PCR-Based Analysis of Mitochondrial DNA Copy Number, Mitochondrial DNA Damage, and Nuclear DNA Damage

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Because of the role that DNA damage and depletion play in human disease, it is important to develop and improve tools to assess these endpoints. This unit describes PCR-based methods to measure nuclear and mitochondrial DNA damage and copy number. Long amplicon quantitative polymerase chain reaction (LA-QPCR) is used to detect DNA damage by measuring the number of polymerase-inhibiting lesions present based on the amount of PCR amplification; real-time PCR (RT-PCR) is used to calculate genome content. In this unit, we provide step-by-step instructions to perform these assays in *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*, *Oryzias latipes*, *Fundulus grandis*, and *Fundulus heteroclitus*, and discuss the advantages and disadvantages of these assays.

Keywords: qPCR • DNA damage • mitochondrial DNA copy number • mitochondrial DNA • *C. elegans*

How to cite this article:
Gonzalez-Hunt, C.P., Rooney, J.P., Ryde, I.T., Anbalagan, C., Joglekar, R., Meyer, J.N. 2016. PCR-based analysis of mitochondrial DNA copy number, mitochondrial DNA damage, and nuclear DNA damage. *Curr. Protoc. Toxicol.* 67:20.11.1-20.11.25. doi: 10.1002/0471140856.tx2011s67

INTRODUCTION

Certain types of DNA lesions (e.g., bulky adducts and single-strand breaks) have the ability to inhibit or block the action of DNA polymerases (Ponti et al., 1991). Some of these types of damage are repaired via nucleotide excision repair (NER), a repair mechanism that mitochondria lack (LeDoux et al., 1992; Scheibye-Knudsen et al., 2015). As a result, mitochondrial DNA (mtDNA) accumulates these lesions at higher rates than nuclear DNA (nucDNA), decreasing levels of DNA replication and transcription and potentially causing deleterious health effects (Niranjan et al., 1982; Stairs et al., 1983; Meyer et al., 2013). Despite active NER and other repair mechanisms, polymerase-stalling lesions in nuclear DNA can also accumulate with aging as repair capacity decreases (Sedelnikova et al., 2004; Meyer et al., 2007).

Mitochondrial genome depletion occurs in many mitochondrial diseases (Suomalainen and Isohanni, 2010; Copeland, 2012), and has also been implicated in the pathogenesis of common diseases like cancer and Parkinson's disease (Yu, 2011; Coskun et al., 2012).

Given the relevance of DNA damage and depletion in the context of organismal health, it is important to develop and improve tools to assess these endpoints. This unit provides updated protocols to measure nuclear and mitochondrial DNA damage and genome copy number using PCR-based assays initially developed in 1992 (Kalinowski et al., 1992).

Current Protocols in Toxicology 20.11.1-20.11.25, February 2016
Published online February 2016 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/0471140856.tx2011s67

The DNA damage assay is able to quantitatively measure the number of polymerase-stalling lesions based on the amount of amplification obtained from a long amplicon quantitative PCR (LA-QPCR; Kalinowski et al., 1992; Hunter et al., 2010; Furda et al., 2012; Furda et al., 2014). To calculate DNA copy number we utilize a real-time PCR (RT-PCR) assay in which, by using a standard curve or comparing average cycle threshold (Ct) values, we can calculate actual or relative DNA content (Venegas and Halberg, 2012; Rooney et al., 2015). This unit also includes updated support protocols with instructions for sample preparation, quantification, and generation of plasmids for copy number calculation using standard curves. Strengths and limitations of the assay are discussed in the Commentary.

BASIC PROTOCOL 1

ANALYSIS OF MITOCHONDRIAL AND NUCLEAR DNA DAMAGE

The purpose of this assay is to measure the number of DNA lesions capable of blocking or inhibiting polymerase activity. An LA-QPCR is run for the sample of interest, and the amount of resulting amplification will be inversely correlated with the number of polymerase-blocking lesions present in the DNA template. Assuming a Poisson distribution of lesions (Ayala-Torres et al., 2000), the amplification for treated samples can be compared with the amplification for control samples, and a relative lesion frequency can be calculated (Furda et al., 2012; Furda et al., 2014). Control or reference samples are defined as having no damage for the purposes of this calculation.

Materials

Nuclease-free H_2O
LongAmp Hot Start *Taq* 2× Master Mix (New England Biolabs)
10 μM primers (see *APPENDIX 3C*; Kramer and Coen, 2000), diluted in 0.1× TE buffer [1 mM Tris·Cl, pH 8 (*APPENDIX 2A*)/0.1 mM disodium EDTA]
Template DNA, purified

0.2-ml PCR tubes
Microcentrifuge and PCR-tube minicentrifuge
96-well format thermal cycler
Dedicated workstation (e.g., PCR hood equipped with UV lamp for sterilization)

Additional reagents and equipment for the polymerase chain reaction (*APPENDIX 3C*; Kramer and Coen, 2000) and quantification of PCR products (Support Protocol 3)

Long-amplicon quantitative polymerase chain reaction (LA-QPCR)

1. UV-sterilize the work area.
2. If running several samples, prepare a fresh master mix immediately before using by adding its components in the following order: nuclease-free water (16 μl per reaction, for a final volume of 50 μl), LongAmp Master Mix (25 μl per reaction), and primers (2 μl of each 10 μM primer working solution per reaction). Gently mix by inverting the tube, then microcentrifuge briefly at maximum speed to bring the solution to the bottom of the tube.

   We set up reactions at room temperature. Avoid high-speed vortexing of the master mix, as the LongAmp Master Mix contains detergents that will generate foam. Always prepare enough master mix for two more reactions than needed (more than two if running a large number of reactions). We have successfully set up 25-μl instead of 50-μl reactions, to decrease costs, by proportionally reducing all component volumes by 50%.

3. Add 15 ng of purified template DNA (5 μl of 3 ng/μl DNA for a 50-μl reaction) or 5 μl of *C. elegans* lysate to each 0.2-ml PCR tube. Also include no-template

and 50% control reactions; they will be used for background subtraction and cycle number optimization (described in more detail below).

No-template and 50% controls must be included for quality-control purposes. Nuclease-free water or 0.1× TE buffer must be used in place of the DNA template for the no-template control. Control template DNA must be diluted 1:1 with nuclease-free water or 0.1× TE buffer and then used as template for the 50% control reactions. To reduce pipetting error, prepare a larger volume (at least 20 µl) of 50% template than needed.

4. Carefully dispense 45 µl of the master mix prepared in step 2 into each PCR tube, avoiding introduction of bubbles or residual liquid on tube walls. Spin down using a PCR-tube minicentrifuge.

5. Set up the thermal cycler with the appropriate reaction conditions. Our standard conditions are: an initial denaturation step of 2 min at 94°C, followed by an optimized number of cycles of a denaturation step of 15 sec at 94°C and a combined annealing/extension step of 12 min at 62° to 68°C (11 min, 30 sec works for *C. elegans*). Include a final extension step of 10 min at 72°C. Refer to Table 20.11.1 for the target-specific PCR parameters we have optimized in the laboratory.

   The recommended annealing/extension temperature range is based on our optimized primers from Table 20.11.1; this temperature will vary based on the primers used (see Table 20.11.2). The optimal cycle number is the one at which the PCR is in the exponential phase i.e., a 50% dilution of a template results in approximately 50% the amplification observed for the 100% template.

6. Place your PCR reactions into the thermal cycler and start the program. Make sure the thermal cycler lid is heated to 99°C throughout the entire PCR reaction time. UV-sterilize the workstation once finished.

7. Keep the reaction products at 4° to 8°C until ready to analyze.

Product quantification and quality check

8. Quantify PCR products following the steps listed in Support Protocol 3. In this case, 10 µl of PCR product are used per well instead of DNA extract (add 90 µl of 1× TE instead of 95 µl to account for the volume difference). Also, the PCR product does not need to be diluted. Refer to Supplemental File 1 (*http://www.currentprotocols.com/protocol/tx2011*) for an example of this procedure.

   Be careful not to open tubes with PCR product in the same room used for setting up the PCR. This prevents cross-contamination of new PCR reactions, which is critical because the same reaction, amplifying the same target, is repeated over and over and the product can volatilize and contaminate an entire room. When running LA-QPCR with new parameters or for the first time, run the PCR products on an agarose electrophoresis gel first to make sure a unique product of the correct size is present (no extra bands). After only one product is observed, perform the product quantification.

9. Average the duplicate fluorescence values for all samples. Subtract the average value of the "no-template control" (blank) sample from all other samples. Make sure that the value of the blank-corrected 50% control signal is 40% to 60% of the blank-corrected control sample signal.

   The fluorescence value for the "no-template control" (blank) should be between 5000 and 7000 (this value will vary based on the plate reader used, but once established will be consistent over time). Higher values are indicative of cross-contamination. If the 50% control is between 40% and 60% of the control sample, this indicates that the PCR reaction is in the exponential phase, meaning that all amplification is directly proportional to the starting amount of template. If this is not the case, the cycle number needs to be increased or decreased in order to achieve exponential amplification.

