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PCR Based Determination of Mitochondrial DNA Copy Number in Multiple Species

JP Rooney[1], **IT Ryde**[1], **LH Sanders**[2], **EH Howlett**[2], **MD Colton**[1], **KE Germ**[3], **GD Mayer**[3], **JT Greenamyre**[2], and **JN Meyer**[1]

[1] Nicholas School of the Environment, Duke University, Durham NC 27708-0328

[2] Pittsburgh Institute for Neurodegenerative Diseases, Department of Neurology, University of Pittsburgh, Pittsburgh, PA 15260

[3] The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX 79401

Summary

Mitochondrial DNA (mtDNA) copy number is a critical component of overall mitochondrial health. In this chapter we describe methods for isolation of both mtDNA and nuclear DNA (nucDNA), and measurement of their respective copy numbers using quantitative PCR. Methods differ depending on the species and cell type of the starting material, and availability of specific PCR reagents.

Keywords

mitochondrial DNA; mtDNA; mtDNA depletion; copy number; QPCR; mitochondrial toxicity; mitochondrial disease

1. Introduction

In humans, the mitochondrial genome (mtDNA) encodes 13 proteins, all of which are components of the electron transport chain (ETC), and are essential for oxidative phosphorylation (OXPHOS) [1]. On average, each cell contains between 10^3 and 10^4 copies of the mitochondrial genome, though this number varies between cell type and developmental stage. mtDNA replication is carried out independent of the cell cycle by the nuclear DNA (nucDNA) encoded polymerase γ, the only DNA polymerase found in the mitochondria [2]. mtDNA replication is regulated, at least in part, by Mitochondrial transcription factor A (Tfam), a component of the mtDNA-protein packaging complex called the nucleoid [3]. Mutations in 9 genes involved in mtDNA replication and nucleotide metabolism cause mitochondrial DNA depletion syndrome (MDS) in humans [4-6]. There are multiple diseases associated with MDS including both Alper's syndrome and progressive external ophthalmoplegia (PEO), as well as other recessive myopathies [4]. mtDNA depletion is also implicated in more common diseases including type 2 diabetes [7], many cancers [8], and neurodegenerative disorders such as Alzheimer and Parkinson Disease [9], though direct causal links have not yet been established. Pharmaceuticals can also block

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mtDNA replication and result in mtDNA depletion, as in the case of the Nucleoside Reverse Transcriptase Inhibitors (NRTIs) used to treat human immune-deficiency virus (HIV). These drugs are nucleoside analogs that block the progression of pol-γ, and can cause mtDNA depletion mediated toxicity [10]. Furthermore, recent work suggests that environmental exposures can alter mtDNA copy number. mtDNA copy number is increased by *in utero* and neonatal exposure to secondhand cigarette smoke in mice [11] and chronic exposure to polycyclic aromatic hydrocarbons in humans [12]. Conversely, exposure to particulate matter during pregnancy reduces mtDNA copy number in placental tissue [13].

This chapter provides protocols for the simultaneous isolation of mitochondrial and nuclear DNA, and measurement of both genome copy numbers from a variety of species. We present three protocols for mtDNA copy number determination. They differ based on availability and optimization of reagents. In *C. elegans*, actual mtDNA copy number per animal can be measured via real time PCR, using a plasmid based mtDNA copy number standard curve [14]. With human samples, real time PCR can also be used, however without a standard curve, such that the measurement of mtDNA copy number is relative to nuclear DNA copy number. A quantitative, non-real time PCR may also be used to measure relative mtDNA copy number in *Drosophila melanogaster*, *Fundulus heteroclitus* (Atlantic killifish), *Fundulus grandis* (Gulf Killifish), *Danio Rerio* (Zebrafish), *Oryzias latipes* (Japanese medaka), Mouse, Rat and Human [15-20]. Real time PCR, species specific primers can be found in Table 1, and non-real time PCR, species specific primers in Table 2.

