

Chapter 12

Measuring mtDNA Damage Using a Supercoiling-Sensitive qPCR Approach

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Abstract

Compromised mitochondrial DNA structural integrity can have functional consequences in mitochondrial gene expression and replication leading to metabolic and degenerative diseases, aging and cancer. Gel electrophoresis coupled with Southern blot and probe hybridization and long PCR are established methods for detecting mtDNA damage. But each has its respective shortcomings: gel electrophoresis is at best semi-quantitative and long PCR does not offer information on the structure. To overcome these limitations, we developed a new method with real-time PCR to accurately quantify the mtDNA structural damage/repair and copy number change. We previously showed that the different mtDNA structures (supercoiled, relaxed circular, and linear) have profound influences on the outcome of the real-time PCR amplification. The supercoiled structure is inhibitory to the PCR amplification, while relaxed structures are readily amplified. We will illustrate the use of this new method by quantifying the kinetics of mtDNA damage and repair in LNCaP prostate cancer cells induced by exogenous H₂O₂ treatments. The use of this new method on clinical samples for spontaneous mtDNA damage level will also be highlighted.

Key words: mtDNA supercoiling, Oxidative damage, DNA repair, Copy number, Real-time PCR.

1. Introduction

Recent studies have shown that alterations in mitochondrial DNA (mtDNA) can induce functional changes that play important roles in metabolic and degenerative diseases, aging and cancer (1). The mitochondrion is responsible for energy production during cellular respiration, and the electron transport chain (ETC) lies at the center of this metabolic function. Situated in the inner membrane, the ETC of the mitochondria supplies energy to the cell through oxidative phosphorylation of ADP to ATP. However, the ETC is

also a major source of reactive oxygen species (ROS) (2, 3). Due to its close proximity and lack of protecting histones, the mtDNA is susceptible to oxidative damage, which can potentially lead to changes in mitochondrial gene expression and somatic mutations in many human cancers (4–8). In the cells, mtDNA is composed of a mixture of supercoiled, relaxed circular and linear forms. The mature mtDNA has a supercoiled structure with an average of 100 negative super-helical turns (9). This supercoiled conformation is susceptible to DNA strand breaks induced by oxidative damage, i.e., a single strand break can lead to the disruption of the supercoiled structure. Because the supercoiled conformation is required for initiation of mtDNA replication and transcription (10–12), maintaining the integrity of the mtDNA structure is crucial to normal mitochondria function.

Several techniques have been developed to study mtDNA damage. Gel electrophoresis is frequently used to detect mtDNA conformational changes. However, this assay is not quantitative. It involves a tedious process coupling of Southern blot and probe hybridization. On the other hand, long PCR allows the quantification of mtDNA damage, but it provides little information on the mtDNA structure. To overcome these limitations, we developed a new approach using real-time PCR to quantify structural damage and copy number change of mtDNA. This method is based on several key findings (13). (a) Supercoiled and relaxed DNA molecules have different efficiencies in PCR amplification. The supercoiled structure inhibits PCR amplification, while relaxed DNA is readily amplified (*see Note 1*). (b) Heat-treatment of DNA templates prior to real-time PCR can be used to artificially introduce strand breaks and relax the mtDNA molecules, allowing quantification of the total amount of mtDNA copies. Thus, this new method is useful for quantifying not only structural damage, repair, and copy number change of mtDNA in stressed cells in culture, but also the level of spontaneous damage of mtDNA from clinical samples. In this chapter, the new approach will be illustrated by studying mtDNA damage responses to acute oxidative stress in prostate cancer cells (LNCaP) treated by H₂O₂.

2. Materials

2.1. Cell Collection for LNCaP (*see Note 2*)

1. PBS-CMF, phosphate buffered saline without CaCl₂, MgCl₂ (GIBCO, Invitrogen cat. No. 20012-027).
2. PBS-CMF/0.5 mM EDTA, to obtain 0.5 mM EDTA, add 500 μ l 0.5 M EDTA to 500 ml to PBS-CMF; 0.5 M EDTA pH8.0 (GIBCO, Invitrogen cat. No. 15575-020).

