As with the mtDNA D-loop, up to a 9-fold difference in ΦX174 DNA quantity was observed among the four DNA extractions and the CV was ~75%. As stated in the preceding paragraph, the CV for the *Tk* amplification between each extraction was only 4.6%. The variation of the ΦX174 DNA, however, was 77%, the same as previously determined for the D-loop. This result demonstrates that the quantity of ΦX174 DNA also varied among extractions of the tissue digest.

Figure 1 depicts the D-loop and ΦX174 DNA quantity calculated for each extraction, normalized to *Tk* and relative to the calibrator. For each DNA extraction, the amounts of mtDNA and ΦX174 DNA remain in proportion to each other, demonstrating that the mtDNA and

ΦX174 DNA behave in a similar fashion during the DNA extraction process. The proportional extraction of mtDNA and ΦX174 DNA was also observed for mouse heart, brain, and liver (data not shown).

Finally, Table 1 shows the relative quantity of the mtDNA for each DNA extraction when compared with ΦX174 DNA as an exogenous reference. Using the D-loop as the mtDNA target and ΦX174 DNA as the reference, the mtDNA copy number varied by a factor of only 1/15 between the DNA extractions. Comparing the variation in mtDNA quantity determined by normalization to *Tk* and ΦX174 DNA, the CV was 74.74% when using *Tk* as the reference, whereas the variation was only 5.92% using ΦX174 DNA as the reference.

For analysis of relative mtDNA quantity in experimental cell cultures and animal tissues, ΦX174 DNA should be introduced early in the DNA extraction process at a known quantity relative to the tissue weight. A simple way to do this is to incorporate ΦX174 DNA into the tissue digestion solution at a fixed concentration and adjust the volume of digestion solution per tissue weight (e.g., 350 μL per 15 mg tissue), as we did in our experiment. The adjusted volume of the tissue digestion mix ensures two things. First, it eliminates any variation in the digestion efficiency due to different volumes of digestion solution per weight of tissue. Second, and most important, adjusting the volume of the tissue digestion solution per tissue weight allows ΦX174 DNA to be added proportionately to each tissue and further used as the reference in determining the mtDNA copy number for the tissues. Using ΦX174 DNA eliminates the need to use

Table 1. Q-PCR results for the mitochondrial D-loop, the nuclear *Tk* gene, and ΦX174 DNA

Sample	D-loop C _t (avg)	<i>Tk</i> C _t (avg)	ΦX174 C _t (avg)	Relative mtDNA quantity ^b normalized to <i>Tk</i>	Relative ΦX174 DNA quantity ^b normalized to <i>Tk</i>	Relative mtDNA quantity ^b normalized to ΦX174
Ext. #1	20.212	26.332	21.270	145.21	77.23	1.88
Ext. #2	21.474	26.377	22.566	62.47	32.45	1.93
Ext. #3	21.427	26.362	22.394	63.87	36.18	1.77
Ext. #4	23.553	26.487	24.727	15.96	7.83	2.04
Calibrator ^a	30.237	29.175	30.384	1	1	1
CV (%)	77.08 ^c	4.6 ^c	77.11 ^c	74.74	74.87	5.92

