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Human Mitochondrial DNA Damage Quantification Kit (qPCR Assay)

Catalog #: MTH-DQ

User Manual
Last Revised: September 18th, 2024

Introduction

The maintenance of mitochondrial genomic integrity is a prerequisite for proper mitochondrial function. Excessive oxidative stress can cause mitochondrial DNA (mtDNA) to be damaged by loss of its supercoiled structure. Accumulation of lesions in mtDNA is believed to be one of the causes of energy crisis in aging tissues as well as with numerous human diseases including neurodegenerative disorders, cardiovascular diseases and cancer. Damage to mtDNA is also a meaningful biomarker for evaluating genotoxicity of drugs and environmental toxins.

The RayBio® Human Mitochondrial DNA Damage Quantification Kit (MTH-DQ) is a Taqman™ probe-based qPCR assay for the specific quantitative measurement of human mitochondrial DNA damage. This kit targets the long mtDNA region (D-Loop gene), which is the most susceptible to damage. In principle, more mtDNA damage or lesions can slow down or block the progression of DNA polymerase, but intact mtDNA does not. Gene-specific Taqman™ probe qPCR is highly sensitive because of the use of “long” PCR methodology that permits the quantitative amplification of fragments of genomic DNA. As a result, very low levels of lesions can be detected, permitting the study of mtDNA damage using genomic DNA from cultured cells, tissue, saliva, urine, spinal fluid, lavage fluid, blood, etc.

Product Use

MTH-DQ is for research use only. It is not approved for application in clinical or *in vitro* diagnostic procedures.

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Storage / Stability

The kit can be stored at -20°C for a period of 12 months prior to opening. After opening, the reagents are valid for at least 6 months if stored at -20°C. Avoid repeated freeze-thaw cycles.

Kit Components

Name	Catalog #	Size / Qty	Description	Storage
2X Probe qPCR Master Mix	MTH-DQ-MIX	1 mL/1 tube	dNTPs, Hot Start *Taq* DNA Polymerase	-20°C
Primer and Probe Mix	MTH-DQ-PP	1 mL/1 tube	Human Long and Short mtDNA Primers and Probes	-20°C
Damaged DNA	MTH-DQ-DDNA	20 µL/1 tube	Damaged Cellular DNA	-20°C
Non-damaged DNA	MTH-DQ-NDDNA	20 µL/1 tube	Non-damaged Cellular DNA	-20°C
Nuclease-Free H_2O	MTH-DQ-H_2O	1 mL/1 tube	Nuclease-Free H_2O	-20°C

Note: Do not mix reagents from different lots.

Additional Materials Required

- Fluorescence PCR instrument capable of reading FAM channel (494 nm maximum absorption, 518 nm maximum emission) and JUN channel (606 nm maximum absorption, 618 nm maximum emission)
- Sterile nuclease-free pipette tips (barrier tips recommended)
- Microfuge tubes
- Compatible PCR Plate or PCR tubes
- Microcentrifuge
- Vortex Mixer

Contact our technical support team for questions about compatibility:
techsupport@raybiotech.com

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Reagent/Sample Preparation

1. Place all reagents and samples on ice to thaw before use. Then, briefly centrifuge to collect contents at the bottom and mix gently with a vortex.

2. DNA Sample preparation: Extract whole genomic DNA or mtDNA from virous cells or tissues. Measure DNA concentration with fluorescent double-strand detection methods (Example: RayBio® Catalog #FQA-DS-48). Adjust the DNA concentration in all the samples to 0.5-50 ng/µL. For optimal quantification, the input DNA amount should be 1-10 ng in 2µL/reaction. Ensure the same amount of DNA loading across all reactions.

Assay Procedure

1. **Calculate Number of Reactions Needed:** The number of reactions to be prepared per PCR run is calculated as: # Reactions = N x (# of replicates) + Non-damaged DNA x 2 + Damaged DNA x 2 + Non-amplification control (Nuclease-Free H_2O) x 2 + 2 (To account for pipetting error), where “N” is the number of samples (plus any replicates).

   Example: For 20 samples with two replicates, total reactions = 20 x 2 + 1 (Non-damaged DNA) x 2 + 1 (Damaged DNA) x 2 + 1 (Nuclease-Free H_2O) x 2 + 2 = 48.