3. Trypsin/EDTA solution: 0.05% trypsin + 0.02% EDTA.
4. RPMI media 1640, with L-glutamine.
5. Fetal Bovine Serum (FBS).
6. Penicillin-streptomycin (10,000 units/ml, 10,000 µg/ml respectively).
7. Complete RPMI medium: remove 50 ml RPMI from 500 ml bottle, add 50 ml FBS (to get 10% FBS) and 5 ml penicillin-streptomycin.
8. Poly-L-lysine (PLL) coated-dish (*see Note 3*).

2.2. DNA Extraction with Genomic-Tip

2.2.1. Cell Culture

1. QIAGEN Blood & Cell Culture DNA Kit Mini. The kit contains the following: C1, G2, QBT, QC, QF buffers, protease, 25 genomic tips.
2. RNase A 2.5 ml (100 mg/ml).
3. Distilled water, DNase and RNase free.
4. 1X Tris/EDTA buffer solution (TE buffer): pH 8.0 ± 0.1, 10 mM Tris, and 1 mM EDTA.
5. Isopropanol (2-propanol) for molecular biology grade, minimum 99%.
6. 70% ethanol.

2.2.2. Snap-Frozen Tissue Samples

1. Refer to material in **Section 2.2.1** for cell culture with the following additional material.
2. Proteinase K (>600 mAU/ml).
3. Glass tissue grinder, ground glass Potter-Elvehjem type (Kontes).
4. Sterile surgical scalpel blades and handles.

2.3. DNA Quantification

2.3.1. Fluorometric Quantification of dsDNA Using PicoGreen[®]

1. 96-well plate, black opaque, fluorometry compatible.
2. Quant-iT[™] PicoGreen[®] dsDNA Assay Kit (Invitrogen, cat. No. P7589). The kit contains PicoGreen dye, 20X TE buffer, and lambda DNA standard (100 µg/ml).
3. Multi-well plate reader with fluorometric capabilities at excitation/emission wavelengths of 480 nm/520 nm respectively (PerkinElmer 1420 Multilabel Counter Victor³V).

2.3.2. Quantification by Nanodrop

1. Nanodrop spectrophotometer instrument.
2. Distilled water, DNase and RNase free.
3. 1X Tris/EDTA buffer solution (TE buffer): pH 8.0 ± 0.1, 10 mM Tris, and 1 mM EDTA.

2.4. Template DNA Preparation and Heat Treatment

1. PCR machine: GeneAmp PCR system 9700 (Applied Biosystems).
2. 1X Tris/EDTA buffer solution, pH 8.0 ± 0.1, 10 mM Tris and 1 mM.

2.5. mtDNA Structural Damage and Repair Analysis Using Real-Time PCR

1. 2X IQTM SYBR[®] Green Supermix 500 × 50 µl reactions (BIO-RAD cat. No. 170-8882).
2. 10 µM primer working solutions (primer sequences listed in **Table 12.1**).
3. Distilled water, DNase and RNase free.

Table 12.1
Primer sequences

Gene	Forward primer 5'–3'	Reverse primer 5'–3'
CO2 (mtDNA)	CCCCACATTAGGCTTAAAAACAGAT	TATACCCCCGGTCGTGTAGCGGT
D-loop (mtDNA)	TATCTTTTGGCGGTATGCACTTTTA ACAGT	TGATGAGATTAGTAGTATGGGA GTGG
β-actin	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG
β-globin	GTGCACCTGACTCCTGAGGAGA	CCTTGATACCAACCTGCCAG

2.5.1. Bio-Rad System

4. MgCl₂ Solution, 50 mM. (BIO-RAD cat. No. 170-8872).
5. 96-well PCR plates, DNase and RNase Free (BIO-RAD cat. No. 2239441).
6. Optical tape for plates (BIO-RAD cat. No. 2239444).
7. Real-time PCR detection system: MyIQTM Single Color Real-Time PCR Detection System with iCyclerTM thermocycler base (BIO-RAD).

