Rev. 1

Human Mitochondrial DNA Damage Quantification qPCR Assay Kit (HMDQ)

Catalog #8978
50 reactions

Product Description

Mitochondrial DNA (mtDNA) is a circular DNA found inside the mitochondria, and it plays a key role in the cells' energy production. Damage to mtDNA can disrupt cellular energy processes, leading to decreased cell growth and apoptosis. mtDNA damage serves as a meaningful biomarker for assessing the genotoxicity of drugs and environmental toxins. ScienCell's Human Mitochondrial DNA Damage Quantification qPCR Assay Kit (HMDQ) operates on the principle that various DNA lesions can impede DNA polymerase progression. Consequently, DNA with fewer lesions amplifies more readily than damaged DNA under identical conditions. Damage levels can be quantified in terms of the lesions per kilobase pair using a Poisson distribution of lesions or the percentage of intact mtDNA of the target sample to the control sample. Additionally, our assay allows for tracking DNA repair kinetics by measuring the restoration of target DNA amplification over time following the removal of the DNA-damaging agent. This assay monitors the integrity of mtDNA directly from total cellular DNA without the need to isolate mitochondria DNA.

The primer sets (Cat. #8978a and Cat. #8978b) recognizes and amplifies sequences in the most conserved regions on human mtDNA, excluding any off-target sequence on nuclear genomic DNA. We utilize 2X LanaRana Long Range PCR Master Mix (cat #MB6098) and Human Long mtDNA Primer Set (Cat. #8978a) to amplify a long 8.9 kb DNA fragment. For amplifying a short 178 bp mtDNA fragment, we use the 2X GoldNStart TaqGreen qPCR Master Mix (Cat. #MB6018a-1) and Human Short mtDNA Primer Set (Cat. # 8978b). Human DNA from Non-Damaged (untreated) and Damaged (200 µM H_2O_2 treated) cells serves as positive and negative controls for the reaction.

Kit Components

Cat #	Component	Quantity	Storage
MB6018a-1	2X GoldNStart TaqGreen qPCR Master Mix, 1 mL	3 vials	-20°C
MB6098	2X LanaRana Long Range PCR Master Mix	1 vial	-20°C
8978a	Human Long mtDNA Primer Set, Lyophilized	1 vial	-20°C
8978b	Human Short mtDNA Primer Set, Lyophilized	1 vial	-20°C
8978c	Nuclease-Free H_2O	10 mL	4°C
8978d	Reference Non-Damaged Human DNA	100 µL	-20°C
8978e	Reference Damaged Human DNA	100 µL	-20°C

Additional Materials Required (Materials Not Included in Kit)

Component	Product Name
DNA isolation kit	SpeeDNA Isolation Kit (ScienCell, Cat #MB6918)
genomic DNA template	Customers' samples
qPCR plate or PCR strip	Plate type or PCR strip based on Customers' qPCR Machine Model

Quality Control
The specificity of the mtDNA long primer set is validated by PCR and gel electrophoresis. The specificity of the mtDNA short primer set is validated by qPCR with melt curve analysis and gel electrophoresis. The mtDNA damage of Reference Damaged Human DNA is determined by qPCR relative to the Reference Non-Damaged Human DNA.

Product Use
HMDQ is for research use only. It is not approved for human or animal use, or for application in clinical or *in vitro* diagnostic procedures. HMDQ can be conducted using genomic DNA from human cultured cells or human tissues.

Shipping and Storage
The product is shipped on dry ice. Upon receipt, store the GoldNStart TaqGreen qPCR master mix (Cat #MB6018a-1) in the dark at -20°C in a manual defrost freezer, the 2X LanaRana Long Range PCR Master Mix (cat #MB6098), primers (Cat #8978a and cat #8978b) and the reference DNA samples (Cat #8978d and #8978e) at -20°C in a manual defrost freezer, and the nuclease-free H_2O (Cat #8978c) at 4°C. Once thawed, do NOT refreeze GoldNStart TaqGreen qPCR master mix (Cat #MB6018a-1), and keep in the dark at 4°C or on ice at all times.

