HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2015 May 06.

Published in final edited form as:

Methods Mol Biol. 2012 ; 920: 111–132. doi:10.1007/978-1-61779-998-3_9.

Analysis of DNA damage and repair in nuclear and mitochondrial DNA of animal cells using quantitative PCR

Amy M. Furda[1], **Amanda Smith Bess**[2], **Joel N. Meyer**[2], and **Bennett Van Houten**[1]

[1]Department of Pharmacology & Chemical Biology, and University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213

[2]Nicholas School of the Environment, Duke University, Durham, NC 27708

Abstract

This chapter was written as a guide to using the long-amplicon quantitative PCR (QPCR) assay for the measurement of DNA damage in mammalian as well as non-mammalian species such as *C. elegans* (nematodes), *Drosophila melanogaster* (fruit flies) and two species of fish (*Fundulus heteroclitus* and *Danio rerio*). Since its development in the early 1990s [1-3], the QPCR assay has been widely used to measure DNA damage and repair kinetics in nuclear and mitochondrial genomes after genotoxin exposure [3-5]. One of the main strengths of the assay is that the labor-intensive and artifact-generating step of mitochondrial isolation is not needed for the accurate measurement of mtDNA copy number and damage. Below we present the advantages and limitations of using QPCR to assay DNA damage in animal cells and provide a detailed protocol of the QPCR assay that integrates its usage in newly developed animal systems.

Keywords

QPCR; DNA damage; DNA repair; mitochondria; mitochondrial DNA; mtDNA damage; mtDNA repair; oxidative stress; nucleotide excision repair; base excision repair

1. Introduction

1.1 Principle of the assay

The idea that many kinds of DNA lesions are able to block DNA polymerase progression is the basis for the QPCR assay [6]. The QPCR assay requires preparing samples with equal amounts of DNA so that the only variable influencing fragment amplification is the number of polymerase-blocking DNA lesions such as single-strand breaks, abasic sites, and bulky adducts [6-8]. In this way, one can compare lesion frequencies of samples based on amplification alone, in which higher amplification corresponds with a lower lesion frequency [1, 9]. By assuming a Poisson distribution of lesions, the amplification of treated samples is compared to amplification of mock treated samples to calculate relative lesion frequency, expressed in lesions per kilobase. As the damage is repaired, amplification is

Corresponding author: Bennett Van Houten, Ph.D.; Hillman Cancer Center, 5117 Centre Avenue Research Pavilion, Suite 2.6, Pittsburgh, PA 15213-1863, UNITED STATES. vanhoutenb@upmc.edu. Telephone: 412-623-7762; Fax: 412-623-7761.

restored and thus, the QPCR assay is useful in measuring the kinetics of DNA repair in both nuclear and mitochondrial genomes after treatment with a DNA damaging agent [2, 3, 10-13]

1.2 Advantages of the assay

Advantages of QPCR in measuring DNA damage and repair include its high sensitivity, its robust function despite low amounts of DNA (nanograms), and its ability to measure DNA damage and repair in specific genes based on primer design. In addition, the DNA extraction procedure for QPCR allows for both nuclear and mitochondrial genomes to be extracted in the same sample without the need for mitochondrial isolation. Because long fragments of DNA (10-15 kb) are amplified, QPCR is highly sensitive to biologically relevant low levels of lesions (approximately 1 lesion per 10^5 bases) [7, 14]. Because QPCR relies on PCR amplification, the assay can be performed on DNA samples containing as little as 1-2 nanograms of genomic DNA, and we have even performed this assay on the equivalent of a single nematode (<1000 cells, and <1 ng DNA) [15]. Thus, the QPCR assay is less demanding on starting material than Southern blots or HPLC, which require at least 10-50 micrograms of DNA for analysis.

Careful adherence to the rules of QPCR primer design (see section 3.5) should allow for any PCR-amplifiable gene or DNA region to be studied with the QPCR technique. In this way, the QPCR assay can allow for a direct comparison of DNA damage sensitivity and repair rate between two genes that are hypothesized to be differentially damaged and repaired. Finally, the DNA extraction procedure allows for the simultaneous extraction of both nuclear and mitochondrial genomes in a single sample and thus, damage sensitivity and repair rates in nuclear and mitochondrial DNA can be compared in one biological sample. In fact, the QPCR assay has been utilized in a wide variety of cells and tissues to compare nuclear DNA (nDNA) damage and repair to mitochondrial DNA (mtDNA) damage and repair after treatment with various genotoxins [16-24].

1.3 Limitations of the assay

The QPCR assay has four main limitations. First, the QPCR assay can only detect lesions that stall or halt DNA polymerase progression, which probably does not include the 8-hydroxydeoxyguanosine (8-OHdG) lesion, one of the most-studied lesions generated during oxidative stress [25]. It is highly unlikely, however, that ROS-producing agents will cause only one type of lesion and in fact it is predicted that 8-OHdG only comprises 10% of H_2O_2-induced DNA damage. Second, although QPCR is able to detect lesions that stall or stop the polymerase, the specific type of lesion cannot be deduced from the QPCR assay alone. An additional concern is that the QPCR assay is not able to detect regio-specific damage in the nDNA that is not within the amplification region of the primer set. Thus, the results can be skewed if the DNA damaging agent specifically targets the amplified region of the genome, or specifically targets a non-amplified region. One study has shown that oxidative lesions seem to occur within promoter regions of specific genes during aging [26]. Of note, limitations similar to the preceding three are common to many other available methods for measuring DNA damage. The last potential issue has only recently been discovered in samples obtained using automated DNA extraction methods (QIAcube), for which it appears

that mtDNA emerges from the extraction mostly in supercoiled form, and we have found it difficult to amplify this DNA due to poor primer access following the denaturing step (supercoiled covalently closed mtDNA rapidly reanneals). However, this issue with mtDNA amplification can be surmounted with use of restriction enzymes to linearize the mtDNA in a region outside of the amplified area. For instance, the HaeII restriction enzyme cuts the mouse mtDNA at a single site outside of the amplification region. The use of restriction enzymes with QIAcube-extracted DNA greatly enhances mtDNA amplification in both mock-treated and treated samples.