2.5.2. Applied Biosystems (ABI) System

1. Power SYBR[®] Fast Green PCR MASTER MIX.
2. 10 µM primer working solutions (primer sequences listed in **Table 12.1**)
3. Distilled water, DNase and RNase free.
4. MicroAmpTM Fast Optical 96-well Reaction Plate with Barcode (0.1 ml).
5. Optical tape for plates (ABI).
6. Real-time PCR detection system: 7500 Fast Real-Time PCR System (ABI).

3. Methods

The supercoiled structure of mtDNA is sensitive to experimental artifacts and can be easily disrupted if not properly handled. It is important to always handle the cells and DNA samples with care. The following protocols are designed to minimize potential artificial DNA damage. This section will go through the logical workflow of the experiment from cell collection (**Section 3.1**) → DNA extraction (**Section 3.2**) of cells (**Section 3.2.1**) or frozen tissues (**Section 3.2.2**) → DNA quantification (**Section 3.3**) → template DNA preparation and dilution (**Section 3.4**) → real-time PCR analysis (**Section 3.5**).

LNCaP cells are used as an example for the cell collection protocol. A QIAGEN Blood & Cell Culture DNA Kit is recommended for DNA extraction. Unlike phenol-based methods (14) (*see Note 4*), this kit employs an ion-exchange system that does not oxidize purines during isolation. The QIAGEN recommended protocol has been modified (*see Note 5*) in order to recover both mtDNA and nuclear DNA. The protocol will also apply for frozen tissue preparation (*see Note 6*).

Following the DNA extraction, the concentrations of the DNA samples will be quantified. It is recommended (*see Note 7*) to perform two rounds of quantifications. The first round will measure the stock concentrations, the second round will measure the precise concentration of 10 ng/ μ l working solutions prepared from the stock solutions. Two methods are recommended: (**Section 3.3.1**) fluorometric quantification of dsDNA using PicoGreen[®] and (**Section 3.3.2**) quantification by Nanodrop[®] spectrophotometer (*see Note 8*).

Prior to the real-time PCR analysis, the half amount of a 1 ng/ μ l DNA solution will be heat-treated. The original and heated 1 ng/ μ l DNA templates will be used for the detection of the mitochondrial markers (CO₂ and/or D-loop) and a 5 ng/ μ l template for nuclear gene markers (β -actin and β -globin). The method used for real-time PCR analysis is based on the relative quantification model suggested by Pfaffl (15).

3.1. Cell Collection (LNCaP)

1. Remove RPMI medium from the dish by aspiration.
2. Add gently 5 ml PBS-CMF/EDTA, incubate for 1 min at room temperature.
3. Remove the PBS-CMF/EDTA and add 2.5 ml of trypsin, incubate for 5 min at 37°C.

4. Resuspend cells by adding 6 ml of RPMI medium to inactivate trypsin; gently aspirate up and down with pipette to get rid of clumps.
5. Transfer cell suspension to a 15 ml tube, and put on ice.
6. Take a small aliquot of cell suspension for cell counting (should be between 2 and 5 million cells).
7. Centrifuge at ~ 180 g for 6 min.
8. Remove medium and resuspend cells in 1 ml PBS-CMF (if the cell suspension has more than 5 million cells, use 2 ml of PBS then split into two 1.5 ml tubes in Step 9).
9. Transfer to 1.5 ml tube, centrifuge at high speed for 2 min.
10. Remove supernatant but not completely, leave just enough to cover the cell pellet. Put on ice.
11. Store at -80°C .

3.2. DNA Extraction with Genomic-Tip for Cell Culture and Snap-Frozen Tissue

This is a modified protocol based on the QIAGEN protocol. Do not use the C1 buffer (*see Note 5*). Prewarm the QF buffer to 50°C in a water bath. Cool the 70% ethanol to -20°C .

3.2.1. For Cell Cultures

Prepare protease solution by adding 1.4 ml distilled water to the powdered protease. The recommended amount of cells for each preparation is 2–5 million.