Table 20.11.1 LA-QPCR primers, Targets, and Conditions Optimized for LongAmp PCR Kit

Species	Genome	Forward primer seq.	Reverse primer seq.	Annealing temp. (°C)	Cycle number	Reference
C. elegans	mt	10.9-kb mito fragment		64	26	This unit; Hunter et al. (2010)
		5′-CCA TCA ATT GCC CAA AGG GGA GT-3′	5′-TGT CCT CAA GGC TAC CAC CTT CTT CA-3′			
	nuc	9.3-kb nuc fragment from the *unc-2* gene		64	29	This unit; Hunter et al. (2010)
		5′-TGG CTG GAA CGA ACC GAA CCA T-3′	5′-GGC GGT TGT GGA GTG TGG GAA G-3′			
M. musculus	mt	10-kb mito fragment		64	17	This unit; Furda et al. (2012)
		5′-GCC AGC CTG ACC CAT AGC CAT AAT AT-3′	5′-GAG AGA TTT TAT GGG TGT AAT GCG G-3′			
	nuc	8.7-kb nuc fragment of the β-globin gene, accession number X14061		62	27	This unit; Ayala-Torres et al. (2000)
		5′-TTG AGA CTG TGA TTG GCA ATG CCT-3′	5′-CCT TTA ATG CCC ATC CCG GAC T-3′			
H. sapiens	mt	8.9 kb mito fragment, accession number J01415		64	17	This unit; Furda et al. (2012)
		5′-TCT AAG CCT CCT TAT TCG AGC CGA-3′	5′-TTT CAT CAT GCG GAG ATG TTG GAT GG-3′			
	nuc	13.5-kb nuc fragment from the 5′ flanking region near the β-globin gene, accession number J00179		64	28	This unit; Furda et al. (2012)
		5′-CGA GTA AGA GAC CAT TGT GGC AG-3′	5′-GCA CTG GCT TAG GAG TTG GAC T-3′			
D. rerio	mt	10.3-kb mito fragment		66	29	This unit; Hunter et al. (2010)
		5′-TTA AAG CCC CGA ATC CAG GTG AGC-3′	5′-GAG ATG TTC TCG GGT GTG GGA TGG-3′			
	nuc	10.7-kb nuc fragment of the AHR2 gene		68	30	
		5′-AGA GCG CGA TTG CTG GAT TCA C-3′	5′-GTC CTT GCA GGT TGG CAA ATG G-3′			
F. heteroclitus	mt	13.1-kb mitochondria fragment, accession number NC_012312		63	22	This unit; Hunter et al. (2010), Jung et al. (2009)
		5′-AAG GAA ACA AGG AGC CGG TA-3′	5′-ACG TAG CGA GAA GGG TTA GG-3′			
	nuc	11.5-kb fragment of the CFTR gene, accession number AY028263		65	29	
		5′-CAG CCG CCC GCA AAT TCT CA-3′	5′-CAG AAT GCG GGC CTT GCT GA-3′			

continued

Table 20.11.1 LA-QPCR primers, Targets, and Conditions Optimized for LongAmp PCR Kit, *continued*

Species	Genome	Forward primer seq.	Reverse primer seq.	Annealing temp. (°C)	Cycle number	Reference
R. norvegicus	mt	12.1-kb mito fragment		66	19	This unit
		5′- TCG CCC CAA CCC TCT CCC TT -3′	5′- TGG GCG GAA TGT TAA GCT GCG T -3′			
	nuc	12.9-kb nuc fragment from chromosome 12 genomic scaffold 5380		66	23	This unit
		5′- CCT GCT GGG CTT GCC TTG GT -3′	5′- AGC AGG GGA GGT GGA TGG GA -3′			

10. As a quality control check, LA-QPCR for each sample needs to be run twice, and the values obtained should to be compared using correlation analysis. Plot the blank-corrected values from one run against the other and calculate the correlation coefficient. If the correlation is good (we recommend $r^2 > 0.9$), the values for replicate PCRs are averaged; if it is poor, a third LA-QPCR run is necessary in order to eliminate outliers. Comparing the values obtained from all three runs using correlation analysis helps identify and remove outliers.

Calculating DNA lesion frequencies

11. Average all copy number values (obtained by following Basic Protocol 2), and divide each sample's copy number value by this average.

    Skip this step if using the logarithmic curve method described in Basic Protocol 2.

12. Divide each sample's blank-corrected fluorescence value by its corresponding ratio obtained in step 11. If using the logarithmic curve method described in Basic Protocol 2, divide by the calculated normalization factors here, instead of the ratios from step 11. This is done to normalize the amount of PCR product to the amount of DNA copies in each template.

13. Average all normalized fluorescence values for the control samples, and divide each normalized sample value by this average. The resulting number is the amplification relative to control.

    Each normalized control value is also divided the by the average of all normalized control values.

14. Take the negative natural logarithm (–ln) of each relative amplification value. The resulting number is the lesion frequency for each sample. We usually represent this value as lesions per 10 kb. In order to do this, multiply the lesion frequency by 10 and divide by the size (in kb) of the LA-QPCR DNA target. Refer to Supplemental Files 1 and 3 (*http://www.currentprotocols.com/protocol/tx2011*) to see lesion number calculations.

    Lesion frequencies need to be calculated for at least two separate LA-QPCR runs of the same sample (technical replicates). We usually collect three different samples from each treated and control group (biological replicates), and perform the experiment at least twice (total $n = 6$ at minimum).

Statistical analysis

15. Take the average of the lesions/10 kb values of biological replicates; this is the level of DNA damage for the treatment or control group.

16. Graph the averaged lesions/10 kb values as mean ± standard error (use standard deviation if interested in representing the distribution of damage levels within a population).

Table 20.11.2 Long- and Short-Amplicon QPCR Primers and Targets (Not Yet Optimized for LongAmp PCR kit)

Species	Genome	Forward primer seq.	Reverse primer seq.	Annealing temp. (°C)	Reference
M. musculus	mt	117-bp mito fragment		60	Ayala-Torres et al. (2000), Furda et al. (2012)
		5′-CCC AGC TAC TAC CAT CAT TCA AGT-3′	5′-GAT GGT TTG GGA GAT TGG TTG ATG T-3′		
	nuc	6.6-kb nuc fragment of the DNA polymerase gene β, accession number AA79582		64	Furda et al. (2012)
		5′-TAT CTC TCT TCC TCT TCA CTT CTC CCC tgg-3′	5′-CGT GAT GCC GCC GTT GAG GGT CTC CTG-3′		
H. sapiens	mt	221-bp mito fragment		62	Furda et al. (2012)
		5′-CCC CAC AAA CCC CAT TAC TAA ACC CA-3′	5′-TTT CAT CAT GCG GAG ATG TTG GAT GG-3′		
	nuc	12.2-kb nuc fragment from region of the DNA polymerase gene β, accession number L11607		64	Furda et al. (2012)
		5′-CAT GTC ACC ACT GGA CTC TGC AC-3′	5′-CCT GGA GTA GGA ACA AAA ATT GCT G-3′		
		10.4-kb nuc fragment encompassing exons 2–5 of HPRT gene, accession number J00205		64	Furda et al. (2012)
		5′-TGG GAT TAC ACG TGT GAA CCA ACC-3′	5′-GCT CTA CCC TGT CCT CTA CCG TCC-3′		
D. melanogaster	mt	151-bp mito fragment		61	Hunter et al. (2010)
		5′-GCT CCT GAT ATA GCA TTC CCA CGA-3′	5′-CAT GAG CAA TTC CAG CGG ATA AA-3′		
		14.2-kb mito fragment		66	
		5′-GCC GCT CCT TTC CAT TTT TGA TTT CC-3′	5′-TGC CAG CAG TCG CGG TTA TAC CA-3′		
	nuc	152-bp nuc fragment		65	
		5′-CGA GGG ATA CCT GTG AGC AGC TT-3′	5′-GTC ACT TCT TGT GCT GCC ATC GT-3′		
		11.5-kb nuc fragment of the β-tubulin gene		67	
		5′-GTA TTC CTG CGC CAG GAG GAT CG-3′	5′-CAG ATG CTG GAG CTG CCT TTG GA-3′		
		10.3-kb nuc fragment of the β-tubulin gene		67	
		5′-GAG GAG CCT TGC GAA CAA CAG CA-3′	5′-CAA TGA CAG CTG CGC CTC GAG AT-3′		
D. rerio	mt	198-bp mito fragment		62	Hunter et al. (2010)
		5′-CAA ACA CAA GCC TCG CCT GTT TAC-3′	5′-CAC TGA CTT GAT GGG GGA GAC AGT-3′		
	nuc	233-bp nuc fragment		60	
		5′-ATG GGC TGG GCG ATA AAA TTG G-3′	5′-ACA TGT GCA TGT CGC TCC CAA A-3′		

continued

Table 20.11.2 Long- and Short-Amplicon QPCR Primers and Targets (Not Yet Optimized for LongAmp PCR kit), *continued*