1.4 Newer applications of the assay

Historically used in mammalian models or systems (e.g., cell culture), the QPCR assay has recently been adapted to non-mammalian laboratory animals including *Caenorhabditis elegans* [27], *Drosophila melanogaster* [28], *Danio rerio* [28], *Trypanosoma cruzi* [29] and *Oryzias latipes* (Meyer, unpublished). These species complement mammalian models with attributes such as short generation times, large numbers of offspring, transparency of embryos and/or adults, ease and cost-effectiveness of maintenance, and powerful genetic tools. In one case (*C. elegans*), the assay has been further improved to permit measurement of DNA damage in single nematodes comprising between 30 and ~1000 cells [15], greatly reducing the number of animals that need to be cultured and permitting analysis of inter-individual variability in response. The assay has also been adapted to the ecological sentinel species *Fundulus heteroclitus* [24] and *Gambusia holbrooki* (Meyer, unpublished), allowing analysis of DNA damage in wild populations inhabiting contaminated sites. For example, Jung et al. [24, 30] found that a population of *F. heteroclitus* inhabiting a creosote-contaminated estuary exhibited elevated levels of mitochondrial and nuclear DNA damage compared to fish from a reference site. In addition to these new non-mammalian species, the QPCR assay was recently used to determine if exogenously-introduced ligase constructs could complement ligase III function in the repair of oxidatively damaged mtDNA [31], to measure DNA damage in white blood cells from patients suffering from the disease Friedreich's ataxia [21], to elucidate the role of tyrosyl-DNA phosphodiesterase (TDP1) in mtDNA repair [32], and to measure mtDNA damage in models of age-related macular degeneration [33, 34], and cataracts [35].

2. Materials

2.1 DNA sample extraction

2.1.1. Materials for manual method of extraction (Qiagen Genomic-tip protocol)

1. Cell pellets of $1x10^6$ cells/pellet or tissue sample
2. QIAGEN Genomic-tips: 20/G for small pellets and tissues <20 mg (QIAGEN, cat. no. 10223), 100/G for tissue samples <100 mg (QIAGEN, cat. no. 10243)
3. Associated buffers with Genomic-tip kit (QIAGEN, cat. no. 19060)(Buffers G2, QBT, QC and QF, RNase A and Proteinase K)
4. Isopropanol

5. Cold 70% ethanol in H_2O
6. 1X Tris-EDTA, pH 8.0 (Fisher; cat. no. BP2473-500)
7. 15-mL conical tubes (four per sample, BD Falcon, BD Biosciences, Bedford, MA)
8. 2.2 mL microcentrifuge tubes (two per sample)
9. Water bath that can be set to 50°C
10. Refrigerated microcentrifuge (Thermo Scientific)

2.1.2. Materials for automated method of extraction (QIAcube)

1. Cell pellets of $1x10^6$ cells/pellet or tissue sample <25 mg
2. 1X PBS (Cellgro)
3. QIAcube (QIAGEN, cat. no. 9001292)
4. QIAcube materials (pipette tips, 2 mL screw-cap sample tubes, rotor adapters, 1.5 mL elution tubes)(QIAcube starter kit, QIAGEN, cat. no. 990395)
5. For human samples, associated buffers and spin columns with the QIAamp DNA mini kit (QIAGEN, cat. no. 51304), and for animal samples, associated buffers and spin columns with the DNeasy blood and tissue kit (QIAGEN, cat. no. 69504)
6. Ethanol, 200 proof

2.2 Pre-PCR DNA quantitation and post-PCR analysis

1. PicoGreen dsDNA quantitation reagent from Molecular Probes (cat. no. P-7581)
2. 1X Tris-EDTA, pH 8.0 (Fisher; cat. no. BP2473-500)
3. Lambda (λ)/HindIII DNA (Gibco; cat. no. 15612-013) to generate a standard curve
4. A fluorescence reader capable of measuring fluorescence with 485 nm excitation and 528 nm emission (BioTek Synergy 2 Multi-Mode microplate reader, BioTek Instruments), black-bottomed 96-well plates (Costar, Corning Inc., Corning, NY, cat. no. 3015), single-channel p10 and p200 pipettes, multichannel pipette and pipet basin
5. 15-mL conical tube (four per sample, BD Falcon, BD Biosciences, Bedford, MA)
6. 0.5-mL autoclaved microcentrifuge tubes (to dilute DNA for PicoGreen reads)

2.3 Restriction digest

1. 225 ng purified DNA
2. Restriction enzyme (mouse: HaeII, New England Biolabs, cat. no. R0107S; human: PvuII, New England Biolabs, cat. no. R3151S; rat: XhoI, New England Biolabs, cat. no. R0146S) and associated components (10X NEBuffer, 100X BSA)
3. ddH_2O
4. 0.2 mL PCR tubes (USA Scientific)

5. Thermocycler or another method of incubating samples at 37°C/56°C

2.4 QPCR

1. GeneAmp XL PCR Kit (Applied Biosystems, cat. no. N8080193)
2. Bovine Serum Albumin (Roche, cat. no. 10-711-454-001)
3. dNTPs (GeneAmp dNTP Mix with dTTP, Applied Biosystems, cat. no. N8080261)
4. Primer oligos
5. 0.2 mL PCR tubes (USA Scientific)
6. A 96-well format thermocycler (Biometra Professional Standard Thermocycler 96, Biometra)

2.5 Dedicated equipment and workstations (see Notes section for additional information)

1. PCR enclosure with UV sterilization and, if possible, an air purifier (Labconco)
2. Distinct, dedicated workstations (preferably in separate laboratories) for: (1) DNA isolation; (2) setting-up the PCR reactions; and (3) post-PCR analysis
3. Pipettes and tips exclusively used for QPCR setup kept apart from pipettes and tips used for pre- and post-QPCR DNA analysis

3. Methods

3.1 DNA extraction

3.1.1 Manual DNA extraction (see Notes section for additional information)

1. To extract DNA from tissues that are very tough, or from organisms with cuticles or exoskeletons, first grind the sample in liquid nitrogen [28]. For softer tissues such as brain and liver, use a motorized pellet pestle to homogenize the sample.
2. For a $1x10^6$ cell pellet (or tissue samples below 15-20 mg), follow the 20/G Genomic-tip protocol for tissues. For larger amounts of tissue (>20 mg), follow the 100/G Genomic-tip protocol. For the purposes of this chapter, the 20/G Genomic-tip protocol will be explained in further detail.
3. Add 2 mL of Buffer G2 and 4 μL RNase A in a labeled 15 mL conical tube and vortex it briefly.
4. Add 100 μL Proteinase K to each tube and vortex it until pellet is completely resuspended.
5. Incubate sample in 50°C water bath for 2 hours and ensure that lysate is clear after incubation. At same time, prewarm Buffer QF in 50°C water bath.
6. While sample is incubating, set up one QIAGEN Genomic-tip 20/G column per sample over a prelabeled 15 mL conical tube and equilibrate the column with 2 mL of QBT buffer.