^aA calibrator sample was run on every Q-PCR plate. The calibrator acted as the basis sample to which all other samples were compared. This allows all the samples to be expressed as an *n*-fold difference relative to the calibrator. To generate the calibrator sample, we used plasmid DNA preparations containing the mitochondrial D-loop region, the nuclear *Tk* region, and ΦX174 DNA. An approximately equivalent number of molecules (~2 × 10⁹) of all three were combined with water in a total volume of 50 mL. Aliquots of 20, 30, and 40 μL were stored as single-use reagents in 0.5-mL microcentrifuge tubes at -20°C. ^bThe comparative Ct method (ΔΔCt method) was used to determine the relative quantity of our target DNA (<http://docs.appliedbiosystems.com/pebi/docs/04303859.pdf>) (12). The ΔΔCt method uses an arithmetic formula, where the amount of target, normalized to a reference and relative to a calibrator, is given by 2^{-ΔΔCt}. The Ct values were determined for the replicate D-loop, ΦX174 DNA, and *Tk* reactions for each DNA extraction and the calibrator sample, and then averaged. The averaged Ct of the reference was subtracted from the averaged Ct of the target for each DNA extraction and the calibrator, giving the ΔCt for each. To derive the relative target quantity, the ΔCt of the calibrator was subtracted from the ΔCt of each extraction to obtain the ΔΔCt value. The ΔΔCt value was then used as the exponent of the base 2 in the formula 2^{-ΔΔCt}. ^cCV was calculated from linear transformed Ct values. Ct (logarithmic value) was converted to a linear value by using the formula 2^{-Ct} (12,13). Ct, 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).

the nuclear genome as a reference, thus avoiding inaccuracies in the quantitation of mtDNA due to differences in DNA extraction efficiencies or differences in the ploidy between tissues.

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References

- Moraes, C.T., S. Shanske, H.J. Tritschler, J.R. Aprille, F. Andreetta, E. Bonilla, E.A. Schon, and S. DiMauro. 1991. mtDNA depletion with variable tissue expression: a novel genetic abnormality in mitochondrial diseases. *Am. J. Hum. Genet.* 48:492-501.
- Arnaudo, E., M. Dalakas, S. Shanske, C.T. Moraes, S. DiMauro, and E.A. Schon. 1991. Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy. *Lancet* 337:508-510.
- Lewis, W., B.J. Day, and W.C. Copeland. 2003. Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. *Nat. Rev. Drug Discov.* 2:812-822.
- Chiappini, F., E. Teicher, R. Saffroy, P. Pham, B. Falissard, A. Barrier, S. Chevalier, B. Debuire, et al. 2004. Prospective evaluation of blood concentration of mitochondrial DNA as a marker of toxicity in 157 consecutively recruited untreated or HAART-treated HIV-positive patients. *Lab. Invest.* 84:908-914.
- Cote, H.C., Z.L. Brumme, K.J. Craib, C.S. Alexander, B. Wynhoven, L. Ting, H. Wong, M. Harris, et al. 2002. Changes in mitochondrial DNA as a marker of nucleoside toxicity in HIV-infected patients. *N. Engl. J. Med.* 346:811-820.
- Miller, F.J., F.L. Rosenfeldt, C. Zhang, A.W. Linnane, and P. Nagley. 2003. Precise determination of mitochondrial DNA copy number in human skeletal and cardiac muscle by a PCR-based assay: lack of change of copy number with age. *Nucleic Acids Res.* 31:e61.
- Biesecker, G., S. Karimi, J. Desjardins, D. Meyer, B. Abbott, R. Bendele, and F. Richardson. 2003. Evaluation of mitochondrial DNA content and enzyme levels in tenofovir DF-treated rats, rhesus monkeys and woodchucks. *Antiviral Res.* 58:217-225.
- Storchova, Z. and D. Pellman. 2004. From polyploidy to aneuploidy, genome instability and cancer. *Nat. Rev. Mol. Cell Biol.* 5:45-54.
- Keighren, M. and J.D. West. 1993. Analysis of cell ploidy in histological sections of mouse tissues by DNA-DNA in situ hybridization with digoxigenin-labelled probes. *Histochem. J.* 25:30-44.
- Gupta, S. 2000. Hepatic polyploidy and liver growth control. *Semin. Cancer Biol.* 10:161-171.
- Guo, W., L. Jiang, S. Bhasin, S.M. Khan, and R.H. Swerdlow. 2009. DNA extraction procedures meaningfully influence qPCR-based mtDNA copy number determination. *Mitochondrion* 9:261-265.
- Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-408.
- Schmittgen, T.D., B.A. Zakrajsek, A.G. Mills, V. Gorn, M.J. Singer, and M.W. Reed. 2000. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal. Biochem.* 285:194-204.

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