Species	Genome	Forward primer seq.	Reverse primer seq.	Annealing temp. (°C)	Reference
C. elegans	mt	195-bp mito fragment		63	Hunter et al. (2010), Meyer et al. (2007)
		5′-CAC ACC GGT GAG GTC TTT GGT TC-3′	5′-TGT CCT CAA GGC TAC CAC CTT CTT CA-3′		
	nuc	225-bp nuc fragment		63	Meyer et al. (2007), Boyd et al. (2010)
		5′-TCC CGT CTA TTG CAG GTC TTT CCA-3′	5′-GAC GCG CAC GAT ATC TCG ATT TTC-3′		
		13.7-kb nuc fragment from the polymerase epsilon gene		68	Hunter et al. (2010)
		5′-AGT CGT TGA ACG CAG TGG TGT CAT-3′	5′-CAG TCT TTC TTC GAC GCA TTC AAC G-3′		
O. latipes	mt	184-bp mito fragment		59	Rooney et al. (2015)
		5′-AAC TCC AAG TAG CAG CTA TGC AC-3′	5′-GAG GGG TAG AAG GCT TAC AAA AA-3′		
	nuc	140-bp nuc fragment		57	
		5′-CTC ACA AAC ATC TTT GCA CTC AG-3′	5′-AGA ACC TCT CTC CAA AAC ATT CC-3′		
F. grandis	mt	206-bp mito fragment		55	
		5′-TTT ACA CAT GCA AGT ATC CG-3′	5′-CCG AAG GCT ATC AAC TTG AG-3′		
	nuc	234-bp nuc fragment		62	
		5′-GCC GCT GCC TTC ATT GCT GT-3′	5′-ATG AGC TGG GTG TGC GCT GA-3′		
F. heteroclitus	nuc	234-bp nuc fragment		62	Jung et al. (2009), Hunter et al. (2010)
		5′-GCC GCT GCC TTC ATT GCT GT-3′	5′-ATG AGC TGG GTG TGC GCT GA-3′		

17. Perform statistical analysis with parametric tests. If comparing two samples, perform a *t* test or one-way analysis of variance (ANOVA). If comparing more than two samples, always perform an ANOVA. If there is more than one independent variable perform a multifactor ANOVA first; if the result is significant then compare desired subsets of the data with post-hoc tests.

In our experience, DNA damage data has always been normally distributed. In the event that your dataset is not, data transformation techniques or non-parametric alternatives to the tests recommended in this step should be used.

ANALYSIS OF MITCHONDRIAL AND NUCLEAR GENOME COPY NUMBER

BASIC PROTOCOL 2

Although the number of genome copies can be measured by amplifying a short target sequence using the quantitative PCR assay described in steps 1 to 10 of Basic Protocol 1 (primers for short-amplicon QPCR are in Table 20.11.2; Furda et al., 2012; Rooney et al., 2015), we currently perform this analysis using RT-PCR instead. This method is particularly advantageous if a standard curve is run along with the samples of interest, because the actual number of copies can be calculated (Bratic et al., 2009; Leung

et al., 2013). Mitochondrial DNA content from purified DNA samples can be calculated without a standard curve by using the comparative Ct method, resulting in a measure of mtDNA content relative to nucDNA copy number (Venegas and Halberg, 2012; Rooney et al., 2015), or, if the goal is to normalize the results from LA- QPCR (Basic Protocol 1, step 12), by creating a logarithmic curve based on the average genome Ct value (see below).

Materials

Nuclease-free H_2O
100,000 copies/μl aliquots of pCR 2.1 plasmid containing cloned species-specific nuclear or mitochondrial gene – if calculating with standard curve (refer to Support Protocol 5)
Power SYBR Green PCR Master Mix (Life Technologies)10 μM primers (see *APPENDIX 3C*; Kramer and Coen, 2000), diluted in 0.1× TE buffer [1 mM Tris·Cl, pH 8 (*APPENDIX 2A*)/0.1 mM disodium EDTA]
Template DNA, purified, or 40 μl young adult (24 hr post-L4) *glp-1* worm lysate (20 worms; 1567 copies/μl; alternative to *C. elegans* nuclear plasmid for standard curve calculations; refer to Support Protocol 1)

Sterile, aerosol filter tips and pipettors dedicated to LA-QPCR
Optical 96-well PCR plate and optical adhesive film
Plate vortexer
Centrifuge
Real-time PCR system

Real-time PCR (RT-PCR)

1. Prepare a serial dilution with which to calculate a standard curve. Using 0.2-ml PCR tubes, proceed as follows. Dilute a 100,000 copies/μl aliquot of the plasmid down to 32,000 copies/μl and 24,000 copies/μl. Serially dilute each preparation 1:1 until a 2000 copies/μl dilution (32,000 copies/μl preparation) and a 3000 copies/μl dilution (24,000 copies/μl preparation) are obtained. If calculating nuclear copy number for worms, and using worm *glp-1* lysate instead of a plasmid, add 40 μl of nuclease-free water to lysate; concentration will now be 784 copies/μl. Serially dilute this preparation 1:1 until getting a 24.5 copies/μl dilution.

   Skip this step if not calculating copy number using a standard curve. Single-use aliquots of plasmids and glp-1 lysates are used to prevent freeze/thaw cycles. Glp-1 worms are used because when grown at 25°C they do not develop a germline, and therefore have a constant number of nuclear DNA copies, as described in Leung et al. (2013). Plotting the Ct values obtained against the known number of copies per standard dilution, and analyzing by logarithmic regression, allows us to calculate the exact number of copies in our sample.

2. If running several samples, prepare a fresh master mix immediately before using by adding its components in the following order: nuclease-free water (8.5 μl per reaction, for a final volume of 25 μl), Power SYBR Green Master Mix (12.5 μl per reaction), and primers (1 μl of each 10 μM primer working solution per reaction). Gently mix and microcentrifuge briefly at maximum speed to bring the solution to the bottom of the tube.

   We set up reactions at room temperature. Always prepare enough master mix for two more reactions than needed (more than two if running a large number of reactions).

3. Aliquot 23 μl of the master mix prepared in step 2 into each well of the PCR plate.

4. Add 2 μl of each plasmid dilution to triplicate wells in the PCR plate, including a 0 copies control (nuclease-free water or 0.1× TE buffer).

   The number of plasmid copies per well for the standard curve would be as follows: 64,000, 48,000, 32,000, 24,000, 16,000, 12,000, 8000, 6000, 4000. If using glp-1 worm lysate, the number of nuclear DNA copies per well for the standard curve would be as follows: 1,568, 784, 392, 196, 98, and 49.

   Skip this step if not calculating copy number with a standard curve.

5. Add 6 ng of purified template DNA (2 μl of 3 ng/μl DNA for a 25 μl reaction) or 2 μl of worm lysate to wells in triplicate. Also include a no-template control reaction.

   A no-template control must be included for quality-control purposes. If running plasmid serial dilutions for a standard curve, then the 0 copies/μl reaction is the no-template control. Nuclease-free water or 0.1× TE buffer must be used in place of the DNA template.

6. Cover plate with optical film and spin, vortex, then spin again (vortex at around 1650 rpm for 30 sec using a plate vortexer; centrifuge at 400 × *g*for 10 sec).

7. Set up a program in the RT-PCR system with the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 60 sec at primer-specific annealing temperature.

   A dissociation curve is also calculated to ensure presence of only one PCR product.

   Refer to Table 20.11.3 for target-specific PCR parameters we have optimized in the laboratory.

8. Place your PCR reactions on the real-time system and start the program.

   We usually perform one RT-PCR run per sample, with triplicate technical and biological replicates, and perform the experiment at least twice (total n = 6 at minimum).

Data analysis

9. Obtain the Ct information from the PCR results. Compare the triplicate values to each other; if any given one varies by more than 0.5 Ct from the others, it can be thrown out of the calculations. Average technical replicates.

10. Plot standard curve Ct values against the known number of copies in each well, and perform a logarithmic regression.

    The resulting equation should be as follows: $Ct = m*ln(x) + b$, where m is the slope, b is the y-intercept and x is the sample's genome copy number. Another way to look at this equation is by isolating the x variable as follows: $x = e^{(Ct - b)/m}$

    Skip this step if not calculating copy number with a standard curve.

11. Use the equation from step 10 and calculate the copy number for each sample. If working with worm lysates as DNA template, obtain the copy number per worm by multiplying the total copy number per sample by the template volume equivalent to one worm (e.g., if 6 worms were picked into 90 μl, then you would multiply by 15), and then divide by the volume of template added to the well (2 μl). Refer to Supplemental File 2 (*http://www.currentprotocols.com/protocol/tx2011*) for example calculations.

    Skip this step if not calculating copy number with a standard curve.