7. After incubation, vortex sample for 10 seconds and apply it to equilibrated column. Allow it to flow freely.
8. Move column to fresh 15 mL conical tube and wash column with 3 × 1 mL wash buffer QC.
9. Move column to fresh 15 mL conical tube and elute DNA with 1 mL elution buffer EF, repeat this one additional time over a fresh 15 mL tube.
10. Precipitate DNA by adding 1 mL eluted DNA + 700 μL isopropanol to a labeled 2.2 mL microcentrifuge tube and inverting tube at least 25 times. Each DNA sample will now have two 2.2 mL tubes from the two elutions. After precipitating DNA, place tubes at −80°C overnight.
11. The following day, thaw sample and centrifuge it at 12000 rpm for 60 minutes at 4°C.
12. Discard the supernatant and resuspend the pellet in 1 mL cold 70% ethanol.
13. Centrifuge sample at 12000 rpm for 45 minutes at 4°C.
14. Discard the supernatant and air-dry samples by inverting opened tubes and placing on paper towel for 10 minutes. Use clean pipette tip to remove droplets from tube until a dry white pellet is all that can be seen in the tube. Add 30-100 μL TE to the tube and combine the pellets in the two 2.2 mL tubes for each sample by taking the TE containing the resuspended pellet and moving it to the sample's other tube and ensuring that the second pellet is in the TE. Pipet up and down slowly and carefully to prevent DNA breakage.

3.1.2 Automated DNA extraction

1. Begin appropriate program in QIAcube for human (QIAamp DNA Mini Kit, blood or body fluid, standard lysis) or animal (DNeasy Blood & Tissue Kit, animal blood or cells, standard lysis). The QIAcube is designed for tissue samples below 25 mg and pellets equal to or below 5×10^6 cells.
2. Follow the on-screen instructions to set up QIAcube for DNA extraction (add reagents to bottles in correct order, label elution tubes, prepare columns and elution tubes in rotor adapters and place in centrifuge, set up tube of proteinase K, load pipette tips).
3. Resuspend cell pellet in 200 μL PBS (human samples) or 100 μL PBS (animal samples) and transfer to a labeled 2 mL screw-cap tube.
4. Place screw-tap tubes in appropriate positions in the QIAcube sample rack and start the protocol.
5. After the protocol is finished, save labeled 1.5 mL elution tubes and place samples at 4°C.

3.2. Restriction digest for mitochondrial analysis of DNA from automated extraction

1. Total volume of all digest components is 50 μL.

2. Calculate volume of DNA to load into each digest. Optimal DNA amount for restriction digest is 225 ng. All samples should have same amount of DNA for restriction digest (225 ng).
3. To calculate amount of ddH_2O to be used in the digest, subtract the volume of DNA to be used in the digest from 39 μL. For example, if 10 μL DNA will be used for the digest, 29 μL ddH_2O will be used. The individual volumes of DNA and ddH_2O are expected to vary between samples, but the total volume of ddH_2O and DNA will always be 39 μL.
4. NEBuffer stock is 10X and is diluted 10X in the digest mix (5 μL).
5. BSA stock is 100X and is first diluted to 10X in ddH_2O and then diluted 10X in the digest mix (5 μL).
6. Add ddH_2O to a labeled 0.2 mL PCR tube, followed by DNA, 5 μL 10X appropriate NEBuffer and 5 μL 10X BSA.
7. Add appropriate restriction enzyme (20 units HaeII for mouse, 10 units PvuII for human, and 20 units XhoI for rat) and place samples in thermocycler. Set thermocycler for 37°C for 60 minutes for mouse DNA, 37°C for 120 minutes for human DNA, and 56°C for 60 minutes for rat DNA. After digest, immediately place samples on ice or at 4°C. Further purification is not necessary prior to QPCR.

3.3 DNA extraction via lysis from a small number of *C. elegans* nematodes

1. Between 1-10 nematodes can be lysed at a ratio of 1 worm per 10 μl of lysis buffer for early-stage larvae to 1 worm per 20-30 μl for gravid adults.
2. Prepare lysis buffer containing 1x rTth XL PCR buffer and 1 mg/ml proteinase K (30% 3.3x rTth XL PCR buffer, 65% deionized, sterile H_2O and 5% 20 mg/ml proteinase K).
3. Aliquot lysis buffer into PCR tubes and place tubes on ice or in a chilled 96-well plate.
4. While observing the PCR tube with a dissecting microscope, use a platinum wire pick or an eyelash to pick nematode(s) into the PCR tube.
5. Immediately after picking the nematode(s) into lysis buffer, place the PCR tube on dry ice or directly at −80°C. Do not allow the nematode(s) to sit in lysis buffer unfrozen for more than five minutes.
6. Place all samples at −80°C for at least ten minutes to disrupt the nematode cuticle. Store samples at −80°C until lysed.
7. To lyse samples, heat to 65°C for five minutes, vortex at high speed for 5 secs, heat for an additional 55 minutes at 65°C, heat to 95°C for 15 minutes, and cool to 4°C.

3.4 Quantitation of DNA template (see Notes section for additional information)

1. For manually-extracted samples, dilute the DNA 1:20 or 1:30 in 1X TE, depending on size of cell pellet. For samples with automated extraction, dilute the DNA 1:10 in 1X TE because the initial DNA yield is lower.
2. Pipet 95 μL 1X TE into wells of a black-bottomed 96-well plate in duplicate for standards and samples.
3. Pipet 5 μL of standards into wells in duplicate (0 ng/μL, 1.25 ng/μL, 2.5 ng/μL, 5 ng/μL, 10 ng/μL, and 20 ng/μL). A standard of 40 ng/μL can also be used for the manually-extracted DNA, which tend to have high initial yields.
4. Pipet 5 μL of diluted DNA samples into wells in duplicate.
5. Thaw PicoGreen reagent in dark at room temperature and then make up PicoGreen solution by adding 5 μL PicoGreen reagent to 1 mL 1X TE.
6. In subdued lighting, pipet 100 μL PicoGreen solution into each well, cover plate, and place in dark for 10 minutes to allow for color development
7. Set up fluorescence reader by setting excitation filter at 485 and emission filter at 528 nM. Add 20 second shaking step before read step. For the Biotek Synergy 5, the sensitivity limit is 75 and shaking of the plate is set at level 3 for 20 seconds.
8. After 10 minute incubation, place plate in fluorescence reader and read sample concentrations. If DNA concentration is higher than the highest standard, dilute DNA further and re-read sample.
9. For manually-extracted samples, calculate volume of sample and 1X TE needed to make a 10 ng/μL concentration of DNA. Once the 10 ng/μL samples are made, load into plate and do PicoGreen read.
10. After acquiring the actual concentrations for the 10 ng/μL samples, calculate the volume of sample and 1X TE needed to further dilute to a 3 ng/μL concentration of DNA required for QPCR. If samples are more dilute we have found that you can run the QPCR assay using less DNA (~1 ng/μL), as long as ALL the samples are diluted equally. It may be necessary to add an additional one to two PCR cycles to increase the signal.
11. For samples extracted with the QIAcube, restriction digest is required for maximum product amplification. Using the initial read of diluted DNA, back-calculate to determine original DNA concentration, and determine the volume of DNA needed for 225 ng total for the restriction digest.