1. Thaw cells on ice, resuspend cells by flicking the tube with fingers.
2. Add 1 ml G2 digestion buffer to tube. Immediately vortex at maximum speed for 25 sec (be consistent for all samples). Do one sample at a time and let the sample rest at room temperature while working with other samples. Centrifuge briefly.
3. Add 3 μl RNase A to each sample. Invert to mix and centrifuge for several seconds.
4. Add 25 μl reconstituted protease. Invert to mix, but do not centrifuge afterwards. Incubate at 50°C for 1 h in water bath. Continue on to step 5.

3.2.2. For Snap-Frozen Tissue Samples

Proceed with one sample at a time. It is important to carry out the following steps as fast as possible since the tissue sample can degrade rapidly after thawing. Do not thaw the samples until needed for cutting or grinding (*see Note 9*). Homogenize gently manually, any motorized assistance will disrupt the mtDNA structure. It is recommended to carry step 1–3 on ice.

1. Prepare a mixture of 1.8 ml G2 buffer + 4 μ l RNase A per sample, mix well.
2. Weigh tissue sample (around 10–50 mg) and cut into smaller piece if needed (*see Note 10*).
3. Add the tissue pieces and \sim 300 μ l of the G2 buffer/RNase A mixture into a glass tissue grinder to homogenize. Grind manually but do not over-grind as this will disrupt the mtDNA structure (*see Note 11*). Collect homogenate into a 2 ml tube. Wash grinder with remaining G2 buffer/RNase A solution and collect to the 2 ml tube.
4. Add 100 μ l proteinase K (*see Note 12*). Invert to mix. Incubate at 50°C for 2 h. Repeat Steps 1–4 for the next sample. Then continue on to Step 5.

DNA extraction with genomic-tips

5. About 15 min before the end of the incubation at step 4, add 2 ml of QBT buffer to each genomic tip and let the buffer equilibrate the tips.
6. Take the samples out of water bath, and vortex digested samples for 10 sec at maximum speed. Then transfer each sample to a genomic-tip.
7. After the samples have flowed through the tip, wash the tip with 3×1 ml of QC buffer.
8. Elute the DNA into a 15 ml tube by adding 910 μ l QF buffer (prewarmed to 50°C) twice into the genomic-tip.
9. Mix the eluted DNA by inverting the tubes and centrifuge at \sim 188 g. Aliquot solution equally into two 2 ml microcentrifuge tubes (\sim 850 μ l).
10. Precipitate the DNA by adding 700–800 μ l of isopropanol to each tube. Mix by inverting and incubate for 10 min at room temperature (*see Note 13*). Centrifuge at 15,000–18,000 \times g for 20 min at 4°C (*see Note 14*).
11. Remove and discard supernatant, leaving the DNA pellet at the bottom of the tube. This pellet contains nuclear and mitochondrial DNA.
12. Wash the DNA pellet with 500 μ l of 70% ETOH (-20°). Centrifuge at 15,000–18,000 g for 10 min at 4°C.
13. Remove and discard the supernatant. Repeat Step 12 once more.
14. Remove the supernatant, three samples at a time, and let the DNA pellet air dry a bit (*see Note 15*).

15. Dissolve DNA pellet with 80–200 μl of TE buffer. Aim for a final concentration of ~ 60 up to ~ 200 ng/ μl .
16. Let the DNA samples sit at 4°C for overnight to ensure that the DNA is completely dissolved.