12. To calculate the mitochondrial genome content of purified DNA samples, the comparative Ct method can be used instead of a standard curve (Venegas and Halberg,

Table 20.11.3 Real-Time PCR Primers and Targets

Species	Genome	Forward primer seq.	Reverse primer seq.	Annealing temp. (°C)	Reference
C. elegans	mt	75-bp mito fragment of nd1 mito		60	Bratic et al. (2009)
		5′-AGC GTC ATT TAT TGG GAA GAA GAC-3′	5′- AAG CTT GTG CTA ATC CCA TAA ATG T -3′		
	nuc	164-bp nuc fragment of cox4		60	Rooney et al. (2015)
		5′-GCC GAC TGG AAG AAC TTG TC-3′	5′-GCG GAG ATC ACC TTC CAG TA-3′		
D. rerio	mt	195-bp *nd1* mito fragment		60	This unit
		5′-CGT TTA CCC CAG ATG CAC CT-3′	5′-GTG CGA TTG GTA GGG CGA TA-3′		
	nuc	90-bp nuc fragment of *vtg2*		60	
		5′-TGG ATA CCT GAC CGA GAG CT-3′	5′-AGA CAA CTC TTA CGG CTG GC-3′		
H. sapiens	mt	107-bp mito fragment of tRNA-Leu(UUR) gene		62	Venegas and Halberg (2012)
		5′-CAC CCA AGA ACA GGG TTT GT-3′	5′-TGG CCA TGG GTA TGT TGT TA-3′		
	nuc	86-bp nuc fragment of β2-microglobulin gene		62	
		5′- TGC TGT CTC CAT GTT TGA TGT ATC T -3′	5′- TCT CTG CTC CCC ACC TCT AAG T -3′		
R. norvegicus	mt	181-bp mito fragment		60	This unit
		5′- CAA ACC TTT CCT GCA CCT CC -3′	5′- AGG CGT TCT GAT GAT GGG AA -3′		
	nuc	144-bp nuc fragment from 3′,5′-cyclic AMP phosphodiesterase (PDE4-1, PDE4-2) gene		62	
		5′-GTT CCC GCC TTC TTC CTC TG-3′	5′-GTT TGC TTG CCG ACT CCT TG-3′		
F. heteroclitus	mt	131-bp mito fragment of 16 S rRNA gene		60	This unit
		5′-AAA ATT AAC GGC CCC AAC CC-3′	5′-CCG AGT TCC TTC TTC CCC TT-3′		
	nuc	234-bp nuc fragment of CFTR gene		60	Jung et al. (2009), Hunter et al. (2010)
		5′-GCC GCT GCC TTC ATT GCT GT-3′	5′-ATG AGC TGG GTG TGC GCT GA-3′		

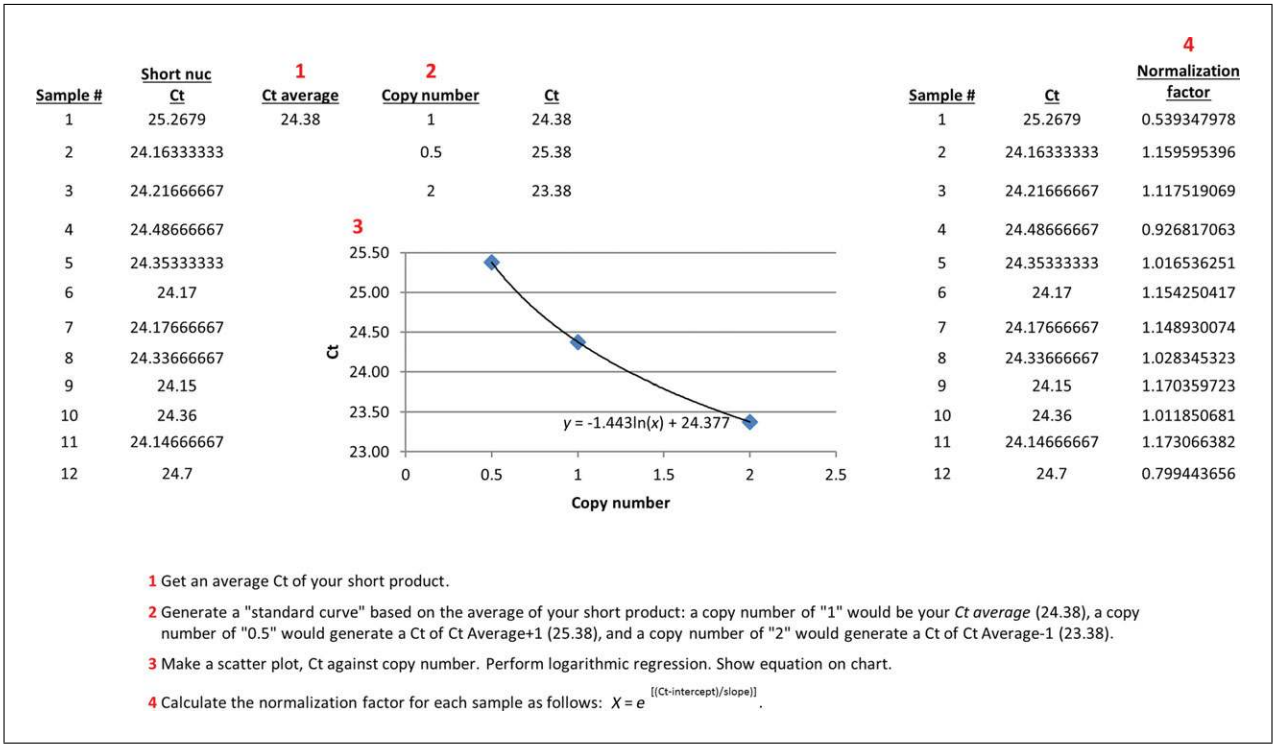
2012). Subtract the mtDNA averaged Ct values from the nucDNA averaged Ct values (both from step 9); this is the ΔCt. Calculate the relative mitochondrial DNA content by raising 2 to the power of ΔCt and then multiplying by 2 (calculate fold difference). Expressed as equations this would be:

$$\Delta Ct = \text{nucDNA Ct} - \text{mtDNA Ct}$$

$$\text{Relative mitochondrial DNA content} = 2 \times 2^{\Delta Ct}$$

Skip this step if calculating copy number with a standard curve. Note that this method only provides mtDNA content relative to nucDNA; if a calculation of actual genome copy number is desired, utilize standard curves.

13. If copy number is being calculated solely for normalization of LA-QPCR, a normalization ratio can be obtained without utilizing standard curves or the comparative Ct method. This is accomplished by creating a logarithmic curve using the Ct values



Sample #	Short nuc Ct	1 Ct average	2 Copy number	Ct
1	25.2679	24.38	1	24.38
2	24.16333333		0.5	25.38
3	24.21666667		2	23.38
4	24.48666667			
5	24.35333333			
6	24.17			
7	24.17666667			
8	24.33666667			
9	24.15			
10	24.36			
11	24.14666667			
12	24.7			

Sample #	Ct	4 Normalization factor
1	25.2679	0.539347978
2	24.16333333	1.159595396
3	24.21666667	1.117519069
4	24.48666667	0.926817063
5	24.35333333	1.016536251
6	24.17	1.154250417
7	24.17666667	1.148930074
8	24.33666667	1.028345323
9	24.15	1.170359723
10	24.36	1.011850681
11	24.14666667	1.173066382
12	24.7	0.799443656

Figure 20.11.1 Calculation of normalization factors for DNA damage assay using logarithmic regression. A standard curve is generated based on the calculated Ct values and corresponding hypothetical copy numbers. Logarithmic regression is performed and the resulting equation is utilized to calculate normalization factors.

for all samples. To do this, average all Ct values for the experiment (after averaging all technical replicates per sample in step 9). Generate a "standard curve" based on the experiment Ct average; designate a copy number of "1" to the *Ct average*. Calculate the Ct for a copy number of "0.5" with the equation Ct = Ct Average +1, and calculate it for a copy number of "2" with the equation Ct = Ct Average –1. Make a scatter plot with these hypothetical values, Ct against copy number. Perform logarithmic regression; the equation will be $Ct = m*\ln(x) + b$, where x is the sample's hypothetical copy number.

Another way to look at this equation is by isolating the x variable as follows: $x = e^{(Ct - b)/m}$. *Calculate x for each sample using the equation from the exponential regression; this is the normalization factor (see Fig. 20.11.1 for a visual representation of this method; refer to Supplemental File 3 (http://www.currentprotocols.com/protocol/tx2011) for example calculations).*

Skip this step if calculating copy number with a standard curve.

14. Perform statistical analysis with parametric tests. If comparing two samples, perform a *t* test or one-way analysis of variance (ANOVA). If comparing more than two samples, always perform an ANOVA. If there is more than one independent variable, perform a multifactor ANOVA first; if the result is significant, then compare desired subsets of the data with post-hoc tests.

15. Data obtained from *C. elegans* lysates is typically best represented graphically as normalized to nucDNA copy number in order to indicate copy number per cell. Normalize mtDNA copy number from each sample by dividing the number of mtDNA copies per worm by the number of nucDNA copies per worm. Graph this data as a mtDNA:nucDNA ratio.

Skip this step if not working with C. elegans lysates.

SUPPORT PROTOCOL 1

DNA TEMPLATE EXTRACTION FROM *C. elegans* (SMALL NUMBER OF WORMS)

Worms of interest are lysed to obtain their DNA and use it as a template for PCR reactions. This method is much faster and less labor-intensive than the traditional batch DNA extraction (Hunter et al., 2010; Furda et al., 2012; Rooney et al., 2015).