Procedures

***Important: Only** use polymerases with hot-start capability to prevent possible primer-dimer formation. **Only** use nuclease-free reagents in PCR amplification.*

1. An overview of the experimental setup is detailed in Figure 1 below.

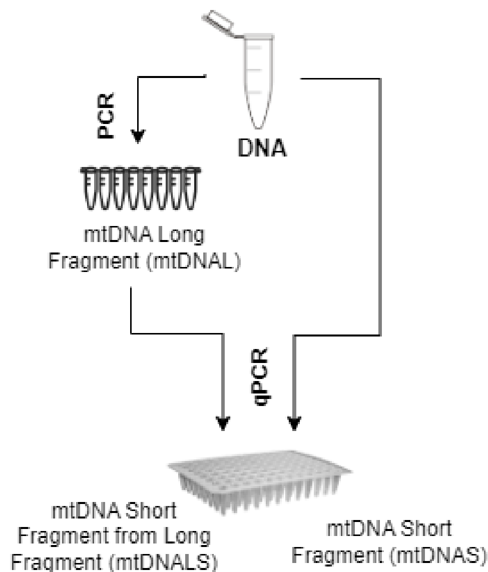

Figure 1- Diagram of the experimental setup. First, perform a PCR amplifying the long mtDNA fragment using the provided Human Long mtDNA Primer Set. Second, perform qPCR using the diluted PCR product with the Human Short mtDNA Primer Set to obtain the Ct value for mtDNALS. Additionally, directly analyze the original DNA samples with qPCR to obtain the Ct value for mtDNAS.

2. **Sample preparation:** Adjust the DNA concentration in all the samples to 1-10 ng/µL. Ensure a consistent amount of DNA loading across all reactions. The same amount of DNA that has been added to the long mtDNA reaction must be added to the short mtDNA reaction since amplification of short mtDNA will be used to normalize the amount of DNA that has been loaded.

3. Allow Cat. #8978a and Cat. #8978b to warm to room temperature.

4. Centrifuge the vials at 1,500x g for 1 minute.

5. Add 200 µl nuclease-free H_2O (Cat. #8978c) to mtDNA long primer set (lyophilized, Cat. #8978a) to make mtDNA long primer stock solution. Aliquot as needed. Store at -20°C in a manual defrost freezer. Avoid repeated freeze-and-thaw cycles.

6. Add 200 µl nuclease-free H_2O (Cat. #8978c) to mtDNA short primer set (lyophilized, Cat. #8978b) to make mtDNA short primer stock solution. Aliquot as needed. Store at -20°C in a manual defrost freezer. Avoid repeated freeze-and-thaw cycles.

7. Aliquot Reference DNA (Cat. #8978d and Cat. #8978e), as needed. Avoid repeated freeze-and-thaw cycles.

8. For each genomic DNA sample and Reference DNA (Cat. #8978d and Cat. #8978e), prepare a PCR reaction with mtDNA long primer stock solution and 2X LanaRana Long Range PCR Master Mix (Cat. #MB6098). Prepare 20 µl PCR reaction for one well as shown in Table 1.

   Optional: Measure DNA concentration with fluorescent double-strand detection methods for greater accuracy.

   Table 1. Long mtDNA PCR reaction

DNA sample (5-15 ng) or Reference DNA (Cat # 8978d or Cat # 8978e) (5 µl)	x µl
Human Long mtDNA Primer Set stock solution (Cat #8978a)	2 µl
2X LanaRana Long Range PCR Master Mix (Cat #MB6098)	10 µl
Nuclease-free H_2O (Cat #8978c)	8-x µl
Total volume	20 µl

9. Seal the PCR reaction tubes. Centrifuge the tubes at 1,500x g for 15 seconds. Refer to Table 2 for PCR program setup.

   Table 2. Long mtDNA PCR program

Step	Temperature	Time	Number of cycles
Initial denaturation	95°C	10 min	1
Denaturation	95°C	15 sec	35
Annealing and extension	66°C	10 min	
Hold	4°C	∞	1

10. Run the PCR program. Then dilute each PCR product 1000-10000x with nuclease free water by serial dilution.

For example, pipette 2 µl of PCR product and mix well with 198 µl of nuclease-free water. Then take 2 µl of this mixture into 198 µl of nuclease-free water to make 10000x dilution. Finally, use 2-5 µl of the diluted PCR product for the subsequent qPCR reaction.

Optional: At this stage PCR products can be analyzed for potential differences in band intensity via gel electrophoresis. The intensity of the band correlates with the amount of intact mtDNA in the sample. Faint band or absence of a band in the sample indicates a high degree of mtDNA damage compared to the control sample (Figure 2). A smeared band may appear above the 8.9 kb band due to the large DNA size and high amount of DNA.