3.5 Primer design and optimization (see Notes section for additional information)

3.5.1 Design long (~10-15 kb) and short (~200 bp) primers for new genomic targets or species

1. Search GenBank to select genomic sequence to be amplified.
2. If applicable, paste the genomic sequence into DNA Duster (http://users.soe.ucsc.edu/~kent/dnaDust/dnadust.html) to remove numbers and spaces.

3. Copy the genomic sequence into primer design software such as Primer 3.
4. Set primer specifications:
    a. Primer length of 20-25 nt
    b. Tm of 68-70°C for long product and 63-65°C for short product (or 60°C if you anticipate utilizing real-time PCR)
    c. GC content of 40-60%
    d. Desired product length (~10-15 kb for long, ~200 bp for short) (it is possible to design primers that amplify a <10 kb region, but decreased amplicon size results in decreased sensitivity)
    e. Return at least three primer options
5. Select at least three forward and three reverse primers.
6. Order primers with standard desalting.
7. Store in −20°C until use.

3.5.2 Selection of optimal primers

1. Spin down lyophilized primers to pull down the pellet.
2. Re-suspend in sterile 0.1x TE (1 mM Tris + 0.1 mM EDTA) to 540 μM.
3. Vortex briefly.
4. Store stock primer solution at −20°C until use. Large volumes may be aliquoted (50 μl or less) in sterile 1.5 ml DNase, RNase free tubes prior to freezing to avoid multiple freeze-thaw cycles.
5. Dilute an aliquot to 10 μM from 540 μM with 0.1x TE. Store 10 μM working solution at 4°C.
6. Run PCR as described below with each primer combination at 63°C and 68°C annealing temperature for short and long primers, respectively. Annealing temperature optimization will be described in 3.5.3.
7. Run all PCR products on an agarose gel (1% and 2% for long and short products, respectively).
8. Pick the primer combination that results in a bright and unique band of the expected size. Very minor non-target products may be eliminated with optimization of magnesium concentration and annealing temperature (see 3.5.3).
9. Confirm the identity of products of expected size by sequencing or restriction digest followed by gel electrophoresis of cut products.

3.5.3 Optimizing primer conditions

1. After picking the optimal primer pair for each product, repeat QPCR at a range of annealing temperatures and magnesium concentrations. Typically a range of 4-6°C

around the designed annealing temperature and 1-1.4 mM (final concentration) $MgO(Ac)_2$ works well. This process is expedited with the use of a thermocycler with gradient capacity.

2. Run products on an agarose gel and, again, select the conditions that yield a bright band of expected size with no secondary products.

3.6 Standard QPCR conditions for animal cells

3.6.1. PCR reaction (see Notes section for more information)—Using the XL PCR kit mentioned in the materials section, the PCR reactions are prepared as follows:

1. To a 0.2 mL PCR tube, add 15 ng of DNA (total).
2. Controls needed include a PCR tube containing 1X TE instead of DNA ("no template" control) and a PCR tube containing 50% DNA amount (DNA diluted 1:1 first).
3. When several samples are being run, a master mix can be made for each primer set, which consists of the following components, added in this order:
   a. sterile Sigma W3500 water (9.6 μL water per reaction when DNA volume is ~5 μL and 8.6 μL water per reaction when DNA volume is ~6 μL)
   b. 1X buffer (15 μL 3.3X buffer per reaction)
   c. 100 ng/μL final concentration of BSA (5 μL of 1 mg/mL stock solution of BSA in ddH_2O per reaction)
   d. 200 μM final concentration of dNTPs (4 μL of 2.5 mM/each nt stock solution per reaction)(see Notes section)
   e. 1.2 mM final concentration of Mg^{++} (2.4 μL of 25 mM $MgO(Ac)_2$ per reaction)
   f. 0.4 μM of each of two primers (2 μL of 10 μM primer working solution per primer per reaction)

   Note: the volumes noted above are per reaction tube. Always make at least two reaction tubes more master mix than needed, or more if a large number of samples will be run.
4. Set up conditions for PCR machine, explained in further detail below (3.6.3).
5. Begin the PCR reaction by a 'hot start'. Bring the reaction mixture to 75°C prior to addition of enzyme (1 unit/reaction, dilute 0.5 μL of the polymerase in 4.5 μL of sterile water for each reaction (see Notes section)) and subsequent cycling.
6. After adding enzyme, close tubes and press continue to run remainder of PCR program.
7. For *C. elegans* extracts from low number of nematodes, the assay is set up in a similar manner, but the DNA concentration is not known, as a fixed number of nematodes is used (see 3.3 under Methods).

3.6.3. Cycle number and optimization

1. Perform cycle tests to determine quantitative conditions for the gene of interest by using a non-damaged sample (15 ng DNA) and a 50% control containing half of the amount of the non-damaged template (7.5 ng DNA). Include a “no template” control (explained above in section 3.6.2) as well.
2. The experimental setup is appropriate if the 50% control shows a 50% reduction of the amplification signal (see Notes section) when quantifying PCR products.

3.6.4 Quantitation of PCR products—Protocol is similar to that of section 3.2 in terms of fluorescence reader protocol, 10 minute incubation step, makeup of PicoGreen solution, and pipetted amounts of DNA, 1X TE, and PicoGreen solution for each well; however, there are some notable differences:

- **a.** The DNA is not diluted, and is taken directly from the PCR tube.
- **b.** Measurements are performed in triplicate wells.
- **c.** No standards are needed, although they can be useful to compare samples from one plate to another if the plate reader settings vary from run to run.
- **d.** Read blank-corrected fluorescence values. The blank is the “no template” control PCR product, explained in section 3.6.2.

3.7 Data analysis

3.7.1 Calculation of lesion frequency

1. Open Microsoft Excel.
2. Put blank-corrected fluorescence values acquired from fluorescence reader in a column.
3. Average the non-treated (undamaged) samples’ blank-corrected fluorescence values.
4. Divide each sample’s blank-corrected fluorescence by the average blank-corrected fluorescence value to get a ratio.
5. Take the negative natural log (-ln) of the ratio to determine the lesion frequency per fragment. We normalize this lesion frequency to number of lesions/10kb.
6. The lesion frequencies ***from at least two separate QPCR runs*** are calculated by combining the average lesion frequency for each biological sample (we use at least two treated and two control samples) with the average lesion frequency.

3.7.2 Normalization to mtDNA copy number

1. Open Microsoft Excel.
2. Put blank-corrected fluorescence values for the small mitochondrial amplicon in a column after the column for large mitochondrial blank-corrected fluorescence values.