2. **Prepare PCR Reaction Mix in Biosafety Cabinet or PCR Hood.** Mix 2X PCR Master Mix with Primers and Probe Mix according to the volumes stated in **Table 1**. Add 18 µL of the PCR Reaction Mix to appropriate wells of a PCR plate or PCR tubes (See **Table 2** for example plate layout).

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Table 1: PCR Reaction Components per Sample Replicate

Component	Damaged DNA Reaction	Non-damaged DNA Reaction	Sample DNA Reaction	Non-amplification control
2X Probe qPCR Master Mix	10 µL	10 µL	10 µL	10 µL
Primers and Probe Mix	8 µL	8 µL	8 µL	8 µL
Damaged DNA	2 µL	-	-	-
Non-damaged DNA	-	2 µL	-	-
Sample DNA	-	-	2 µL	-
Nuclease-Free H_2O	-	-	-	2 µL
Total Volume	**20 µL**	**20 µL**	**20 µL**	**20 µL**

Table 2: Example layout on a 96-well plate for 20 Samples (only half of the plate shown)

Damaged DNA-1	Damaged DNA-2	Sample 6-1	Sample 6-2	Sample 14-1	Sample 14-2
Non-damaged DNA-1	Non-damaged DNA-2	Sample 7-1	Sample 7-2	Sample 15-1	Sample 15-2
Nuclease-Free H_2O-1	Nuclease-Free H_2O-2	Sample 8-1	Sample 8-2	Sample 16-1	Sample 16-2
Sample 1-1	Sample 1-2	Sample 9-1	Sample 9-2	Sample 17-1	Sample 17-2
Sample 2-1	Sample 2-2	Sample 10-1	Sample 10-2	Sample 18-1	Sample 18-2
Sample 3-1	Sample 3-2	Sample 11-1	Sample 11-2	Sample 19-1	Sample 19-2
Sample 4-1	Sample 4-2	Sample 12-1	Sample 12-2	Sample 20-1	Sample 20-2
Sample 5-1	Sample 5-2	Sample 13-1	Sample 13-2		

3. **Load Samples.** Add 2 µL of appropriate DNA samples (Damaged DNA, Non-damaged DNA, Sample DNA, Nuclease-free H_2O) to designated wells of the PCR plate or tubes. Pipette up and down at least 5 times to mix.

4. **Seal the PCR Reaction Plate or Tubes Tightly.** Centrifuge the plate or tubes for 15 seconds at low speed to collect contents at the bottom and remove the bubbles. Then, run the plate immediately.

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5. **PCR Amplification**

   Set the Sample Number, Damaged DNA, Non-damaged DNA, and Non-amplification control according to your 96-well plate setup. Select FAM and JUN channels. Ensure the "reference fluorescence dye" (passive reference) is set to "None".

   Refer to **Table 3** for Taqman™ PCR program setup (Use the Applied Biosystems QuantStudio 5 Real-Time PCR System as an example).

Table 3. mtDNA damage PCR program

Step		Temperature (°C)	Time	Temperature Ramp Rate	Number of Cycles
Step 1	Remove Carry-over Contamination	50	2 min	1.6°C / sec	1
Step 2	Initial Denaturation	95	10 min	1.6°C / sec	1
Step 3	Denature	95	10 sec	1.6°C / sec	40
	Anneal, extend, and detect fluorescence	60	60 sec	1.6°C / sec	

6. **Results and Export.** In the analysis settings, select automatic baseline, or select according to your lab validation. Then, open and export the results as an Excel file.

7. **mtDNA Damage Quantification.** Use the comparative ΔΔCq (Quantification Cycle Value) method in the formula of $(1 - 2^{-\Delta\Delta Cq})$ X 100% for mtDNA damage quantification.

Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed
2. Load the PCR reaction mix and samples
3. Seal the PCR reaction tubes or plate
4. Set up the PCR Amplification
5. Export the results
6. Calculate the mtDNA Damage

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Typical Data

Calculate the mean Cq value for each set of duplicate damaged DNA, non-damaged DNA and samples. The non-amplification control should be “Undetermined”. Then, use the comparative ΔΔCq (Quantification Cycle Value) method in the formula of = $(1 - 2^{-\Delta\Delta Cq}) \times 100\%$ for mtDNA damage quantification.