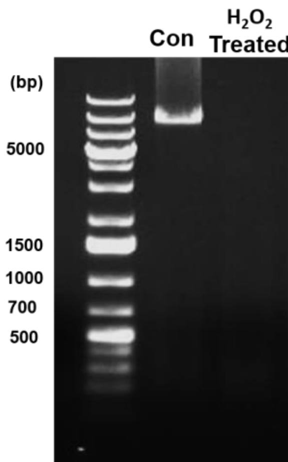

Figure 2. Gel electrophoresis of PCR product.
DNA from Control (Un-Damaged) and 200 µM H2O2 treated for 15 minutes (Damaged) samples tested with the PCR to amplify mtDNAL, 8.9 kb mtDNA fragment. Diluted mtDNAL PCR products were tested with gel electrophoresis.

11. Prepare qPCR reactions for each diluted PCR product, DNA sample and Reference samples (Cat. #8978d and cat #8978e), in triplicate with mtDNA short primer set and GoldNStart TaqGreen qPCR master mix. qPCR reaction (20 µl) for one well is shown in Table 3.

    Note: This kit provides materials for 50 Long mtDNA PCR reactions and 300 Short mtDNA qPCR reactions. We strongly recommend performing the Long mtDNA reaction in duplicate for each sample. For maximum reliability, run Short mtDNA qPCR **in triplicate** for diluted PCR product and original DNA. So, each sample run for Long mtDNA reaction requires 6 reactions of Short mtDNA reaction.

    Table 3. Short mtDNA qPCR Reaction

DNA sample or diluted PCR product	x µl
Human short mtDNA Primer Set Stock Solution (Cat#8978b)	2 µl
2X GoldNStart TaqGreen qPCR master mix (Cat #MB6018a-1)	10 µl
Nuclease-free H_2O (Cat #8978c)	8-x µl
Total volume	***20 µl***

12. Seal the qPCR reaction wells. Centrifuge the plates or tubes at 1,500x g for 15 seconds.

13. Refer to Table 4 for qPCR program setup. The 2X GoldNStart TaqGreen qPCR master mix (Cat. #MB6018a-1) contains SYBR®Green as the reporter dye and does not contain a ROX passive reference dye. If the qPCR instrument being used has a "ROX passive reference dye" option, please deselect this option.

Table 4. Short mtDNA qPCR program

Step	Temperature	Time	Number of cycles
Initial denaturation	95°C	10 min	1
Denaturation	95°C	15 sec	35
Annealing	66°C	15 sec	
Extension	72°C	45 sec	
Data acquisition	Plate read		
Optional	*Melting curve analysis*		1