Materials

Worm lysis buffer (see recipe)
Worms of interest
Platinum wire worm pick

0.2-ml PCR tubes
Ice or cryogenic 96-well plate (PCR cooler)
−80°C freezer
96-well format thermal cycler or heat block

1. Aliquot 90 µl of worm lysis buffer into each PCR tube. Place PCR tubes with buffer on ice or on a cryogenic 96-well plate.
2. Pick six worms (L4 stage or later) and place into each PCR tube. Immediately place tubes with worms in the −80°C freezer. Do not leave worms on buffer unfrozen for more than 5 min.

   If using worms younger than L4 stage, pick 9 worms per 90 µl of lysis buffer instead.
3. Place tubes with worms in the −80°C freezer for at least 10 min in order to disrupt the nematode's cuticle.
4. Set up a program in the thermal cycler to lyse the worms as follows: 65°C for 1 hr, then 95°C for 15 min, and once finished hold at 4° to 8°C.
5. Remove PCR tubes from the −80°C freezer and place in the thermal cycler. Start program. Make sure lid is set to be heated to 99°C.
6. Use worm lysate as template for LA-QPCR immediately or store at −80°C until further use.

   In our experience, worm lysate cannot be stored at 4°C if it is intended for use in LA-QPCR. Storing at −80°C, even if this exposes the lysate to multiple freeze/thaw cycles, preserves the integrity of the DNA better than storing it at 4°C. This is not an issue if the lysate is going to be used solely for short amplicon PCR or RT-PCR.

SUPPORT PROTOCOL 2

DNA TEMPLATE EXTRACTION FROM *C. elegans* (LARGE NUMBER OF WORMS) OR ANIMAL TISSUE

Traditionally, DNA is extracted from the cells or tissue of interest and purified before use in PCR reactions. Extracting DNA from cells can be done easily following standard procedures and commercial kits that result in very high molecular weight DNA that is not oxidized during extraction; we recommend the Qiagen Genomic-tip 20/G kit, using a 1×10^6 cell pellet and following the kit's tissue protocol (the cell culture protocol isolates the nuclei and discards the mtDNA). However, extracting DNA from other samples like animal tissue or large numbers of *C. elegans* requires special care to preserve adequate DNA integrity for LA-QPCR. Before using the extraction kits, these samples must also be snap frozen first and manually ground (or homogenized if using fresh soft tissue; Hunter et al., 2010; Furda et al., 2012).

Materials

Worms or tissue of interest

K medium (see recipe)
20% glycerol solution (for animal tissues)
Liquid nitrogen
Genomic-tip 20/G kit (Qiagen) including:
- Buffer G2
- Buffer QBT
- Buffer QC
- Buffer QF
- RNase A
- Proteinase K
- Isopropanol
- 70% ethanol

15-ml screw-cap conical tubes
Orbital shaker
Mortar and pestle
1- to 2-ml cryotubes
Handheld homogenizer (for softer animal tissue)
Spatula, sterile

1. Wash off 3000 to 5000 worms from a culture dish with K medium into a 15-ml screw-cap conical tube and centrifuge 2 min at 2200 × *g*, room temperature. Carefully remove supernatant and refill the tube with 10 ml of K medium. Place tube on shaker for 20 min in order to allow worms to clear their guts.

   Skip this step if extracting DNA from animal tissue.

2. Centrifuge tube for 2 min at 2200 × *g*, room temperature. Perform a wash by carefully removing supernatant, refilling tube with K medium and then centrifuging again using the same parameters. Repeat washing step, this time leaving a small volume of the supernatant behind.

   Skip this step if extracting DNA from animal tissue.

3a. *For worms:* Using a Pasteur pipet, resuspend worms in leftover supernatant and freeze them by dripping the suspension directly into a mortar containing liquid nitrogen.

   Skip this step if extracting DNA from animal tissue. Frozen worm pellets can be placed in cryotubes and stored at −80°C until further processing.

3b. *For animal tissue:* Snap freeze tissue samples (up to 20 mg per sample; optional for soft tissues) by placing the tissue in a cryotube with 20% glycerol solution and storing it at −80°C.

4. Cool mortar and pestle by packing dry ice underneath the mortar, pouring liquid nitrogen on the mortar and pestle, and letting it evaporate.

5. Place about six frozen worm pellets (step 3a) or the frozen tissue sample (step 3b) on the chilled mortar and carefully grind with the pestle until a squeaking sound is heard.

   If using fresh soft tissue, homogenize it directly in buffer G2 and RNase A following the protocol provided by the Genomic-tip kit.

6. Using a sterilized spatula, scoop up powder from mortar into a 15-ml conical tube with buffer G2 and RNase A as described in the protocol provided by the Genomic-tip kit.

7. Continue following the protocol provided by the Genomic-tip kit in order to isolate DNA.

It is possible to extract DNA from cultured cells or tissue with an automated protocol for an instrument such as the QIAcube (Qiagen), but in this case mtDNA must be digested prior to amplification. Refer to Furda et al. (2012) and see Support Protocol 4.

SUPPORT PROTOCOL 3

DNA QUANTIFICATION

An important step in ensuring a successful PCR is accurately measuring the amount of template DNA. The same amount of template must be used for all samples and all runs. This quantification is not necessary if using worm lysates, as a precise number of age-synchronized worms were picked into the lysis buffer (in principle, it would similarly be possible to base LA-QPCR on small nucDNA quantification, although we have not optimized this approach). PicoGreen dye is used, as it fluoresces >1,000-fold brighter when bound to DNA, and has a 25 pg/ml limit of detection.

Materials

λ DNA/Hind III Fragments (Invitrogen)
1× TE buffer: 10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)/1 mM EDTA
0.1× TE buffer 1 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)/0.1 mM EDTA
Extracted DNA samples
50-μl aliquots (stored at −20°C) of Quant-iT PicoGreen dsDNA reagent (Molecular Probes)

96-well white- or black-bottomed plates
Fluorescence plate reader capable of measuring 485 nm excitation and 528 nm emission

1. Prepare a DNA serial dilution to calculate a standard curve by diluting λ DNA/Hind III fragments at 15, 10, 5, 2.5, 1.25, and 0 ng/μl in 1× TE buffer.

   We recommend preparing large volumes (e.g., 2 ml) of the standard curve solutions and using them repeatedly in order to reduce variability among measurements. Store at 4°C.

2. Dilute the DNA extracts 1:10 in 1× TE buffer in order to get the DNA concentration in the range of the standard curve.

   Depending on the amount of worms, cells, or tissue used for extraction, it might be necessary to further dilute the DNA if the values are too high, or use the undiluted extract if the values are too low.

3. Add 95 μl of 1× TE buffer to each well to be used in the 96-well plate (add 90 μl to standard wells).

   The number of wells needed is the number of samples and standards in duplicate.

4. Add 5 μl of each sample and 10 μl of each standard to the wells containing 1× TE buffer, in duplicate.

5. Turn the overhead lab lights off to prepare PicoGreen solution. Remove a PicoGreen aliquot from the −20°C freezer and allow it to thaw at room temperature. Once thawed, prepare a solution of 5 μl PicoGreen per 1 ml of 1× TE buffer.

   You need 100 μl of this solution per well undergoing quantification. Make excess PicoGreen solution to account for pipetting loss.

6. Add 100 μl of PicoGreen solution to each well to be analyzed. Cover plate with aluminum foil and incubate at room temperature for 10 min.

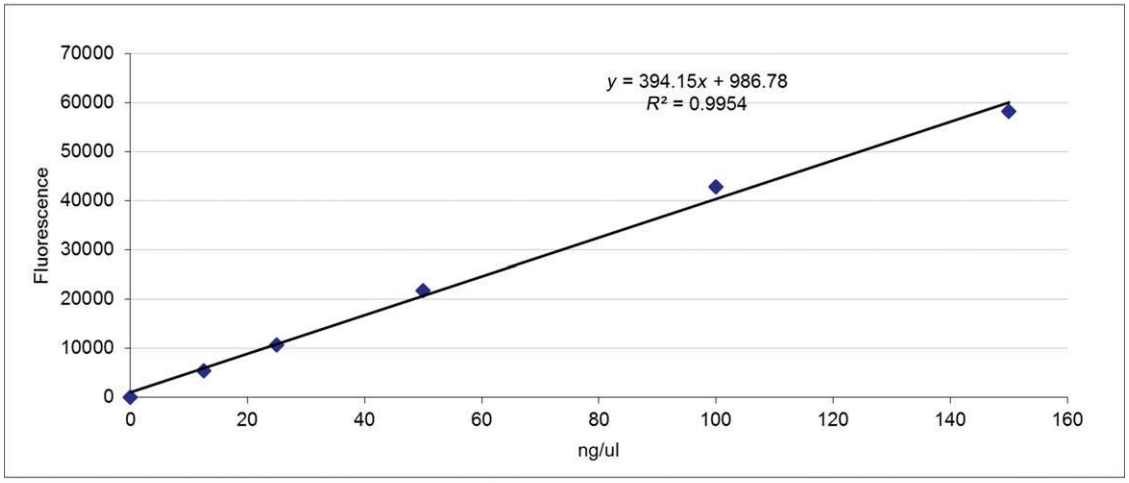
   PicoGreen solution is best added with a multichannel pipettor.