2. Materials

2.1 DNA Isolation

2.1.1 *C. elegans*, small number

1. Worm Lysis Buffer: Standard 1x PCR Buffer in nuclease-free H_2O, 1ug/mL Proteinase K.
2. Platinum worm pick
3. Thin walled PCR tubes Thermal cycler or heat block
4. Dry ice and/or -80°C freezer

2.1.2 C. elegans , large numbers or animal tissue

1. K medium: 31.5mM KCl, 51mM NaCl in ddH_2O (*C. elegans*)
2. RNAlater Solution (animal tissue)
3. 15 mL conical tubes
4. Liquid nitrogen
5. Dry ice
6. Mortar and pestle (*C. elegans* and tough animal tissue such as muscle)
7. Handheld homogenizer (softer animal tissue such as liver)
8. Qiagen G/20 Genomic Tips Kit

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9. Isopropanol
10. 70% ethanol
11. Glass Pasteur pipets
12. 1.7 mL microcentrifuge tubes
13. 50°C water bath
14. Refrigerated microcentrifuge
15. Tabletop centrifuge with 15mL conical tube buckets

2.1.3 Cultured Cells

1. Either the Qiagen G/20 Genomic Tips Kit and associated buffers (see above), or
2. A QIAcube for automated DNA isolation, with the QIAmp DNA Mini Kit for human samples or the DNeasy Blood and Tissue Kit (Qiagen) for animal samples [12].
3. Pellets of approximately 1×10^6 cells

2.2 DNA Quantification (See Note 1)

1. Pico Green dsDNA quantification reagent
2. Lambda/HindIII DNA standard curve
3. 1x TE buffer: 10mM Tris-HCL pH 8.0, 1mM EDTA
4. Fluorescent plate reader with excitation filter at 480nm and an emission filter at 520nm (485nm and 528nm also work well)
5. Black or white bottom 96 well plate

2.3 Real Time PCR

1. SYBR Green PCR Master Mix
2. Standard 96 well PCR plate with optically clear sealing film
3. Real Time PCR System (ABI 7300)
4. ABI Prism 7300 Sequence Detection Software
5. Primers, species and target genome specific, see table 1
6. Nuclease free H_2O

2.4 Quantitative, Non-Real Time, PCR

1. Standard thermal cycler

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[1]DNA from small scale worm lysis does not need to be quantified prior to PCR, however, quantification is required for purified DNA from large scale preparations.

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2. KAPA Long Range Hot Start DNA Polymerase Kit (KAPABiosystems), (optimized for Human samples, see note 2), or:
3. GoTaq Flexi PCR Kit (Promega), (optimized for *F. grandis* samples, see note 2)
4. 0.2mL PCR tubes
5. PCR hood with germicidal lamp for sterilization
6. Primers, species and target genome specific, see table 2
7. All materials from section 2.2 DNA Quantification
8. 0.1 mg/mL bovine serum albumin in nuclease free H_2O.
9. 10mM dNTPs Mix
10. Nuclease Free H_2O
11. Dedicated pipettes and sterile aerosol pipet tips for QPCR set up
12. Different set of pipettes and regular tips for post-PCR analysis
13. Distinct workstations for setting up and post-PCR analysis (See note 3)

3. Methods

3.1 DNA Isolation

3.1.1 *C. elegans*, small number of worms

1. Using a platinum worm pick, transfer 6 individual, L4 stage or later *C. elegans* into 90μL of 1x worm lysis buffer pre-aliquoted into thin walled PCR tubes (See Notes 4 and 5), and freeze on dry ice (or at -80°C) immediately. If using dry ice, once all samples are picked transfer to -80°C for at least 10 minutes. This is usually done in triplicate for each sample and data are averaged (See Note 6).
2. Thaw samples, vortex briefly and spin to collect contents at the bottom of the tube. In a standard thermal cycler or heat block, heat to 65°C for 1 hour, followed by 95°C for 15 minutes, and then hold at 8°C. This crude worm lysate will be used as template DNA for the real time PCR reactions and **does not need to be quantified**.

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[2]The GeneAmp XL PCR kit from Applied Biosystems that was previously used for the QPCR assay has been discontinued. We have now optimized the QPCR assay using KAPA LongRange Hot Start DNA Polymerase with Human samples, and the GoTaq Flexi PCR kit with *F. grandis* samples. We expect these protocols to be easily adaptable to the other species that we provide primers for. It is also likely that other PCR kits will work with this protocol, though some optimization may be required (discussed in further detail in Section 3.4 and note 17).