3.3. DNA Quantification (see Note 7)

3.3.1. Fluorometric Quantification of dsDNA Using PicoGreen[®]

3.3.1.1. First Round of Quantification of the Stock DNA Samples

Initial quantification

1. Thaw the PicoGreen[®] dye in the dark at room temperature.
2. Mix DNA samples by gently flicking the tube. Centrifuge briefly.
3. Prepare the lambda DNA standard. The recommended 2X dilution series range from 20 to 0.625 ng/ μl (6 standards).
4. Aliquot 99 μl of TE buffer and 1 μl of DNA sample into a single well. Repeat for duplicate well and go to next sample. It is recommended to first prepare a mix of 198 μl TE buffer and 2 μl DNA in a microcentrifuge tube and then aliquot 100 μl of the mixture into two wells.
5. Aliquot 90 μl TE buffer and 10 μl of the lambda standard into a well. Repeat for duplicate well and go to next lambda standard.
6. Prepare PicoGreen[®] solution: aliquot 5 μl of stock PicoGreen[®] into 0.995 ml TE buffer; 100 μl of this mixture is required for each well (keep in dark).
7. Aliquot 100 μl of PicoGreen[®] solution into each well, then mix by pipetting up and down.
8. Incubate in the dark at room temperature for 10 min.
9. Perform fluorometric measurement using a multiplate reader at excitation/emission lengths of 480 nm/520 nm.
10. Calculate the concentration of the stock DNA samples from the standard curve.
11. Prepare a 10 ng/ μl working solution from each stock DNA sample (volume: around 200 μl).

3.3.1.2. Second Round of Quantification of the 10 ng/ μl DNA Samples

Precise quantification

1. Repeat the steps from “First Round of Quantification”. Add 90 μl TE buffer and 10 ng/ μl of DNA sample into each well this time.
2. Measure the concentration of 10 ng/ μl DNA working solutions. 1 and 5 ng/ μl DNA templates will be prepared from each working solution.

3.3.2. Quantification by Nanodrop[®] (For model ND-1000) (see Note 8)

1. Choose the nucleic acid assay option from the Nanodrop software.
2. Initialize the machine with distilled water. Pipette 1 μ l of water onto reading pedestal. Click OK.
3. Wipe off water with clean tissue. Make a blank reading with 1 μ l TE buffer. Click Blank.
4. Place 1 μ l of DNA sample and click measure. After each reading, wipe off with clean tissue.
5. Take triplicate measurements of each sample and use the average of the results.
6. Prepare 10 ng/ μ l working solutions from stocks. Quantify again to get precise measurements.

3.4. Template DNA Preparation and Heat Treatment

1. Prepare 5 ng/ μ l templates in TE buffer from the 10 ng/ μ l working solutions (volume: 200 μ l).
2. Prepare 1 ng/ μ l templates in TE buffer from the 5 ng/ μ l templates (volume: 100 μ l).
3. Aliquot 50 μ l of 1 ng/ μ l DNA into a PCR tube and proceed with heat-treatment in Step 4. The remaining half will serve as the original template.
4. Perform heat treatment on 50 μ l of 1 ng/ μ l DNA by following this PCR protocol: 95°C for 6 min and 10°C for cool down.
5. 5 ng/ μ l DNA will be used for quantifying nuclear markers, and 1 ng/ μ l original and heated DNA will be used for mitochondrial markers.
6. Prepare a five-point standard using the stock DNA of the control sample. The recommended standard is a 5X dilution series ranging from 40 to 0.064 ng/ μ l (see Note 16).

3.5. mtDNA Structural Damage and Repair Analysis Using Real-Time PCR

To ensure optimal reproducibility, always prepare a master mix containing the SYBR Green Supermix and primers, and use aliquots of this mixture with individual DNA samples. As an example, LNCaP cells are treated with exogenous H₂O₂ to measure mtDNA damage during exposure and repair activity during recovery (**Fig. 12.1**). The following protocol is performed in 30 μ l reaction in triplicate using the Bio-Rad system.

1. Mix samples and standards gently by flicking. Centrifuge briefly.
2. Prepare and label 0.5 ml tubes for each sample and standard.