Template for quantification of DNA using picogreen dye

Standard (ng DNA)	*read1*	*read 2*	mean	Corrected mean
150	58307	59480	*58893.5*	**58190.5**
100	42701	44361	*43531*	**42828**
50	22257	22707	*22482*	**21779**
25	11340	11514	*11427*	**10724**
12.5	6083	6173	*6128*	**5425**
0	679	727	*703*	**0**

slope= **394.1505116**

intercept= **986.7837209**



DNA samples **3ng/µl (100 µl)**

read 1	read 2	mean	corrected mean	conc. (ng/µl) assuming 5 µl added	*DNA(µl)*	*TE(µl)*
33192	33464	33328	32625	16.1	18.7	***81.3***
31130	31017	31073.5	30371	14.9	20.1	***79.9***
32391	32028	32209.5	31506.5	15.5	19.4	***80.6***
30701	30640	30670.5	29967.5	14.7	20.4	***79.6***
35726	35521	35623.5	34920.5	17.2	17.4	***82.6***
38694	38334	38514	37811	18.7	16.1	***83.9***
34613	34270	34441.5	33738.5	16.6	18.1	***81.9***
33907	33319	33613	32910	16.2	18.5	***81.5***
34646	33987	34316.5	33613.5	16.6	18.1	***81.9***
34588	34346	34467	33764	16.6	18.0	***82.0***

Figure 20.11.2 Example of DNA quantification template. The fluorescence values for the standard curve are plotted against the standard concentrations and correlation analysis is performed. Using the equation, the actual DNA concentration of the samples can be calculated. Based on this concentration, the DNA can be diluted with 0.1× TE buffer to bring the concentration down to 3 ng/µl.

7. Set up the plate reader. Set filters to 485 nm excitation and 528 nm emission and add a 20-sec shaking step before the reading step. Measure fluorescence.

8. Calculate the DNA concentrations of the samples by comparing sample fluorescence values to the standard curve values (Fig. 20.11.2; refer to Supplemental File 4 (*http://www.currentprotocols.com/protocol/tx2011*) for example calculations). Plot the fluorescence readings for the standard curve against the standard curve concentrations and perform correlation analysis. Use the resulting equation to calculate the DNA concentration in the samples of interest. If the fluorescence reading or concentration for a sample is higher than the highest standard, dilute sample further and measure again. If sample signal is too low (close to the 0 or 1.25 ng/μl standard readings), use undiluted samples and measure again.

9. Dilute desired amount of DNA extract in 0.1× TE buffer so that the concentration is 3 ng/μl.

   The DNA is now ready to use for PCR assays.

   The final dilution that results in a concentration of 3 ng/μl should be carried out using a preceding concentration of no more than 15 ng/μl to ensure accurate measurement and dilution. If the preceding dilution is more concentrated, carry out as many additional dilutions as necessary to obtain a penultimate concentration of no more than 15 ng/μl.

   To save time and reagents, and especially if some samples have very high concentrations of DNA, it is also possible to carry out the initial (rough) quantification using a Nanodrop or similar spectrophotometer, and then carry out the first dilution and proceed using PicoGreen as described above. Nanodrop and PicoGreen methods do not give identical values, however, so this initial quantification should be considered an estimate.

SUPPORT PROTOCOL 4

LINEARIZATION OF MITOCHONDRIAL DNA FOLLOWING AUTOMATED DNA EXTRACTION

As mentioned earlier, it is possible to extract DNA from samples in a fully automated manner by using a QIAcube from Qiagen. However, after QIAcube extraction the mtDNA appears to be mostly in the supercoiled conformation, making primer access difficult (Furda et al., 2012). Utilizing restriction enzymes to linearize the mtDNA in a region outside the amplification target can alleviate this.

Materials

225 ng purified DNA
Nuclease-free H_2O
Restriction enzyme (*Hae*II for human, *Pvu*II for mouse, and *Xho*I for rat, and corresponding CutSmart or NEBuffer buffer; New England Biolabs)

0.2-ml PCR tubes
96-well format thermal cycler

1. Calculate the volume of the purified DNA needed to perform the digest (225 ng are needed; refer to Support Protocol 3).

   Final digestion volume will be 50 μl per sample.

2. Calculate the amount of nuclease-free water needed in order to bring the volume up to 44 μl (subtract the value obtained in step 1 from 44 μl)

3. Pipet the calculated amount of purified DNA and nuclease-free water into a PCR tube. Also add 5 μl of the appropriate buffer.

4. Add 1 μl of the enzyme and incubate in a thermal cycler following the time and temperature parameters described in the enzyme supplier protocol.

5. Place samples in ice or store at 4°C immediately following incubation.

No further purification is needed for PCR assays.

GENERATING STANDARD CURVE PLASMIDS FOR MITCHONDRIAL AND NUCLEAR GENOME COPY NUMBER ANALYSIS

SUPPORT PROTOCOL 5

In order to measure the absolute mitochondrial and nuclear genome copy numbers by RT-PCR, we have generated a standard curve by cloning the respective short (100- to 250-bp) target sequences into the pCR2.1 plasmid. We adopted the restriction enzymes cloning method for our work. Bacterial glycerol stocks are available from us upon request.

Materials

Genomic DNA (Support Protocol 2)
Nuclease-free H_2O
Taq DNA polymerase with standard *Taq* buffer
dNTP mix (2.5 mM of each nucleotide)
Magnesium chloride ($MgCl_2$)
Forward and reverse primers (see Table 20.11.4; also see *APPENDIX 3C*; Kramer and Coen, 2000)
QIAquick PCR Purification Kit (Qiagen)
pCR2.1 plasmid DNA (bacterial stocks available from us upon request)
Restriction enzymes (*Hin*dIII and *Xho*I and associated NEBuffer; New England Biolabs)
Gel extraction kit (available from various molecular biology suppliers)
T4 DNA ligase and associated buffer (New England Biolabs)
Heat shock–competent *E. coli* (Any recA$^-$ cloning strain)
SOC medium (see recipe)
LB agar plates containing 100 μg/ml ampicillin (see recipe)
LB broth containing 50 μg/ml ampicillin or 50 μg/ml kanamycin (see recipe)

Sterile, aerosol filter tips and pipets dedicated to PCR
NanoDrop2000 UV-Vis Spectrophotometer
0.2-ml PCR tubes
Thermal cycler with heated lid
Heat block

Additional reagents and equipment for the polymerase chain reaction (PCR; *APPENDIX 3C*; Kramer and Coen, 2000), agarose gel electrophoresis (Voytas, 2000)

Perform PCR

1. Obtain genomic DNA from animal tissue as described in Support Protocol 2. Quantify the extracted DNA using a NanoDrop2000 UV-vis spectrophotometer at 260 nm.

2. Design primers with modifications made to the 5′-end of the forward and reverse primers: restriction site addition and 5′-extension to the restriction site. Refer to Table 20.11.4 for some species-specific cloning primers used in our work.

   The restriction site should be the same or provide the same sticky end to the choice of restriction enzymes used to digest the multiple cloning site of the cloning vector. For our cloning strategy, we selected two sticky-end cutters that create different 5′-overhangs: HindIII which recognizes the hexamer AAGCTT, and XhoI, which recognizes the hexamer CTCGAG. To increase the efficiency of the restriction enzyme, the end fragment of the primer DNA is cleaved and the 5′ end of the restriction site is extended by 4 to 6 random nucleotides.

Table 20.11.4 Cloning PCR Primers and Conditions

Species	Genome	Target gene	Forward primer seq.[a]	Reverse primer seq.[a]	Amplicon (bp)	Annealing temp. (°C)	Reference
C. elegans	mt	nd-1	NR	NR	NR	NR	Bratic et al. (2009)
	nuc	cox-4	5′-ATCT AAGCTT GCC GAC TGG AAG AAC TTG TC-3′	5′-ATAG CTCGAG GCG GAG ATC ACC TTC CAG TA-3′	184	60	This unit
H. sapiens	mt	tRNA-Leu(UUR)	5′-ATCTAAGCTT GCC TTC CCC CGT AAA TGA TA-3′	5′-ATAG CTCGAG AGG AAT GCC ATT GCG ATT AG-3′	215	55	This unit
	nuc	B2M	5′-ATCT AAGCTT TGC TGT CTC CAT GTT TGA TGT ATC T-3′	5′-ATAG CTCGAG TCT CTG CTC CCC ACC TCT AAG T-3′	106	62	This unit
F. heteroclitus	mt	16S rRNA	5′-ATAG AAGCTT AAA ATT AAC GGC CCC AAC CC-3′	5′-ACTT CTCGAG CCG AGT TCC TTC TTC CCC TT-3′	131	64	This unit
	nuc	CFTR	5′-ATAG AAGCTT GCC GCT GCC TTC ATT GCT GT-3′	5′-AATT CTCGAG ATG AGC TGG GTG TGC GCT GA-3′	234	65	Hunter et al. (2010)
D. rerio	mt	nd-1	5′-ATCT AAGCTT CGT TTA CCC CAG ATG CAC CT-3′	5′-ATAG CTCGAG GTG CGA TTG GTA GGG CGA TA-3′	215	60	This unit
	nuc	vtg2	5′-ATCT AAGCTT TGG ATA CCT GAC CGA GAG CT-3′	5′-ATAG CTCGAG AGA CAA CTC TTA CGG CTG GC-3′	110	60	This unit

[a]Underlined regions denote restriction sites.