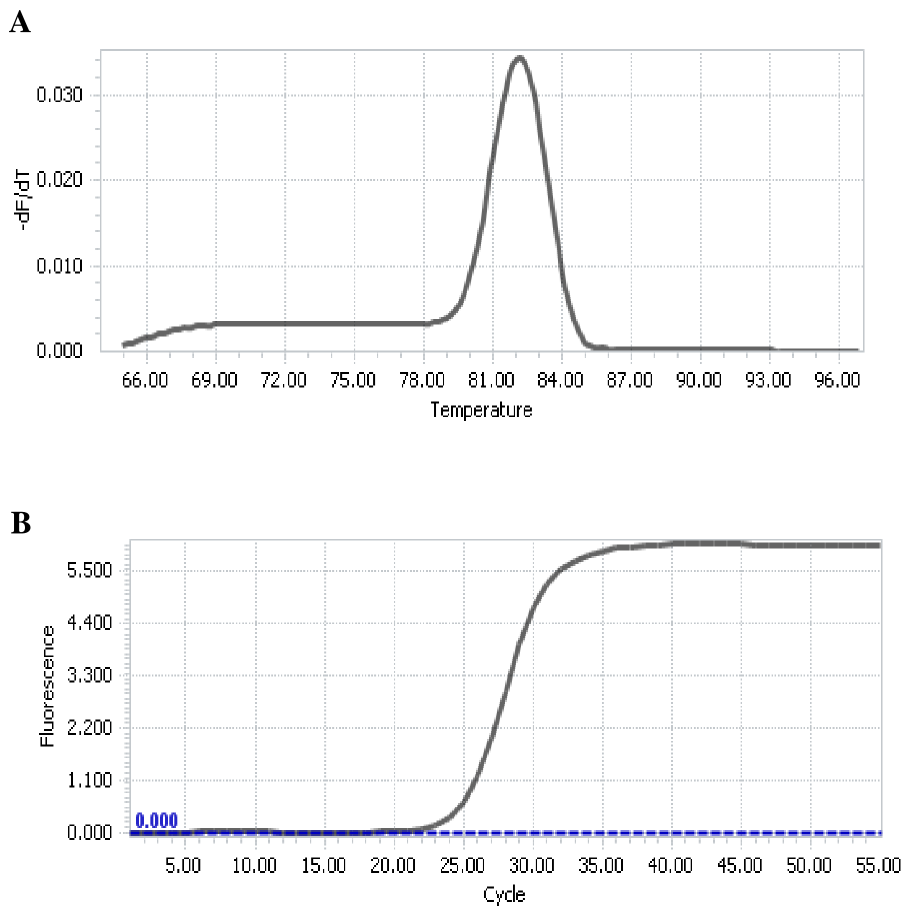

Figure 3. A. A typical amplification curve showing the amplification of a qPCR product. **B**. A typical melting peak of a qPCR product.

Quantification Method: Comparative $\Delta\Delta Cq$ (Quantification Cycle Value) Method

Note: Please refer to your qPCR instrument's data analysis software for data analysis. The method provided here serves as guidance for quick manual calculations.

1. For ΔCq (mtDNALS) is the quantification cycle number difference of mtDNALS between the damaged (target) and the un-damaged (control) DNA samples.

   ΔCq (mtDNALS) = Cq (mtDNALS, damaged (target) sample) - Cq (mtDNALS, un-damaged (control) sample)

2. For mtDNAS ΔCq (mtDNAS) is the quantification cycle number difference of mtDNAS between the damaged (target) and the un-damaged (control) samples.

   ΔCq (mtDNAS) = Cq (mtDNAS, damaged (target) sample) - Cq (mtDNAS, un-damaged (control) sample)

   Note: the value of ΔCq (mtDNAS and mtDNALS) can be positive, 0, or negative.

3. $\Delta\Delta Cq$ = ΔCq (mtDNALS) - ΔCq (mtDNAS)

4. Relative mtDNA damage of the damaged (target) sample to the un-damaged (control) sample (fold)

   $= 2^{-\Delta\Delta Cq}$

5. Percentage of intact mtDNA of the damaged (target) sample to the un-damaged (control) sample

   $= 2^{-\Delta\Delta Cq} / (2^{-\Delta\Delta Cq} + 1) \times 100$

6. F= Frequency of lesions per kilo base pair (kb) of DNA
   d= size of the long DNA length being amplified in kilobases (kb). In this kit, mtDNA long fragment is 8.9 kb.

   $F = -\ln (2^{-\Delta\Delta Cq})/d$

Example Calculations: Comparative ΔΔCq (Quantification Cycle Value) Method

Table 3. Cq (Quantification Cycle) values of mtDNALS and mtDNAS qPCR obtained from the Human DNA samples, HUVECS treated with 200 μM H_2O_2 for 15 minutes.

Primer set	*Reference Damaged DNA*	*Reference Un-damaged DNA*
mtDNAS	14.56	15.58
mtDNALS	21.63	15.30

1. ΔCq (mtDNAS) = Cq (mtDNAS damaged reference sample) - Cq (mtDNAS, reference undamaged sample)

   = 14.56-15.58

   = -1.02

2. ΔCq (mtDNALS) = Cq (mtDNALS damaged reference sample) - Cq (mtDNALS, reference un-damaged sample)

   = 21.63 – 15.30

   = 6.33

3. ΔΔCq = ΔCq (mtDNALS) - ΔCq (mtDNAS)

   = 6.32- (-1.02)

   = 7.35

4. Relative of intact mtDNA of the damaged reference sample to the un-damaged reference sample (fold)

   $= 2^{-\Delta\Delta Cq}$

   $= 2^{-(7.35)}$

   = 0.0061

5. Percentage of intact mtDNA of the damaged reference sample to the un-damaged reference sample

   $= 2^{-\Delta\Delta Cq}/(2^{-\Delta\Delta Cq}+1)x100$

   =0.0061/(0.0061+1)x100

   =0.6%

6. F= Frequency of lesions per kilo base pair (kb) of DNA

   d= size of the long DNA length being amplified in kilobases (kb)

   $= -\ln(2^{-\Delta\Delta Cq})/d$

   $= -\ln(2^{-(7.35)})/8.9$

   = 0.57

Conclusions: In conclusion, for every kilobase of mtDNA there is an estimated 0.57 lesions.