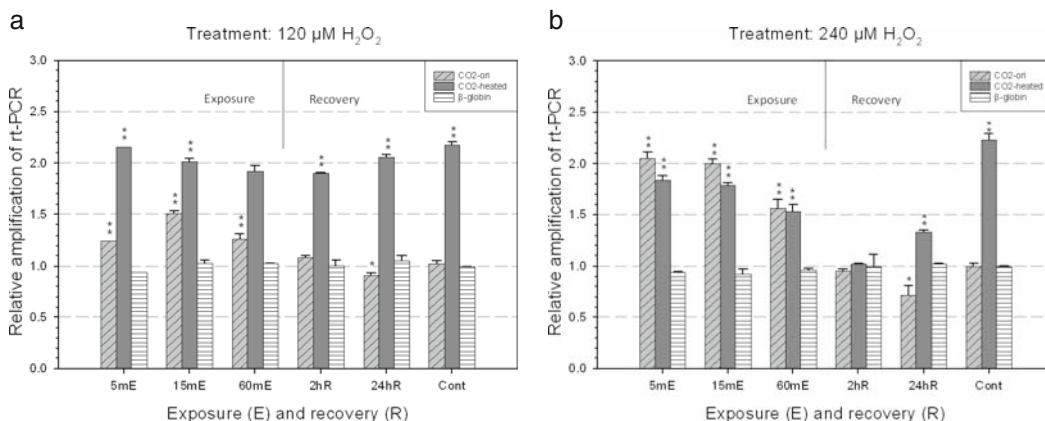


Fig. 12.1. MtDNA damage responses to oxidative damage in LNCaP prostate cancer cells induced by exogenous H_2O_2 treatment. Two concentrations (120 and 240 μM) of H_2O_2 were used to induce oxidative stress in LNCaP cells. For exposure, LNCaP cells were treated for 5, 15, and 60 min to induce DNA damage. For recovery, LNCaP cells were first treated for 60 min, and then allowed to recover in fresh medium for 2 and 24 h. The CO_2 mitochondrial marker was normalized with the nuclear marker β -actin; a second nuclear marker β -globin was shown to indicate the lack of structural change in the target nuclear genes. The *diagonally striped bars* represent the relaxed mtDNA fraction in the original DNA template. The *solid gray bars* represent the total mtDNA content in the pre-heated mtDNA template. Changes in relaxed mtDNA fraction and total mtDNA content correspond to mtDNA damage and copy number change, respectively, in treated samples. **(A)** At low concentration of H_2O_2 (120 μM), LNCaP cells showed early increase of mtDNA structural damage from 5 to 60 minE when compared to control, but the damage was repaired 24 h after the treatment. The total mtDNA content remained stable. **(B)** While at a higher concentration of H_2O_2 (240 μM), LNCaP showed acute early mtDNA structural damage, reflecting complete disruption of mtDNA structure in the original template. The damage was not repaired 24 h after the treatment, resulting in a significant loss of mtDNA content as indicated by decreases of total mtDNA signals in pre-heated templates. Statistics performed by one-way ANOVA, compared to original fraction of control (* = $p < 0.05$, ** = $p < 0.01$) (see **Note 21**).

3. Prepare the total amount of master mix needed by adding the reagents in this specific order (**Table 12.2**), based on a six paired samples (i.e., for six each original and heated 1 ng/ μ l DNA) and five standards.
4. Aliquot 5.4 μ l of DNA template into a 0.5 ml tube, add 84.6 μ l of the master mix. Mix well by pipetting up and down. Put tube on ice until ready to load into wells.
5. Repeat Step 4 for each sample and standard.
6. Aliquot each template mixture into three wells (30 μ l per well). Be careful to deposit the aliquot at the bottom of the well without creating bubbles. Avoid cross-contamination of the wells. Blank solutions without DNA will serve as negatives.
7. Seal the plate with optical tape. If necessary, centrifuge the plate briefly to get rid of bubbles.

Table 12.2
(see Note 17)

Template points (triplicate, 30 μ l/well)	Reactions	Reagents	1X	54X
5 DNA standards	15X	Distilled H ₂ O	10.8 μ l	583.2 μ l
6 Original 1 ng/ μ l DNA	18X	50 mM MgCl ₂	0.6 μ l	32.4 μ l
6 Heated 1 ng/ μ l DNA	18X	→ 10 μ M Forward primer	0.9 μ l	48.6 μ l
1 Blank	3X	10 μ M Reverse primer	0.9 μ l	48.6 μ l
	54X	2X SYBR Mix (Bio-Rad)	15 μ l	810 μ l
			28.2 μ l	1,522.8 μ l