3. Average all small mitochondrial blank-corrected fluorescence values.
4. Divide each sample's small mitochondrial blank-corrected fluorescence values by the average of all samples to get a ratio.
5. Divide the large mitochondrial blank-corrected fluorescence value by its corresponding small mitochondrial blank-corrected fluorescence ratio to get a "normalized" large mitochondrial blank-corrected fluorescence value.
6. Average the non-treated (undamaged) samples' "normalized" large mitochondrial blank-corrected fluorescence values.
7. Divide each sample's "normalized" large mitochondrial blank-corrected fluorescence values by the average non-treated blank-corrected fluorescence value to get a ratio.
8. Take the negative natural log (-ln) of the ratio to determine the lesion frequency per fragment. Now the lesion frequencies for mtDNA damage are normalized to mtDNA copy number.

3.8 Statistical analysis

3.8.1 Quality control

1. Ensure that 50% controls are adequate (generally between 40 and 60%).
2. Compare blank-corrected fluorescence values for replicate QPCR reactions by correlation analysis. If the correlation is poor, perform a third PCR. Comparison of the three values in the context of the correlation provides the basis for removal of outliers.

3.8.2 Data compilation and assessment

1. Combine lesion values for QPCR replicates (we do at least two separate QPCR runs). These are considered analytical replicates and do not contribute to the "n" of the experiment. However, combining two or more replicates is important in minimizing technical noise between QPCR runs.
2. Test normality of the data (e.g., with the Kolmogorov-Smirnov Normality test). In our experience, DNA damage data has always been normally distributed. If this were not the case, graphical presentations (e.g., box-and-whisker plots) and statistical analyses (e.g., Kruskal Wallis test) should be substituted for those described below.

3.8.3 Data presentation

1. Present the data graphically as mean ± standard error (unless the goal is an understanding of population distribution of DNA damage levels, in which case standard deviations would be more appropriate).
2. For repair studies, it may be clearer to present the data using a "percent removal" format.

3.8.4 Other statistical considerations

1. Error values are generated for control samples by comparing each individual control to the average of all controls.
2. If only two samples are being compared, a t-test or 1-factor ANOVA can be used to test for statistical significance.
3. If more than two samples are being compared, ANOVA should be used.
4. If there is more than one variable (e.g., dose, chemical), then an initial (global) multifactor ANOVA should be used, and post-hoc comparisons of subsets of the data should only be carried out if warranted by significant global ANOVA results.

Notes

Reagent storage conditions

The extraction reagents and buffers for manual and automated extraction, including 1X TE, 1X PBS, and isopropanol, are stored at room temperature. DNA and diluted primers at a concentration for QPCR are kept at 4°C. The GeneAmp XL PCR kit and its reagents, the BSA stock and working stocks, the dNTP stocks and working stocks, restriction enzymes and associated NEbuffer and BSA, PicoGreen reagent, diluted (λ) DNA standards, and primer stocks are stored at −20°C. Initial DNA extracts can be kept at 4°C temporarily but should be moved to −80°C for long-term storage. Cell pellets are stored at −80°C. Ensure that all components kept at −20°C and −80°C are thawed completely before use.

Extraction

1. DNA template integrity is essential for the reliable amplification of long PCR targets [36]. Although various kits are commercially available for DNA isolation, procedures that involve phenol extraction should be avoided due to potential introduction of artifactual DNA oxidation. The manual and automated procedures we use for DNA extraction (QIAGEN) give rise to templates of relatively high molecular weight and highly reproducible yield.
2. When using the manual genomic-tip protocol, the tissue protocol is used irrespective of whether tissue or cells are being studied, since the protocol for DNA extraction of cultured cells involves isolation of nuclei and hence loss of mtDNA.
3. When extracting DNA manually, vortex the samples well prior to lysis and again before adding them to the columns. This vortexing does not affect the subsequent amplification of the DNA.

DNA quantitation

1. The success of QPCR is absolutely dependent upon the accurate quantitation of the DNA present in the samples [3].
2. The accuracy of the data obtained with the PicoGreen assay is comparable to or can exceed the reproducibility that is accomplished with ^{32}P radiolabeled nucleotides

(Chen, Y. and Van Houten, B., unpublished observation) followed by subsequent agarose gel electrophoresis.

3. The diluted (λ) DNA standard is kept at −20°C and the diluted DNA samples are kept at 4°C. For long term storage, we routinely store the concentrated samples at −20°C and try to avoid many cycles of freeze/thaw.

4. If samples are still highly concentrated after the first dilution (i.e. well above 10 ng/μL), we recommend an additional round of quantitation. This ensures accuracy of the concentration of the final 3 ng/μL solution.

QPCR

1. **Separate work stations:** In order to avoid carryover of PCR products into the next subsequent reactions, separate work stations must be set up, preferably in different laboratory spaces. We house our PCR thermocyclers in a separate room and set up all PCR reactions in a dedicated UV-equipped hood. Once the PCR run is underway, the tubes are never again opened in that room.

2. **Avoidance of cross-contamination:** In addition to high quality reagents, the most important factor for the success of QPCR is the diligent avoidance of sample cross-contamination with PCR products. We use sterile technique for all steps. The constant use of disposable gloves when handling samples and reagents is essential to avoid the introduction of nucleases, foreign DNA, or other contaminants that can cause degradation of the template or inhibition of the polymerase during cycling. Additionally, we have found that it is extremely important to have distinct, dedicated workstations for different steps of the procedure, preferably in physically separate laboratories. It is of extreme importance not to open the PCR tubes after the last cycle in the same laboratory where the reactions were set up. Small DNA quantities can volatilize and contaminate other reactions, particularly if the tube is still hot, and completed reactions contain very high numbers of PCR products. The inclusion of a blank sample (where no DNA is added) helps to assure that no contamination has occurred with spurious DNA or PCR products. This sample should give no DNA band, if checked on gel, nor high fluorescence signal (as gauged by PicoGreen).

3. **Primers:** To simply compare DNA damage between genomes, a single long nuclear and mitochondrial product are sufficient. However, to detect differential damage or repair (potentially in coding and non-coding regions), primers can be designed for multiple gene targets. Organism-specific websites (e.g., WormBase for *Caenorhabditis elegans*) can be rich sources of information about relative expression levels of different genes. A single short product is sufficient since this is used for copy number normalization and does not reflect lesion frequency. Avoid highly repetitive regions of the nuclear genome. Gels illustrating primer analysis and other aspects of primer design troubleshooting are presented in Meyer [37]. If the full genome sequence of the species is known, it is best to use in-silico PCR to check for possible amplification of additional targets. This could in some cases escape detection even by restriction digest, especially in the case of duplicated

genes. Any primer design software can be used as long as it allows for primer/product conditions (i.e. annealing temperature, primer length, product length, GC content) to be specified. Primer 3 has been a reliable source of good primers for QPCR and this free web program can be found at http://frodo.wi.mit.edu/primer3/. Primers can be suspended in sterile 0.1X TE, 1X TE or de-ionized water. The use of 0.1X TE rather than 1X TE is a precautionary measure to reduce the amount of EDTA such that it does not affect PCR through chelation of Mg^{2+}. Aliquots of the stock primer solution can be made after suspension and before freezing in order to avoid multiple freeze/thaw cycles. If frozen, primer stocks should be completely thawed prior to use. Once a 540 μM primer aliquot is diluted to a working concentration of 10 μM it is kept at 4°C and is stable for several months. Primers should be renewed from time to time to maintain amplification. When first developing the assay in your laboratory it is essential to assess the PCR product of your primer set by agarose gel electrophoresis to verify the size of the product and to assure that no other spurious products are generated.