Example Calculations:

	Sample 1			Non-damaged DNA			Damaged DNA		
Channel	Rep-1	Rep-2	Average	Rep-1	Rep-2	Average	Rep-1	Rep-2	Average
JUN	17.32	17.69	**17.51**	16.95	17.21	**17.08**	Undetermined	Undetermined	None
FAM	16.84	16.64	**16.74**	16.61	17.69	**17.15**	16.61	17.04	16.82

ΔΔCq = ΔCq (JUN) - ΔCq (FAM)

= [JUN (Cq, Sample 1) – (Cq, Non-damaged DNA)] - [FAM (Cq, Sample 1) – (Cq, Non-damaged DNA)]

= [17.51 - 17.08] - [16.74 - 17.15]

= 0.84

The mtDNA damage of Sample 1:

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

= $(1- 2^{-0.84}) \times 100\%$

= 0.4414 x 100%

= 44.14 %

Result: The average mtDNA damage of Sample 1 is 44.14%

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A. Sensitivity

The minimum detectable concentration of DNA is 0.1 ng. For optimal quantification, the input DNA amount should be 1-10 ng in 2 µL/reaction.

B. Specificity

The non-human and non-amplification control samples have higher Cq values than human DNA or are undetermined, which means there is high specificity for mtDNA amplification.

Species	Input DNA (1ng)	FAM			JUN		
		Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
Human DNA	A549	16.012	16.255	16.410	14.343	14.505	14.599
	293T	17.686	17.586	16.786	15.747	15.409	16.035
	Hela	17.158	17.099	17.713	17.083	17.710	17.097
Non-Human DNA	*E.coli*	31.549	31.596	29.885	Undetermined		
	BHK	30.809	30.915	30.421	Undetermined		
	VERO	32.221	31.982	31.791	Undetermined		
	SF9	33.237	30.542	32.677	Undetermined		
	CHO	33.475	31.688	33.351	Undetermined		
Non-amplification control	Nuclease-free H_2O	Undetermined			Undetermined		

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C. Reproducibility

CV<5% for Cq value and CV<15% for mtDNA damage when the input DNA ≥ 0.3 ng / reaction.

293T (ng)	Channel	Cq value						mtDNA damage (%)					
		Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	CV%	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	CV%
100	FAM	11.77	11.74	11.99	11.91	12.17	**1.34**	20.99	17.67	22.97	28.50	26.77	**16.70**
	JUN	12.66	12.64	12.64	12.67	13.32	**2.08**						
30	FAM	13.20	12.68	12.89	13.34	12.77	**1.95**	28.47	29.64	32.39	26.97	29.61	**6.04**
	JUN	14.24	13.81	13.73	14.08	13.97	**1.32**						
10	FAM	14.50	14.32	14.28	13.84	14.55	**1.75**	27.54	29.39	23.95	34.17	29.14	**11.43**
	JUN	15.52	15.44	14.95	14.73	15.75	**2.47**						
3	FAM	15.75	15.48	15.02	16.23	16.12	**2.81**	31.26	23.23	26.05	30.25	25.04	**11.36**
	JUN	16.85	16.48	15.73	17.03	17.23	**3.19**						
1	FAM	17.26	17.33	17.22	17.14	16.90	**0.85**	24.32	32.25	28.90	30.69	32.02	**9.84**
	JUN	18.22	18.51	17.98	17.95	18.15	**1.11**						
0.3	FAM	18.64	18.41	18.67	19.03	18.20	**1.49**	23.22	26.51	23.99	25.99	20.80	**8.51**
	JUN	19.58	19.48	19.34	19.75	19.23	**0.93**						
0.1	FAM	19.72	19.50	19.35	18.88	19.57	**1.48**	45.97	58.81	83.47	87.68	64.17	**22.88**
	JUN	21.17	21.41	22.21	22.19	21.74	**1.91**						
0.03	FAM	20.30	20.04	19.77	19.94	20.42	**1.18**						
	JUN	Undetermined											
Non-damaged DNA	FAM	16.33	16.36	16.79	16.78	16.35	1.30						
	JUN	16.89	16.98	17.07	17.07	17.05	0.40						
Damaged DNA	FAM	16.66	16.99	16.66	16.89	16.58	0.95						
	JUN	Undetermined											
Non-amplification control	FAM	Undetermined											
	JUN	Undetermined											