8. PCR program for mtDNA markers on the Bio-Rad system: cycle 1 (1X), 95.0°C for 1.5 min; cycle 2 (30X), step 1 at 95.0°C for 20 sec, step 2 at 61.0°C for 30 sec; cycle 3 (1X), 95.0°C for 1 min; cycle 4 (1X), 55.0°C for 1 min, cycle 5 (40X) 55.0°C for 10 sec with an increase of 1.0°C after each repeat for collecting melt curve data. Enable real-time data collection at cycle 2 step 2 (for ABI system, see Note 18).
9. At least one nuclear DNA marker is analyzed to serve as a reference gene for the analysis. To run nuclear gene, calculate the amount of each reagent based on triplicates of five standards, six 5 ng/ μ l DNA samples, and one blank (36X). The PCR program is the same as in Step 8, except for 40X at cycle 2 (for ABI system, see Note 19).
10. The data can be analyzed with any relative expression software tool (REST) which is based on the Pfaffl formula:

$$R = \frac{\left(\text{Efficiency}_{\text{target gene}}\right)^{\Delta CP_{\text{target}}(\text{Mean Control} - \text{Mean sample})}}{\left(\text{Efficiency}_{\text{reference gene}}\right)^{\Delta CP_{\text{ref}}(\text{Mean Control} - \text{Mean sample})}}$$

Assign a nuclear gene as the reference gene and use the original 1 ng/ μ l from the control sample as the calibrator for analysis (Fig. 12.2) (see Note 20).

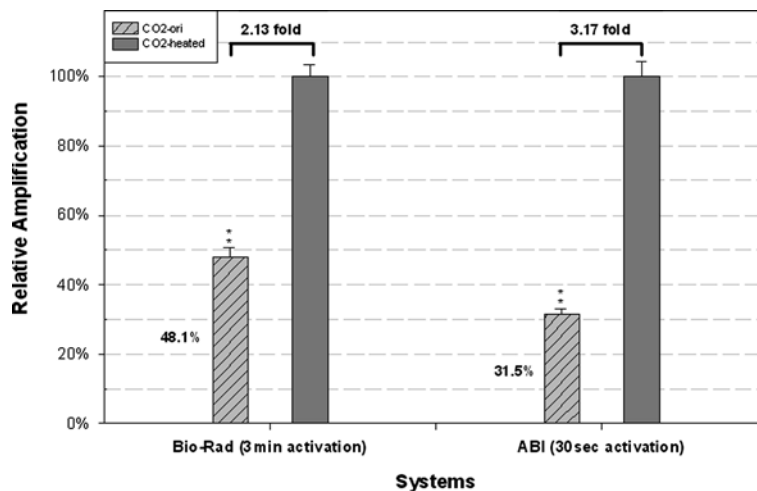


Fig. 12.2. **Baseline levels of mtDNA damage in non-treated prostate cancer cells analyzed by two qPCR systems: Bio-Rad vs ABI.** The baseline level of damage and the spontaneous damage are two distinct features. The baseline level of damage is the amount of mtDNA damage that is being detected by the systems. Artifacts introduced during the sample preparation and the initial heat-activation of real-time PCR can increase the baseline level of damage. On the other hand, spontaneous damage represents the actual amount of endogenous mtDNA damage of non-treated samples. The mtDNA structure is heat sensitive, and consequently the initial enzyme activation step at 95°C required by the real-time PCR protocol can potentially introduce artificial damage to the mtDNA structure. By shortening this step, it is possible to reduce this artificial damage. This figure compares the relative amplification of non-treated C4-2 (an isogenic variant of LNCaP) DNA using Bio-Rad and ABI systems at 3 min and 30 sec of initial 95°C activation, respectively. The Bio-Rad system detected 48.1% of damaged mtDNA, while the ABI system detected 31.5% of damage. A reduction of 34.5% was observed between the two systems, representing the removal of substantial amount of artificial damage by the ABI system. As such the baseline level of damage detected with the ABI system is more representative of the spontaneous level of mtDNA damage. The differences observed between the two systems can be attributed to the shorter initial heat-activation time as well as the different enzyme chemistry used. Statistics were performed with *t*-test, the two groups were found significantly different (Bio-Rad (3 min), $n = 4$; ABI (30 s), $n = 3$; ** = $p < 0.01$) (see **Note 22**).