4. **dNTPs:** A solution of 10 mM of total dNTPs is prepared (2.5 mM of each nucleotide) and is stored as 100 μL aliquots in −20°C to minimize degradation. Higher misincorporation frequency for the enzyme and reduction in effective magnesium concentration can occur if dNTPs exceed 200 μM in the QPCR reaction.

5. ***rTth* polymerase:** Increasing amounts of the thermostable polymerase beyond 2.5 units per reaction can increase the production of nonspecific amplification products.

6. **Magnesium:** The optimal concentration must be determined for each set of primers and template. The *rTth* polymerase is extremely sensitive to magnesium; we advise that amplification of the fragment of interest be evaluated using varying quantities of Mg^{++}, starting from 0.9 mM and increasing by 0.1 increments.

7. **Quantitative aspect of amplification:** The usefulness of the QPCR assay for the detection of DNA damage requires that amplification yields be directly proportional to the starting amount of template. These conditions must be met by keeping the PCR in the exponential phase. During each set of amplifications we routinely amplify a control sample in which only 50% of the template is added to the QPCR. It is best to dilute control samples to 50% and add 5 μL of this dilution, rather than pipetting 2.5 μL into the reaction tube. Depending on the DNA quality and the products being amplified, relative amplification ranging from 40-60% is considered acceptable. Any experiments that are outside this range are not satisfactory and the entire set of reactions is discarded. It may be necessary to re-optimize the PCR by varying the number of cycles to establish a linear response to increasing template concentrations from 1.25 ng to 30 ng. Once the optimal number of cycles is identified, always run this 50% control as a quality control.

8. **Optimization of thermal conditions:** Another concern when performing QPCR is finding the optimal thermal conditions for amplification of your target gene. As mentioned before, QPCR in our laboratory is routinely performed using hot start,

which produces cleaner PCR products because it prevents nonspecific annealing of primers to each other, as well as to template, before enzyme addition. Keep in mind that the melting temperature of the primers and the annealing temperature used in the PCR determine how stably and specifically the primers hybridize to the DNA template. Thus, it is important to check this parameter with suitable software beforehand, and annealing temperatures must be experimentally optimized. Table 2 shows the most favorable conditions for animal DNA amplifications currently used in our laboratory.

9. **Normalization to mtDNA copy number:** As noted above, if DNA is extracted using an automated system employing the QIAcube, linearization of the mtDNA is necessary to get an accurate level of mtDNA. This is accomplished using digestion of the samples with a restriction enzyme (discussed in further detail in section 2.3 in materials and section 3.2 in methods).

References

1. Kalinowski DP, Illenye S, Van Houten B. Analysis of DNA damage and repair in murine leukemia L1210 cells using a quantitative polymerase chain reaction assay. Nucleic Acids Res. 1992; 20(13): 3485–94. [PubMed: 1630919]
2. Salazar JJ, Van Houten B. Preferential mitochondrial DNA injury caused by glucose oxidase as a steady generator of hydrogen peroxide in human fibroblasts. Mutat Res. 1997; 385(2):139–49. [PubMed: 9447235]
3. Yakes FM, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc Natl Acad Sci U S A. 1997; 94(2):514–9. [PubMed: 9012815]
4. Santos JH, et al. Cell sorting experiments link persistent mitochondrial DNA damage with loss of mitochondrial membrane potential and apoptotic cell death. J Biol Chem. 2003; 278(3):1728–34. [PubMed: 12424245]
5. Mandavilli BS, Boldogh I, Van Houten B. 3-nitroproprionic acid-induced hydrogen peroxide, mitochondrial DNA damage, and cell death are attenuated by Bcl-2 overexpression in PC12 cells. Molecular Brain Research. 2005; 133(2):215–223. [PubMed: 15710238]
6. Ponti M, et al. Measurement of the sequence specificity of covalent DNA modification by antineoplastic agents using Taq DNA polymerase. Nucleic Acids Res. 1991; 19(11):2929–33. [PubMed: 2057351]
7. Ayala-Torres S, et al. Analysis of gene-specific DNA damage and repair using quantitative polymerase chain reaction. Methods. 2000; 22(2):135–47. [PubMed: 11020328]
8. Santos JH, et al. Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells. Methods in Molecular Biology. 2006; 314:183–199. [PubMed: 16673882]
9. Jennerwein MM, Eastman A. A polymerase chain reaction-based method to detect cisplatin adducts in specific genes. Nucleic Acids Res. 1991; 19(22):6209–14. [PubMed: 1956780]
10. Chen KH, et al. Up-regulation of base excision repair correlates with enhanced protection against a DNA damaging agent in mouse cell lines. Nucleic Acids Res. 1998; 26(8):2001–7. [PubMed: 9518496]
11. Ballinger SW, et al. Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. Circ Res. 2000; 86(9):960–6. [PubMed: 10807868]
12. Mandavilli BS, Ali SF, Van Houten B. DNA damage in brain mitochondria caused by aging and MPTP treatment. Brain Res. 2000; 885(1):45–52. [PubMed: 11121528]
13. Ballinger SW, et al. Hydrogen peroxide causes significant mitochondrial damage in human RPE cells. Experimental Eye Research. 1999; 68(6):765–772. [PubMed: 10375440]