[3]Maintaining distinct pre- and post-PCR workstations and pipettes helps to reduce the possibility of contamination of the pre-PCR workstation, and therefore new PCR reactions, with PCR product. This is especially important when the same PCR target will be amplified repeatedly from many different samples, as is often the case with experiments that use these protocols. We use a PCR hood equipped with a UV sterilizing lamp for reaction assembly, and completed reactions are never opened in this room. This is not a concern with real time PCR as samples are not processed post-PCR.

[4]6 worms in 90μL is the standard condition we routinely use, however different numbers of worms can be used. For worms at or past the L4 stage, we use a ratio of 1 worm per 15μL lysis buffer, and for younger worms we pick 1 worm per 10μL buffer.

[5]We culture worms on K-media plates, as opposed to the standard NGM, because K-media supports thinner bacterial lawns, therefore resulting in less transfer of bacteria when picking worms for PCR.

[6]Samples are picked in triplicate and each sample is amplified in triplicate, resulting in 9 individual real time PCR reactions per data point. For example, if comparing ethidium bromide treated worms with controls, we would pick 3 tubes, each with 6 worms in 90μL lysis buffer, from the treated group (EtBr 1, 2 and 3) and the same from the control group (C1, 2 and 3). After lysis, each of these samples would be amplified via real time PCR in triplicate, resulting in 9 PCR reactions for each treatment group.

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This lysate can also be used for the non-real time quantitative PCR, if real time PCR is not available.

3.1.2 *C. elegans*, large number of worms [15] or animal tissue (skip steps 1 and 2)

1. Wash worms off of bacterial plate with K medium into a 15ml conical tube, pellet at 2200xG for 2 minutes, remove medium and resuspend in 10ml fresh medium. Gently rock tubes for 20 minutes to allow worms to clear gut contents. Pellet at 2200xG for 2 minutes and wash 2x with fresh medium.
2. Resuspend worm pellet in a small volume of medium (residual medium left after wash) with a glass pipette and drip worm suspension directly into liquid nitrogen.
3. Frozen worm pellets can be stored at -80°C indefinitely, animal tissue can be stored at -80°C in RNAlater. As a guide, roughly 10-15mg of *F. grandis* liver tissue is sufficient for DNA isolation.
4. Grind frozen worm pellets or tough tissue samples to a fine powder in a liquid nitrogen cooled mortar and pestle (see note 7). A squeaking sound is heard when worms are sufficiently ground. Alternatively, if the tissue is not tough (i.e. liver tissue) it can be manually homogenized in pre-aliquoted buffer G2 with RNAse A.
5. Scoop the powder into pre-aliquoted buffer G2 with RNAse A, as per the Qiagen 20/G Genomic Tips Handbook tissue protocol.
6. Follow the Qiagen 20/G Genomic Tips Tissue protocol for DNA isolation.

3.1.3 Cell culture samples [15]

1. Standard DNA isolation methods can be used. We routinely use the Qiagen 20/G Genomic Tips Kit or, for automated DNA isolation, the QIAcube with the QIAamp DNA Mini kit or DNeasy Blood and Tissue Kit can be used (see note 8).

3.2 DNA Quantification [15]

1. DNA from large scale worm preparations, cultured cells or animal tissue needs to be quantified prior to real time or quantitative non-real time PCR. DNA from the small-scale worm lysis protocol does not.
2. Prepare a DNA concentration standard curve by diluting Lambda/HindIII DNA to 150ng/μL, 100ng/μL, 50ng/μL, 25ng/μL, 12.5ng/μL and 0ng/μL in TE buffer (See note 9).
3. Dilute DNA samples 1:10 in 1x TE buffer (See note 10).

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[7]When grinding frozen worm pellets, we typically pack the outside of the mortar with dry ice, and then chill the mortar and pestle with a small amount of liquid nitrogen. Once the liquid nitrogen has boiled off the worm pellets are added to the mortar and ground. Care should be taken in the initial few “grinds” as the larger pellets have a tendency to “jump” out of the mortar.