4. Notes



1. The supercoiled structure inhibits the binding of oligonucleotide primers, preventing further amplification. In contrast, the relaxed forms (open circular and linear) allow effective primer binding and subsequent DNA polymerization. Thus, the relaxed forms of mtDNA are better substrates for PCR than supercoiled mtDNA.
2. Listed materials for prostate cancer LNCaP cells. Substitute with appropriate reagents and buffers for cell lines to be used.
3. LNCaP cells detach easily in regular culture dishes. Prepare PLL coated dishes by adding 3 ml of 1% PLL solution to a 100 mm dish. Incubate for 5 min, then remove all solution from the dish. Dry in fume hood overnight.

4. There are other methods for DNA extraction such as Trizol. It is shown that the Trizol procedure disrupts most structural features from the mtDNA (data not shown).
5. The C1 step removes cellular organelles including mitochondria during preparation, leading to loss of mtDNA.
6. Fresh snap-frozen tissue samples are suitable for structural analysis, but not paraffin-embedded tissues due to crosslinking.
7. The 2-step DNA quantification is optional but recommended. Since this will limit the dynamic range of the samples during real-time PCR amplification, leading to reproducible quantification.
8. A conventional spectrophotometer can also be used for the two-step DNA quantification. We recommend using the Nanodrop spectrophotometer which requires only 1–2 μ l of DNA sample for precise measurement.
9. It is not recommended to repeatedly freeze and thaw the same samples as this can degrade the quality of the DNA. If the tissue sample is to be used on separate occasions, cut the tissue into smaller pieces and thaw only what is needed.
10. Work fast. It is not recommended to thaw the tissue sample until it is ready to be homogenized in the glass homogenizer. If the tissue sample is too hard to cut, thaw on wet ice.
11. After homogenization, there will still be residue pieces of tissue that are not completely homogenated. Do not continue grinding because this will disrupt the DNA structure. Transfer everything to a 2 ml tube. Proteinase K will digest the remaining pieces.
12. Proteinase K is used instead of the protease included in the kit because the former is more efficient in digesting the remaining tissue in the homogenate.
13. Unless the sample is exposed to harsh treatments during experiments, the precipitated DNA should look like long intertwined DNA fibers.
14. The DNA fiber is mostly nuclear DNA; mtDNA is too small to be seen in suspension. The high-speed centrifugation is needed to bring down the mtDNA and to form a pellet with nuclear DNA.
15. Do not over-dry as this will make it harder to dissolve in TE buffer.
16. A standard is needed to take into consideration the PCR efficiency when using Pfaffl's relative quantification model. The standard is not required for the $\Delta\Delta C_t$ method, but the efficiencies of different markers have to be optimized and validated.

17. It is recommended to add an additional 3–4 μl of distilled water to the final mixture to compensate for the loss due to pipetting.
18. PCR program for mtDNA markers on the ABI system: cycle 1 (1X), 95.0°C for 30 sec; cycle 2 (30X), step 1 at 95.0°C for 3 sec, step 2 at 61.0°C for 30 sec; cycle 3 (1X) add melt curve. Enable real-time data collection at cycle 2 step 2.
19. PCR program for nuclear DNA on the ABI system: cycle 1 (1X), 95.0°C for 30 sec; cycle 2 (40X), step 1 at 95.0°C for 3 sec, step 2 at 61.0°C for 30 sec; cycle 3 (1X) add melt curve. Enable real-time data collection at cycle 2 step 2.
20. There are various REST tools that are freely available on the Internet: REST-XL, REST-MCS, and others.
21. Similar results were obtained by analyzing with the D-loop mitochondrial marker.
22. Similar results were observed with other cell lines, frozen tissues, and blood leukocyte samples. The Power SYBR[®] Fast Green PCR MASTER MIX from ABI and its 7500 Fast Real-Time PCR System platform made it possible to cut down the initial 95°C activation time to 30 sec (*see Note 18*). This should be taken into consideration when analyzing clinical samples where small differences in spontaneous damage levels need to be measured.

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