14. Van Houten B, et al. Development of long PCR techniques to analyze deletion mutations of the human hprt gene. Mutat Res. 1998; 403(1-2):171–5. [PubMed: 9726017]
15. Boyd WA, et al. Nucleotide excision repair genes are expressed at low levels and are not detectably inducible in Caenorhabditis elegans somatic tissues, but their function is required for normal adult life after UVC exposure. Mutat Res. 2010; 683(1-2):57–67. [PubMed: 19879883]
16. Wang AL, et al. Autophagy and exosomes in the aged retinal pigment epithelium: possible relevance to drusen formation and age-related macular degeneration. PLoS One. 2009; 4(1):e4160. [PubMed: 19129916]
17. Wang AL, et al. Increased mitochondrial DNA damage and down-regulation of DNA repair enzymes in aged rodent retinal pigment epithelium and choroid. Mol Vis. 2008; 14:644–51. [PubMed: 18392142]
18. Acevedo-Torres K, et al. Mitochondrial DNA damage is a hallmark of chemically induced and the R6/2 transgenic model of Huntington's disease. DNA Repair (Amst). 2009; 8(1):126–36. [PubMed: 18935984]
19. Bonner M, Kmiec EB. DNA breakage associated with targeted gene alteration directed by DNA oligonucleotides. Mutat Res. 2009; 669(1-2):85–94. [PubMed: 19463835]
20. Khurana RN, et al. Mitochondrial oxidative DNA damage in experimental autoimmune uveitis. Invest Ophthalmol Vis Sci. 2008; 49(8):3299–304. [PubMed: 18450595]
21. Haugen AC, et al. Altered gene expression and DNA damage in peripheral blood cells from Friedreich's ataxia patients: cellular model of pathology. PLoS Genet. 2010; 6(1):e1000812. [PubMed: 20090835]
22. Chatterjee A, et al. Targeting of mutant hogg1 in mammalian mitochondria and nucleus: effect on cellular survival upon oxidative stress. BMC Cancer. 2006; 6:235. [PubMed: 17018150]
23. Duxin JP, et al. Human Dna2 is a nuclear and mitochondrial DNA maintenance protein. Mol Cell Biol. 2009; 29(15):4274–82. [PubMed: 19487465]
24. Jung D, et al. Effects of benzo[a]pyrene on mitochondrial and nuclear DNA damage in Atlantic killifish (Fundulus heteroclitus) from a creosote-contaminated and reference site. Aquat Toxicol. 2009; 95(1):44–51. [PubMed: 19726093]
25. Wu LL, et al. Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin Chim Acta. 2004; 339(1-2):1–9. [PubMed: 14687888]
26. Lu T, et al. Gene regulation and DNA damage in the ageing human brain. Nature. 2004; 429(6994):883–891. [PubMed: 15190254]
27. Meyer JN, et al. Decline of nucleotide excision repair capacity in aging Caenorhabditis elegans. Genome Biol. 2007; 8(5):R70. [PubMed: 17472752]
28. Hunter SE, et al. The QPCR assay for analysis of mitochondrial DNA damage, repair, and relative copy number. Methods. 2010; 51(4):444–51. [PubMed: 20123023]
29. Cabrera G, et al. DNA repair BER pathway inhibition increases cell death caused by oxidative DNA damage in Trypanosoma cruzi. J Cell Biochem. 2011
30. Jung D, et al. The long amplicon quantitative PCR for DNA damage assay as a sensitive method of assessing DNA damage in the environmental model, Atlantic killifish (Fundulus heteroclitus). Comp Biochem Physiol C Toxicol Pharmacol. 2009; 149(2):182–6. [PubMed: 18706522]
31. Simsek D, et al. Crucial role for DNA ligase III in mitochondria but not in Xrcc1-dependent repair. Nature. 2011; 471
32. Das BB, et al. Role of tyrosyl-DNA phosphodiesterase (TDP1) in mitochondria. Proc Natl Acad Sci. 2010
33. Karunadharma PP, et al. Mitochondrial DNA damage as a potential mechanism for age-related macular degeneration. Invest Ophthalmol Vis Sci. 2010
34. Lin H, et al. Mitochondrial DNA damage and repair in RPE associated with aging and age-related macular degeneration. Invest Opthalmol Vis Sci. 2011
35. Zhang Y, et al. Expression changes in DNA repair enzymes and mitochondrial DNA damage in aging rat lens. Molecular Vision. 2010; 16:1754–1763. [PubMed: 20808729]
36. Cheng S, et al. Template integrity is essential for PCR amplification of 20- to 30-kb sequences from genomic DNA. PCR Methods Appl. 1995; 4(5):294–8. [PubMed: 7580917]

37. Meyer JN. QPCR: a tool for analysis of mitochondrial and nuclear DNA damage in ecotoxicology. Ecotoxicology. 2010; 19(4):804–11. [PubMed: 20049526]

Table 1

Gene Targets and Primer Pairs for QPCR

Human		
13.5 kb fragment from the 5’ flanking region near the β-globin gene, accession number J00179		
48510	5’-CGA GTA AGA GAC CAT TGT GGC AG-3’	sense
62007	5’-GCA CTG GCT TAG GAG TTG GAC T-3’	antisense
12.2 kb region of the DNA polymerase gene β, accession number L11607		
2372	5’-CAT GTC ACC ACT GGA CTC TGC AC-3’	sense
3927	5’-CCT GGA GTA GGA ACA AAA ATT GCT G-3’	antisense
10.4 kb fragment encompassing exons 2-5 of HPRT gene, accession number J00205		
14577	5’-TGG GAT TAC ACG TGT GAA CCA ACC-3’	sense
24997	5’-GCT CTA CCC TGT CCT CTA CCG TCC -3’	antisense
8.9 kb mitochondria fragment, accession number J01415		
5999	5’-TCT AAG CCT CCT TAT TCG AGC CGA-3’	sense
14841	5’-TTT CAT CAT GCG GAG ATG TTG GAT GG-3’	antisense
221 bp mitochondria fragment		
14620	5’-CCC CAC AAA CCC CAT TAC TAA ACC CA -3’	sense
14841	5’-TTT CAT CAT GCG GAG ATG TTG GAT GG-3’	antisense
Mouse		
8.7 kb fragment of the β-globin gene, accession number X14061		
21582	5’-TTG AGA CTG TGA TTG GCA ATG CCT -3’	sense
30345	5’-CCT TTA ATG CCC ATC CCG GAC T-3’	antisense
6.6 kb fragment of the DNA polymerase gene β, accession number AA79582		
Chr8, $_{23735019}$	5’-TAT CTC TCT TCC TCT TCA CTT CTC CCC TGG-3’	sense
Chr8, $_{23741702}$	5’-CGT GAT GCC GCC GTT GAG GGT CTC CTG-3’	antisense
10 kb mitochondria fragment		
3278	5’-GCC AGC CTG ACC CAT AGC CAT AAT AT-3’	sense
13337	5’-GAG AGA TTT TAT GGG TGT AAT GCG G-3’	antisense
117 bp mitochondria fragment		
13597	5’-CCC AGC TAC TAC CAT CAT TCA AGT-3’	sense
13688	5’-GAT GGT TTG GGA GAT TGG TTG ATG T-3’	antisense
Rat		
12.5 kb fragment from the clusterin (TRPM-2) gene, accession number M64733		
5781	5’-AGA CGG GTG AGA CAG CTG CAC CTT TTC-3’	sense
18314	5’-CGA GAG CAT CAA GTG CAG GCA TTA GAG-3’	antisense
13.4 kb mitochondria fragment		
13559	5’-AAA ATC CCC GCA AAC AAT GAC CAC CC-3’	sense
10633	5’-GGC AAT TAA GAG TGG GAT GGA GCC AA-3’	antisense
211 bp mitochondria fragment		
14678	5’-CCT CCC ATT CAT TAT CGC CGC CCT TGC-3’	sense