3. Perform a PCR to obtain sufficient amounts of template DNA by amplifying the insert region from genomic DNA. First prepare the reaction mix by adding the components in the following order (per reaction):

   Nuclease-free water (for a final volume of 50 μl)
   10 μl 5× *Taq* polymerase buffer
   5 μl dNTP mix (2.5 mM of each nucleotide)
   3 μl 50 mM $MgCl_2$
   1.5 μl 10 μM forward primer
   1.5 μl 10 μM reverse primer
   0.5 μl 2.5 U/μl *Taq* polymerase
   5 μl (200 to 500 ng) template DNA (step 1).

   Mix the above reagents in a 0.2-ml PCR tube and briefly spin down to remove any air bubbles.

   Reactions are set-up at room temperature. Since the amplified products are subjected to several downstream applications, it is advisable to scale up the reaction numbers or reaction volume.

4. Perform the PCR reaction in a thermal cycler with a heated lid using the conditions:

1 cycle:	2 min	94°C	(initial denaturation)
30-35 cycles:	15 sec	94°C	(denturation)
	30 sec	62°-65°C	(annealing/extension)
1 cycle:	10 min	72°C	(final extension)
1 cycle:	indefinite	8°C	(hold).

Perform PCR reaction clean-up

5. Purify the amplified DNA fragment(s) from the reaction mixture using a commercial PCR purification kit such as Qiagen's QIAquick PCR purification kit prior to setting up the restriction enzyme digestion reaction.

6. Double digest the plasmid and insert DNA separately by mixing the restriction digestion reactions in separate 1.7-ml microcentrifuge tubes in the following order (per reaction):

   Nuclease-free H_2O (to a final volume of 50 μl per reaction)
   5 μl 10× restriction enzyme buffer (NEBuffer)
   10 μl (2 μg) pCR2.1 plasmid DNA
   or
   insert DNA (up to 40 μl of the PCR product from step 5 can be used for each 50-μl reaction)
   2 μl (10 to 20 U) *Hin*dIII and *Xho*I (or other appropriate restriction enzymes for the double digest).

   Both enzyme digestion reactions can be carried out simultaneously if the enzymes work equally in the commercially available buffers. For reference, check the New England Biolabs double-digest finder to decide on the most compatible buffer. The restriction enzyme is added last and mixed gently by pipetting the solution up and down.

7. Incubate the reaction mix at 37°C in a heat block, filled with water, for up to 2 hr.

8. Purify the digested vector and insert DNA from the small DNA fragment (product of digestion) by running the reaction mix in a 2% agarose preparative gel (Voytas, 2000) at 100 V for 45 min in 1× TE buffer.

9. Extract the respective products (vector and insert DNA) from the agarose gel using a commercial gel extraction kit.

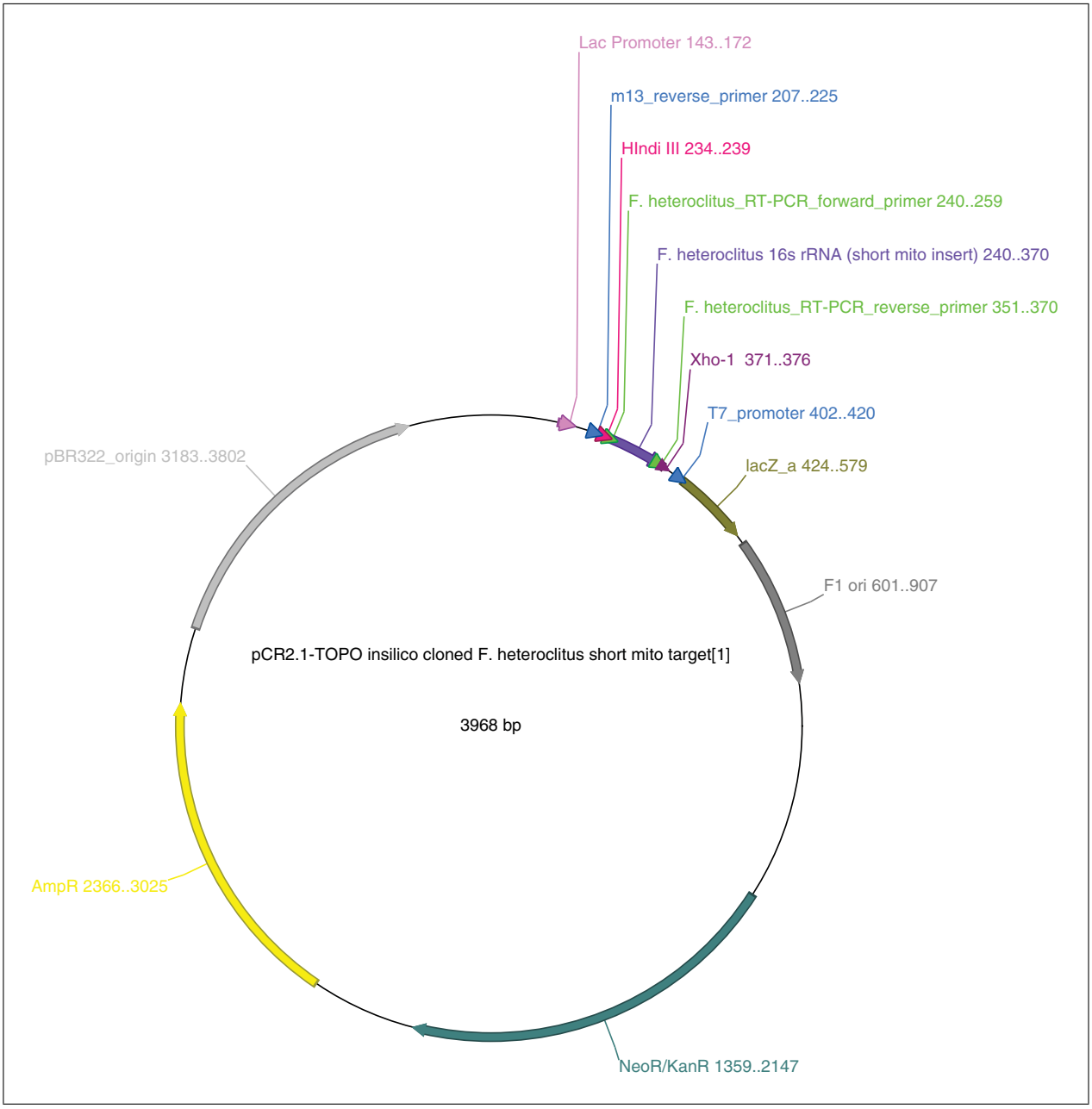


Figure 20.11.3 Example of an in silico cloned short product target in pCR2.1 plasmid. Figure shows cloned pCR2.1 containing the *F. heteroclitus* short mito target (131 bp, 16s rRNA) inserted at the multiple cloning site (MCS) using unique restriction enzymes *Hin*dIII and *Xho*I. The target was PCR amplified using primers that contained these restriction elements at the 5′ end of the forward and reverse primers. Sequencing primers, M13_reverse and T7_Promoter, are highlighted in blue.

10. Ligate the digested and purified insert and linear vector DNA using bacteriophage T4 DNA ligase. Set up a standard sticky-end ligation reaction by adding the ingredients in the following order (per reaction) to a 0.2-ml PCR tube:

    Nuclease-free H_2O (to a final volume of 20 µl per reaction)
    2 µl 10× ligase buffer
    2 µl ~50 ng/µl digested plasmid DNA from step 6
    Digested insert DNA (step 6; in appropriate molar ratio to vector DNA; appropriate volume for respective ratio)
    1 µl 20 NEB U/µl T4 DNA ligase

Mix the contents gently by pipetting the solution up and down followed by a brief spin and incubate at 16°C overnight in a thermal cycler.

Reactions are set up with vector to insert DNA in a molar ratio of 1:1, 1:2, and 1:4, along with a control reaction containing the digested vector DNA only to determine the self-ligated non-recombinant background.

11. Transform the ligation mix into chemically competent *E. coli* cells by heat shock. Briefly, thaw *E.coli* cells (50 µl per reaction) on ice water. Add 5 to 10 µl of the ligation mix and mix gently. Incubate the mixture for 30 min on ice water. Heat shock the cells for 45 sec at 42°C and place them immediately on ice. Add 1 ml of SOC medium and incubate at 37°C for 1 hr. Pellet the culture by centrifuging 1.5 min at 2348 × *g*, room temperature. Remove 800 µl of the supernatant and plate out the re-suspended cultures on LB agar plates containing 100 µg/ml ampicillin. Incubate the plates overnight at 37°C.

    We use commercially available competent E.coli cells to enable uptake of the circular vector DNA. As to the E. coli strain, use any recA-cloning strain such as TOP10 or DH5a. The negative control i.e., the vector only ligation mixture and a positive control i.e., an uncut known vector is also used.