[8]We routinely linearize the Human mitochondrial DNA from QIAcube preparations by digesting with the PvuII restriction enzyme prior to analysis [15]. This is done not for the short product copy number PCR, but to optimize a long range PCR for DNA damage detection. However, we measure copy number on these linearized templates. While we do not expect this digestion to be necessary for copy number analysis, this has not been exhaustively tested.

[9]We prepare larger stocks of the standard curve dilutions that are routinely re-used, to reduce variability. Store at 4°C.

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4. Add 5μL of DNA and 95μL of 1x TE buffer into 2 duplicate wells of a black or white 96 well plate (suitable for fluorescence measurements).
5. Add 5μL of each Lambda/HindIII standard and 95μL 1x TE into 2 duplicate wells.
6. The following 3 steps should be done in low light conditions (See note 11). Prepare Pico Green working solution (100μL of working solution needed per well) by adding 5μL Pico Green reagent per 1ml TE buffer (See Note 12).
7. Add 100μl Pico Green working solution to each well and incubate at room temperature in the dark for 10 minutes.
8. Measure the fluorescence of each sample with excitation at 480nm and emission at 520nm.
9. Determine DNA sample concentrations by comparing fluorescence values to those of the standard curve. If the DNA concentrations are far above the range of the curve, re-dilute the DNA and measure again (see note 13).
10. Dilute the sample to 3ng/μL in TE buffer.

3.3 Real Time PCR

3.3.1 *C. elegans* samples with standard curve

1. Prepare the standard curve (see note 14) as follows: Thaw an aliquot of the mtDNA copy number standard curve plasmid [14] (50,000 copies/μL), and dilute to 32,000 copies/μL. Serially dilute 1:1 down to 4,000 copies/μL. Add 2μL of each dilution and a 0 copies/μL control (TE buffer or water) to separate wells in the 96 well PCR plate to be used in the copy number PCR. A typical standard curve contains 64,000, 32,000, 16,000, 8,000, 4,000, and 0 copies per well.
2. Proceed with real time PCR setup as for all other samples.

3.3.2 All other real time PCR samples, without standard curve [21]

1. Assemble the PCR reactions as follows: combine 2μL of template DNA (standard curve dilution, worm lysate, or 3ng/μL isolated DNA), 2μL of mtDNA target specific primer pair (400nM final concentration each, see Note 15), 12.5μL SYBR Green PCR Master Mix and 8.5μL H_2O in 1 well of the 96 well PCR plate. Each

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[10] Depending on the number of worms or cells, or the amount of tissue used for DNA extraction this dilution will change. The aim is to get the DNA concentration in the range of the standard curve for an accurate measurement of concentration.

[11] Overhead lab lights are turned off and shades are pulled down. During the 10 minute incubation the plates are covered with aluminum foil or placed in a drawer.

[12] Picogreen working solution should be prepared in excess of what is needed to account for loss during pipetting. We pour the working solution into a reservoir and add 100μL to each well of the plate using an 8-channel micro-pipette, and typically make between 500μL and 1000μL excess per full 96 well plate.

[13] As stated in note 8, the aim is to get the DNA concentrations in the range of the standard curve. If the DNA concentration is too high, further dilute and measure again. If too low, the undiluted samples can be measured.

[14] The standard curve for *C. elegans* copy number allows us to calculate *actual* mtDNA copy number per worm. Most methods can only determine copy number on a per nuclear copy basis. The standard curve plasmid is pCR2.1 with a 75 bp fragment of the nd-1 gene, containing the real time PCR primer target sequence, cloned into the multiple cloning site [14]. Based on the molecular weight of the plasmid (MW=4006) and its concentration, we can accurately determine the number of plasmids (and therefore the number of nd-1 primer targets) per μL. We store this plasmid at a concentration of 100,000 copies per μL in single use aliquots (to avoid freeze thaw cycles) at -20°C. We routinely make duplicate serial dilutions of the standard curve and include both sets of dilutions in our real time PCR reactions to ensure accuracy.

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sample is amplified in triplicate and the data are averaged (see Note 16). Repeat this step in separate wells using nuclear DNA specific primers.

2. When analyzing a large number of samples with the same primer pair, a master mix is made containing the reagents that are common to all reactions (SYBR Green Master Mix, H_2O, and primers) and aliquoted into individual reactions.