14885	5’-GTC TGG GTC TCC TAG TAG GTC TGG GAA-3’	antisense
Caenorhabditis elegans[1]		
13.7 kb fragment from the polymerase epsilon gene		
	5’-AGT CGT TGA ACG CAG TGG TGT CAT-3’	sense
	5’-CAG TCT TTC TTC GAC GCA TTC AAC G-3’	antisense
9.3 kb fragment from the unc-2 gene		
unc2f1	5’-TGG CTG GAA CGA ACC GAA CCA T-3’	sense
unc2r1	5’-GGC GGT TGT GGA GTG TGG GAA G-3’	antisense
225 bp nuclear fragment		
1334	5’- TCC CGT CTA TTG CAG GTC TTT CCA-3’	sense
1536	5’- GAC GCG CAC GAT ATC TCG ATT TTC-3’	antisense
10.9 kb mitochondrial fragment		
1528	5’- CCA TCA ATT GCC CAA AGG GGA GT -3’	sense
12442	5’-TGT CCT CAA GGC TAC CAC CTT CTT CA-3’	antisense
195 bp mitochondrial fragment		
12272	5’-CAC ACC GGT GAG GTC TTT GGT TC -3’	sense
12442	5’-TGT CCT CAA GGC TAC CAC CTT CTT CA-3’	antisense
Drosophila melanogaster		
11.5 kb fragment of the β tubulin gene		
	5’-GTA TTC CTG CGC CAG GAG GAT CG-3’	sense
	5’-CAG ATG CTG GAG CTG CCT TTG GA-3’	antisense
10.3 kb fragment of the β tubulin gene		
	5’-GAG GAG CCT TGC GAA CAA CAG CA -3’	sense
	5’-CAA TGA CAG CTG CGC CTC GAG AT-3’	antisense
152 bp nuclear fragment		
	5’-CGA GGG ATA CCT GTG AGC AGC TT-3’	sense
	5’-GTC ACT TCT TGT GCT GCC ATC GT-3’	antisense
14.2 kb mitochondrial fragment		
	5’-GCC GCT CCT TTC CAT TTT TGA TTT CC-3’	sense
	5’-TGC CAG CAG TCG CGG TTA TAC CA-3’	antisense
151 bp mitochondrial fragment		
	5’-GCT CCT GAT ATA GCA TTC CCA CGA-3’	sense
	5’-CAT GAG CAA TTC CAG CGG ATA AA-3’	antisense
Fundulus heteroclitus (Atlantic killifish)		
11.5 kb fragment of the CFTR gene, accession number AY028263		
	5’-CAG CCG CCC GCA AAT TCT CA-3’	sense
	5’-CAG AAT GCG GGC CTT GCT GA-3’	antisense
234 bp nuclear fragment		
	5’-GCC GCT GCC TTC ATT GCT GT-3’	sense
	5’-ATG AGC TGG GTG TGC GCT GA-3’	antisense

9.4 kb mitochondria fragment, accession number CN984995		
	5’-TTG CAC CAA GAG TTT TTG GTT CCT AAG ACC-3’	sense
	5’-GAT GTT GGA TCA GGA CAT CCC AAT GGT GCA-3’	antisense
264 bp mitochondria fragment		
	5’-ATC TGC ATG GCC AAC GCC TA-3’	sense
	5’-GGC GGT GCC AGT TTC CTT TT-3’	antisense
Danio rerio (zebrafish)		
10.7 kb fragment of the AHR2 gene		
	5’-AGA GCG CGA TTG CTG GAT TCA C-3’	sense
	5’-GTC CTT GCA GGT TGG CAA ATG G-3’	antisense
233 bp nuclear fragment		
	5’-ATG GGC TGG GCG ATA AAA TTG G-3’	sense
	5’-ACA TGT GCA TGT CGC TCC CAA A-3’	antisense
10.3 kb mitochondria fragment		
	5’-TTA AAG CCC CGA ATC CAG GTG AGC -3’	sense
	5’-GAG ATG TTC TCG GGT GTG GGA TGG-3’	antisense
198 bp mitochondria fragment		
	5’-CAA ACA CAA GCC TCG CCT GTT TAC-3’	sense
	5’-CAC TGA CTT GAT GGG GGA GAC AGT-3’	antisense

[1] Primers and PCR conditions for additional nuclear targets are listed in Meyer et al. (2007)

Table 2

PCR Conditions for Animal Targets

Target	Amplicon Size	Cycles	Temp (°C)	Primer concentration (μm, each)	$MgO(Ac)_2$ concentration (mM)
Human					
β-globin	13.5 kb	27	64	0.4	1.3
Polymerase β	12.2 kb	26	64	0.4	1.2
HPRT	10.4 kb	29	64	0.4	1.3
Large Mito	8.9 kb	19	64	0.4	1.2
Small Mito	221 bp	19	60	0.4	1.1
Mouse					
β globin	8.7 kb	25	65	0.4	1.1
Polymerase β	6.6 kb	25	64	0.4	1.2
Long Mito	10 kb	20	64	0.4	1.2
Short Mito	117 bp	18	60	0.4	1.1
Rat					
Clusterin	12.5 kb	28	65	0.4	1.0
Long Mito	13.4 kb	20	65	0.4	1.2
Short Mito	211 bp	20	60	0.4	1.1
C. *elegans*					
Polymerase ε	13.7 kb	21	68	0.4	1.2
unc-2	9.3 kb	20	68	0.4	1.2
Polymerase ε	225 bp	23	63	0.4	1.2
Long Mito	10.9 kb	16	66	0.4	1.2
Short Mito	195 bp	18	63	0.3	1.2
D. melanogaster					
β tubulin	11.5 kb	22	67	0.4	1.2
β tubulin	10.3 kb	22	67	0.4	1.2
Short Nuclear	152 bp	24	65	0.4	1.2
Long Mito	14.2 kb	17	66	0.4	1.2
Short Mito	151 bp	19	61	0.4	1.2
F. heteroclitus (Atlantic killifish)					
CFTR	11.5 kb	24	68	0.4	1.1
CFTR	234 bp	24	62	0.4	1.2
Long Mito	9.4 kb	16	65	0.4	1.2
Short Mito	264 bp	24	62	0.4	1.2
D. rerio (zebrafish)					
AHR2	10.7 kb	24	69	0.4	1.05
AHR2	233 bp	27	60	0.4	1.2

Target	Amplicon Size	Cycles	Temp (°C)	Primer concentration (μm, each)	$MgO(Ac)_2$ concentration (mM)
Long Mito	10.3 kb	19	68	0.4	1.2
Short Mito	198 bp	21	62	0.4	1.2

Temperature is the annealing temperature and is based on the melting temperature of the oligonucleotide pair.