12. Pick and culture the *E. coli* transformants in 2 ml of LB broth containing either ampicillin (50 µg/ml) or kanamycin (50 µg/ml) antibiotics for a maximum of 16 hr in a 37°C incubator shaker.

    We use a sterile aerosol filter tip to pick the transformed colonies from the LB plate. We also use a sterile round-bottom polystyrene test tube with snap cap to culture bacteria

13. Isolate the cloned plasmid from *E. coli* using a commercially available plasmid purification kit following the manufacturer's protocol.

    We use the Qiagen QIAprep Spin Miniprep Kit (50); however, for this purpose any commercially available kit can be used.

14. Analyze the transformants, after obtaining the plasmid, for the presence of the right insert either by restriction analysis or sequencing using M13 and or T7 sequencing primers (see Fig. 20.11.3).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****.*

K medium

2.36 g potassium chloride (KCl)
3.0 g sodium chloride (NaCl)
1.0 liter distilled deionized H_2O
Autoclave to sterilize
Store at room temperature, indefinitely, under sterile conditions

LB broth containing 50 µg/ml ampicillin or kanamycin

Per liter:
10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH
Autoclave
Cool to ~50°C
Add ampicillin or kanamycin to 50 µg/ml
Store up to 1 month at room temperature

LB plates containing 100 μg/ml ampicillin

Per liter:
10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH
15 g agar
Autoclave
Cool to ~50°C
Add ampicillin to 100 μg/ml
Store in the dark up to 2 weeks at 4°C

SOC medium

1 g yeast extract
4 g tryptone
0.4 ml 5 N NaCl
0.5 ml 1 N KCl
2 ml 1 N MgCl
2 ml 1 N $MgSO_4$

Make up the total volume to 150 ml with distilled deionized water and autoclave. Dissolve 0.72 g of glucose in 50 ml distilled deionized water and filter sterilize the solution. Add the entire sterile glucose solution to the autoclaved solution once cooled. Store at room temperature.

Worm lysis buffer, 3.3×

Prepare in nuclease-free H_2O:
82.5 mM tricine (pH 8)
264 mM potassium acetate
36.2% (w/v) glycerol
7.425% (v/v) DMSO
Store at −20°C indefinitely

Adapted from Cheng (2001).

COMMENTARY

Background Information

The LA-QPCR assay has been used to detect DNA damage, traditionally from cultured cells and model organisms, in the laboratory for more than 20 years (Kalinowski et al., 1992; Meyer, 2010). To date, it has been optimized for a variety of species, including humans, rats, mice, yeast, fruit fly, nematodes, plants, fish, and others (Meyer et al., 2007; Jung et al., 2009; Hunter et al., 2010; Castro et al., 2012; Colton et al., 2014; Furda et al., 2014; Kumar et al., 2014). It consists of amplifying a long target in the genome of interest (~10 kb) and quantifying the PCR product. If polymerase-stalling lesions are present, the amplification product will be less than expected; comparing the level of amplification in the treated samples to the control samples allows us to estimate the lesion frequency. For this to be possible, the amplification must be exponential; the amount of PCR product has to be directly proportional to the amount of starting template (Meyer, 2010; Furda et al., 2012).

There are several advantages to using this assay. First, little template and therefore little sample is needed (we have used as little as 50 to 100 pg per sample, using individual nematodes). Second, the assay is highly specific, because it is primer based, allowing analysis of the species, genome, and even genomic regions of interest. The DNA extraction methods described here isolate both mitochondrial and nuclear DNA; this allows us to measure damage in both genomes for each sample without separation of genomes that can lead to artifactual differences (Meyer, 2010; Furda et al., 2012). Different regions of nuclear genome

can be analyzed, in order to compare region-specific damage or repair (e.g., Van Houten et al., 2000; Meyer et al., 2007; however, note that not all of the targets described in those papers have been re-optimized for the current polymerase). Third, the assay detects many kinds of damage, providing an integrative although not complete measure of damage present. However, there are several limitations to this assay as well. Only lesions that stall the DNA polymerase can be detected, and it cannot discern among those lesions. For nucDNA damage measurements, there is the possibility that the region amplified is not representative of the entire genome. The lesion frequencies are calculated by using the control lesion levels as a damage-free baseline; however, of course, in fact all templates have some level of damage. Finally, if the template is obtained by batch extraction, lesion measurements cannot be made at the individual cell level (Meyer, 2010).

Traditionally, quantitative PCR is also used to measure genome copy number, by amplifying a small region (~200 bp) of the genome. This small target is unlikely to have DNA lesions and is therefore a good representation of the genome content present (Santos et al., 2006; Furda et al., 2012). These values are used to normalize the amplification from LA-QPCR to the actual amount of starting copies, which can vary dramatically for mtDNA from cell to cell. It is also helpful when amplifying *C. elegan*s lysates, as it can account for errors in picking (wrong number of worms per lysate) and in worm size in the case of growth-variable populations (Hunter et al., 2010). The method presented in this protocol is based on RT-PCR instead and, if standard curves are run alongside samples, it has the advantage of allowing us to calculate the actual number of mitochondrial and nuclear genome copies. If standard curves are not available, we use the comparative Ct method, and as a result the measure of mtDNA content is relative to nucDNA content (Bratic et al., 2009; Venegas and Halberg, 2012; Rooney et al., 2015). If the goal is to normalize results from LA-QPCR, and standard curves are not available, we employ a method that utilizes logarithmic regression to calculate normalization factors per sample.

Critical Parameters

The biggest concern when performing these PCR-based assays is obtaining high-quality DNA that has not been sheared, oxidized, or otherwise damaged. Standard DNA extraction techniques do not yield high-molecular-weight DNA that is suitable for the LA-QPCR assay, although such DNA can be used for copy-number analysis. Similarly, phenol/chloroform-based extraction methods cause DNA oxidation that may obscure the damage of interest. Further discussion of these concerns, and examples of how to analyze DNA integrity, are presented in Meyer (2010).

Another major concern is cross-contamination of newly isolated template DNA with PCR products generated in previous PCR reactions. To avoid this, high-quality reagents must be used and gloves worn at all times. UV-irradiate the work area before and after sample preparation. Use dedicated pipets and tips, and designate a PCR-only workstation. Never open the completed reactions in the same room where the dedicated PCR workstation is located. Always include a no-template control to monitor for contamination.

Another parameter to keep in mind is pipetting consistency and accuracy. Small changes in volume can greatly increase variability in the PCR results, as can introduction of air bubbles and creation of microenvironments in residual drops on tube walls or caps.

The normality of the data obtained must be verified, and parametric or non-parametric statistical tests should be run based on this analysis.

Troubleshooting

The most common problem encountered is contamination. If, despite taking precautions, the results from the no-template controls suggest the presence of contamination, the first thing to do is replace the reagents. Particularly, economical reagents that are aliquotted and kept in the workstation, like nuclease-free water and TE buffer, need to be exchanged for fresh replacements. It is also important to decontaminate all work areas and instruments (e.g., PCR hood, pipettors, racks, etc.).

If designing primers for a new species, or if the optimization of cycle number or other PCR parameters is problematic, please refer to Hunter et al. (2010), Furda et al. (2012), and Meyer (2010) for suggestions. Follow our PCR parameter recommendations, keeping in mind that all PCR conditions may require some re-optimization in the context of different thermal cyclers and other equipment.

Using the hot-start master mix version of the PCR kits greatly reduces the chances of problems arising, and reduces noise and

variability. We have not yet optimized our PCR parameters for the regular, non-master-mix kit.

Anticipated Results

These assays yield genome copy number values and DNA damage values for nuclear and mitochondrial genomes. Examples of results obtained with these assays are available as supplemental files. For the DNA damage assay, we expect the data to be normally distributed. However, this needs to be corroborated for every new experiment. In the event that the data are not normally distributed, nonparametric statistical tests should be used to analyze the data. It is important to keep in mind that the limit of detection for the DNA damage assay is ~1 lesion per 10^5 bases (Meyer, 2010), although this value depends on the number of replicates and variability between them. Lesion frequencies below this value cannot be considered DNA damage. The assay can be used to measure DNA repair by examining damage levels over time, as long as there is not significant cell division that results in production of new, undamaged nucDNA or mtDNA replication. Either event will dilute the number of lesions/10 kb by increasing the number of total bases, confounding measurement of damage removal or repair.

Time Considerations

Manual DNA purification from tissue takes 1 to 2 days, depending on the number of samples and how quickly they elute, with some down time during incubations and elutions. Worm lysis can take up to 2 hr, depending on the number of samples. DNA quantification (pre- or post-PCR) takes approximately 45 min. Setting up the LA-QPCR or RT-PCR can take an hour or more depending on the number of samples. Running the PCR program varies depending on the number of cycles, but is frequently 6 to 8 hr for the LA-QPCR assay.

Acknowledgments

We thank Aleksandra Trifunovic for sharing the mtDNA copy number plasmid for *C. elegans* with us. This work was supported by NIEHS R01-ES017540-01A2 and P42ES010356.

Conflicts Of Interest

The authors declare that there are no conflicts of interest.

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