3. Cycle in an ABI 7300 Real Time PCR System (or comparable system) as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and annealing temperature (primer specific, table 1) for 60 seconds. A dissociation curve is also calculated for each sample to ensure presence of a single PCR product.

3.3.3 Quantitative (Non-Real Time) PCR

1. For samples from C. elegans, D. melanogaster, F. heteroclitus, F. grandis, O. latipes, D. rerio, Mouse, Rat, and Human, relative mtDNA content can be measured in a quantitative, non-real time, PCR reaction in which the PCR product is quantified after completion of the reaction. Nuclear copy number can also be measured using this protocol, and primers are listed in table 2 for some nuclear targets, however this is not often necessary (See note 17).

2. Extra control reactions are necessary for this protocol. Be sure to include a "50% control" that contains control template DNA (or worm lysate) diluted 1:1 with H_2O or TE buffer prior to being added to the reaction (it is not advised to simply add ½ the volume of the control sample), and a "No Template control" that contains only H_2O or TE buffer in place of template DNA.

3. Initially, it is vital to determine the appropriate cycle number for the reaction (see note 18). Listed in table 2 are approximate cycle numbers that will serve as good starting points for cycle number optimization. The cycle number is correct when the "50% control" reaction results in 40-60% of the PCR product of the undiluted control reaction.

4. Specific reaction conditions for 2 PCR kits are presented below. The first uses the KAPABiosystems LongRange Hot Start PCR kit, and has been optimized with Human samples. The second used the GoTaq Flexi Kit from Promega and has been optimized for *F. grandis* samples (See note 2 for more detailed information).

5. Using the KAPA LongRange Hot Start kit, the reactions are prepared as follows:

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[15]Individual primers stocks (100μM) are kept at -20°C, and working dilutions (25μM) at 4°C. When assembling PCR reactions, working dilutions are mixed 1:1 and further diluted to 5μM each. 2μL of this primer mix is added to each PCR reaction, resulting in final primer concentrations of 400nM each.

[16]As stated in note 4, each sample is analyzed in 3 separate real time PCR reactions, and the results of these 3 reactions are averaged.

[17]In the quantitative, non-real time PCR for all samples other than *C. elegans*, the concentration of total DNA is known. Total DNA concentration is based almost entirely on nuclear DNA, and the same amount of total DNA is added to each PCR reaction. For this reason, we effectively start each PCR reaction with the same number of nuclear DNA copies, and the values obtained for relative mitochondrial DNA content do not need to be normalized prior to comparison between samples.

[18]PCR product is created proportionally to the amount of specific target sequence in the template during the exponential phase of the reaction. Therefore, for quantitative results, the reaction must be stopped while in this exponential phase. Assuring that the "50% control" sample results in 40-60% of the PCR product of undiluted control will also assure that the reaction is in the exponential phase [12, 13].

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a. A master mix is made if several samples are being run simultaneously, which consists of the following components, added in this order:

i. 24.5 µL nuclease-free H_2O (for a final volume of 50µL)

ii. 10 µL of 5X buffer solution (vortex at this stage)

iii. 1 µL of BSA in nuclease-free H_2O (0.1mg/mL stock, 2ng/µL final)

iv. 1 µL of dNTPs (10mM stock, 200µM final)

v. 2.5 µL of each primer working solution (10µM stock, 0.5µM final, except *O. latipes*, 7.5 µM stock)

vi. 3.5 µL of $MgCl_2$ (25mM stock, 1.75mM final)

vii. 0.5µL KAPA LongRange Hot Start DNA Polymerase (2.5U/µL)

viii. Vortex and spin

b. Aliquot the master mix into the appropriate number of tubes, and add 15ng purified DNA (5µL if diluted to 3ng/µL) or 5µL of worm lysate as template to the pre-aliquoted master mix. Also, add 5µL of "50% control" and "No Template control" to the appropriate tubes.

c. The PCR amplification profile is as follows: 94°C for 3 mins, followed by the optimized number of cycles of 94°C for 15sec, annealing temperature (table 2) for 45sec, and 72°C for 45sec. To complete the profile perform a final extension for 10 min at 72°C.