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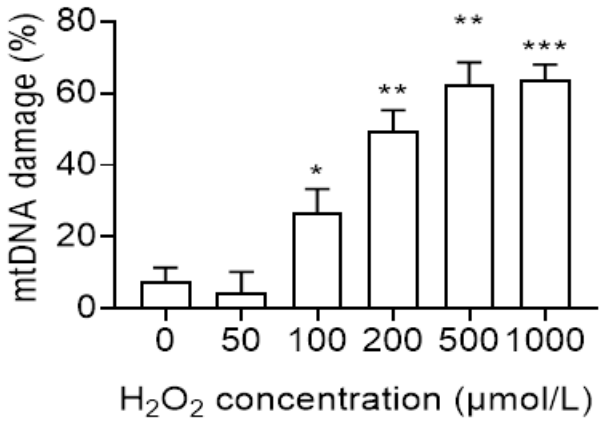
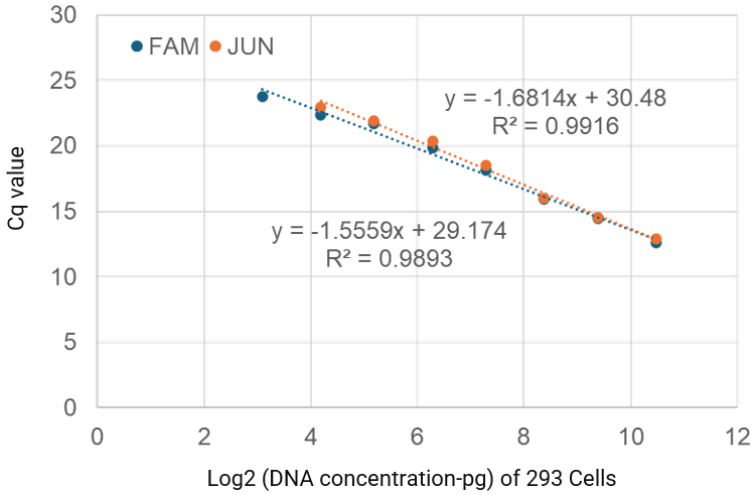
Example of H_2O_2-induced mtDNA damage



H_2O_2-induced mtDNA lesions. Quantification of mtDNA damage (%) by PCR amplification of total DNA isolated from Hela cells exposed to 0 – 1000 µM hydrogen peroxide (H_2O_2) for 60 minutes showing a steadily increasing mtDNA damage over H_2O_2 concentration in all tested mtDNA regions. Error bars designate standard deviation (four independent experiments). The input DNA amount is 1 ng/reaction for all samples.

Example standard curves of FAM and JUN amplification



Standard curves of FAM and JUN amplification for different DNA concentrations (From 30 pg - 100,000 pg/reaction) of Hela cells detected by this kit.

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Important General Notes

1. Each kit has passed strict Q.C testing. However, results may vary slightly from lab to lab. Intra-assay variance among kits from different batches might arise from aforementioned factors.

2. Kits from different batches may differ slightly in detection range, sensitivity and developing time. Please perform the experiment exactly according to the instructions in this manual.

3. To minimize cross-contamination during qPCR, please refer to the following 5 tips:

- Always wear gloves and change them frequently.
- Use aerosol-resistant pipette tips.
- Apply the three-room rule, where there is a dedicated area for nucleic acid extraction, one for reaction set up for the qPCR reaction, and one for the qPCR cycler.
- All surfaces in the PCR area should be routinely decontaminated with DNase/RNase killer solutions to prevent cross contamination.
- Always use DNase/RNase-free water to perform your experiments.

4. Use pipettes calibrated for low volumes (such as P2 or P10) for absolute accuracy. Also, using the right pipettes ensures reproducibility between replicates.

5. Dilute your samples (less may be more). qPCR is highly sensitive, and less template often gives a more accurate measurement. For optimal quantification, the input DNA amount should be 1 - 10 ng in 2 µL/reaction.

6. Check and double-check the program on the qPCR cycler. This is important if you are using a shared instrument. Even if you have your own template file set up, double check your run cycle before hitting start. Other researchers may have made small changes to your cycling template (e.g. annealing temperature, hot start activation time) without your knowledge. Therefore, we advise verifying that the program you are about to launch is unchanged.

7. To achieve reproducible results, the operation of every step in the assay should be controlled (i.e. DNA isolation method, DNA input concentration, etc.). Furthermore, a preliminary experiment before every assay for each batch is recommended.