6. Using the GoTaq Flexi Kit, the reactions are assembled as follows:

a. A master mix is made if several samples are being run simultaneously, which consists of the following components, added in this order:

i. 9.5 µL nuclease free water (for a final volume of 25µL).

ii. 5µL of 5x Colorless GoTaq Flexi Buffer (vortex at this stage)

iii. 1µL of PCR Nucleotide mix (10mM stock, 400µM final)

iv. 1µL of each primer solution (10µM stock, 0.4µM final)

v. 1µL $MgCl_2$ (25mM stock, 1mM final)

vi. 0.5µL GoTaq DNA polymerase (5U/µL)

vii. Vortex and spin

b. Aliquot the master mix into the appropriate number of tubes, and add 60ng purified DNA (6µL if diluted to 10ng/µL) as template to the pre-aliquoted master mix.

c. The PCR amplification profile is as follows: 94°C for 2 minutes, followed by the optimized number of cycles of 94°C for 30 sec, annealing temperature (table 2) for 30 sec, and 72°C for 1 minute. To complete the profile perform a final extension for 5 minutes at 72°C.

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7. The primers provided in table 2 have been tested and verified to result in a single, specific, PCR product, however when first optimizing the assay it is recommended to check the specificity of the PCR products on an agarose gel, as conditions may vary slightly based on laboratory equipment and PCR kits used. It is critical to obtain a single product to ensure accurate quantification in step 8.
8. Quantify the resulting PCR products (similar to section 3.2). Add 10μL of each PCR product and 90μL of TE buffer to each of 2 duplicate wells of a white or black 96 well plate. Also, add 10μL of the DNA concentration standard curve (see section 3.2) and 90μL of TE buffer to each of 2 duplicate wells of the same plate.
9. Follow steps 6, 7, and 8 of section 3.2 for fluorescent quantification of DNA. The standards are not used to calculate DNA concentration in this protocol, but are useful to assure that the samples fall within the linear range of the instrument and can be helpful when comparing samples on different plates.

3.5 Data Analysis

3.5.1 *C. elegans* samples, real time PCR with standard curve

1. Obtain mtDNA cycle threshold (C_T) values from the Real Time PCR software, and average the C_T values for the triplicate reactions. If any of the triplicate C_T values vary by more than 0.5 units from the others they should be removed prior to analysis.
2. Obtain the C_T values for the standard curve reactions and perform a logarithmic regression with C_T on the Y-axis and copy number on the X-axis (see note 19).
3. Compare the C_T values of the samples with those of the standard curve to determine the copy number per PCR reaction. We use the following equation:
   a. $e^{[(\text{Sample CT} - \text{slope})/\text{y-intercept}]}$ = mtDNA copy number per reaction
4. Assuming worms were picked at a ratio of 1 worm per 15μL and 2μL were used as template, multiply this number by 7.5 to get mtDNA copy number per worm (See note 20).

3.5.2 H. sapiens samples, real time PCR without standard curve [15]

1. Obtain both mtDNA and nucDNA C_T values from Real Time PCR software, and average the C_T values from triplicate reactions.
2. To determine the mitochondrial DNA content, relative to nuclear DNA use the following equations:
   a. C_T = (nucDNA C_T – mtDNA C_T)
   b. Relative mitochondrial DNA content = 2×2^{CT}

[19] When plotting the standard curve C_T values, assure that all values fall on the regression line. If a single sample is not on the line, it can be removed. Also, visually inspect the C_T values for accuracy. Each time the starting DNA concentration is reduced by half the C_T should increase by 1.

[20] If we pick 6 worms in 90μL of lysis buffer, we assume that 15μL of this lysate is roughly equal to 1 worm, therefore copy number per 15μL = copy number per worm. This calculation can be adjusted based on the ratio of worms to volume of lysis buffer.

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3.5.3 Quantitative, non-real time PCR

1. Obtain the fluorescence values from the plate reader software.
2. Blank subtract - subtract the "No Template Control" values from all other PCR product values (Not standard curve values).
3. Average the values of the duplicate wells.
4. Assure that the "50% control" values fall between 40 and 60% of the undiluted control values.
5. The fluorescence values can be used directly to compare relative mitochondrial DNA content between samples.

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Table 1

Real Time PCR primers and conditions

Species	Genome	Target Gene	Forward Primer Seq 5′-3′	Reverse Primer Seq 5′-3′	Amplicon (bps)	Annealing temperature	Reference
C. elegans	mt	nd-1	AGC GTC ATT TAT TGG GAA GAA GAC	AAG CTT GTG CTA ATC CCA TAA ATG T	75	62	[14]
	nuc	Cox-4	GCC GAC TGG AAG AAC TTG TC	GCG GAG ATC ACC TTC CAG TA	164	62	this chapter
H. sapiens	mt	tRNA-Leu(UUR)	CAC CCA AGA ACA GGG TTT GT	TGG CCA TGG GTA TGT TGT TA	107	62	[21]
	nuc>	B2-microglobulin	TGC TGT CTC CAT GTT TGA TGT ATC T	TCT CTG CTC CCC ACC TCT AAG T	86	62	

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Table 2

Quantitative, non-Real Time PCR primers and conditions

Species	Genome	Forward Primer Seq 5′-3′	Reverse Primer Seq 5′-3′	Amplicon (bps)	Annealing[a] temperature	Cycle[b] Number	Reference
Mouse	mt	CCC AGC TAC TAC CAT CAT TCA AGT	GAT GGT TTG GGA GAT TGG TTG ATG T	117	60	18	[17]
Rat	mt	CCT CCC ATT CAT TAT CGC CGC CCT TGC	GTC TGG GTC TCC TAG TAG GTC TGG GAA	211	60	21	[17]
Human	mt	CCC CAC AAA CCC CAT TAC TAA ACC CA	TTT CAT CAT GCG GAG ATG TTG GAT GG	221	60	18	[17]
D. melanogaster	mt	GCT CCT GAT ATA GCA TTC CCA CGA	CAT GAG CAA TTC CAG CGG ATA AA	151	61	19	[16]
	nuc	CGA GGG ATA CCT GTG AGC AGC TT	GTC ACT TCT TGT GCT GCC ATC GT	152	65	24	
D. rerio	mt	CAA ACA CAA GCC TCG CCT GTT TAC	CAC TGA CTT GAT GGG GGA GAC AGT	198	62	21	[16]
	nuc	ATG GGC TGG GCG ATA AAA TTG G	ACA TGT GCA TGT CGC TCC CAA A	233	60	27	
C. elegans	mt	CAC ACC GGT GAG GTC TTT GGT TC	TGT CCT CAA GGC TAC CAC CTT CTT CA	195	63	18	[16, 18]
	nuc	TCC CGT CTA TTG CAG GTC TTT CCA	GAC GCG CAC GAT ATC	225	63	23	[18, 19]

Species	Genome	Forward Primer Seq 5′-3′	Reverse Primer Seq 5′-3′	Amplicon (bps)	Annealing[a] temperature	Cycle[b] Number	Reference
			TCG ATT TTC				
O. latipes	mt	AAC TCC AAG TAG CAG CTA TGC AC	GAG GGG TAG AAG GCT TAC AAA AA	184	59	22	this chapter
	nuc	CTC ACA AAC ATC TTT GCA CTC AG	AGA ACC TCT CTC CAA AAC ATT CC	140	57	26	
F. grandis	mt	TTT ACA CAT GCA AGT ATC CG	CCG AAG GCT ATC AAC TTG AG	206	55	25	this chapter
	nuc	GCC GCT GCC TTC ATT GCT GT	ATG AGC TGG GTG TGC GCT GA	234	62	25	
F. heteroclitus	mt	ATC TGC ATG GCC AAC GCC TA	GGC GGT GCC AGT TTC CTT TT	264	62	24	[16, 20]
	nuc	GCC GCT GCC TTC ATT GCT GT	ATG AGC TGG GTG TGC GCT GA	234	62	24	

[a]Annealing temperature and cycle number are suggested starting points and may need optimization based on differences in laboratory equipment and PCR kit used.

[b]Annealing temperature and cycle number are suggested starting points and may need optimization based on differences in laboratory equipment and PCR kit used.

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