

Mouse Mitochondrial DNA Damage Quantification Kit (qPCR Assay)

Catalog #: MTM-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 diseases including neurodegenerative disorders, cardiovascular diseases and cancer. Damage to mtDNA is also a meaningful biomarker for evaluating genotoxicity of drugs and environmental toxins. Mice have become the most ubiquitous model organism used to study human disease. Mice are strikingly similar to humans at the genomic level and the pathophysiology of disease in mice is similar to that of humans. Moreover, mice are a cost-effective and efficient tool to enhance research and drug testing.

The RayBio[®] Mouse Mitochondrial DNA Damage Quantification Kit (MTM-DQ) is a Taqman[™] probe-based qPCR assay for the specific quantitative measurement of mouse mitochondrial DNA damage. This kit targets the long mtDNA region (D-Loop gene), which is the most susceptible to damage. 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, blood, etc.

Product Use

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

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	MTM-DQ-MIX	1 mL/1 tube	dNTPs, Hot Start <i>Taq</i> DNA Polymerase	-20°C
Primer and Probe Mix	MTM-DQ-PP	1 mL/1 tube	Mouse Long and Short mtDNA Primers and Probes	-20°C
Damaged DNA	MTM-DQ-DDNA	20 µL/1 tube	Damaged Cellular DNA	-20°C
Non-damaged DNA	MTM-DQ-NDDNA	20 µL/1 tube	Non-damaged Cellular DNA	-20°C
Nuclease-Free H ₂ O	MTM-DQ-H ₂ O	1 mL/1 tube	Nuclease-Free H ₂ O	-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

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 H₂O) 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 H₂O) 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).

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 ₂ O	-	-	-	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 ₂ O-1	Nuclease-Free H ₂ O-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		

- Load Samples.** Add 2 µL of appropriate DNA samples (Damaged DNA, Non-damaged DNA, Sample DNA, Nuclease-free H₂O) to designated wells of the PCR plate or tubes. Pipette up and down at least 5 times to mix.
- 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.

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	50	2 min	1.6°C / sec	1
Step 2	95	10 min	1.6°C / sec	1
Step 3	95	10 sec	1.6°C / sec	40
	60	60 sec	1.6°C / sec	

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

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 $\Delta\Delta Cq$ (Quantification Cycle Value) method in the formula of $= (1 - 2^{-\Delta\Delta Cq}) \times 100\%$ for mtDNA damage quantification.

Example Calculations:

Channel	Sample 1			Non-damaged DNA			Damaged DNA		
	Rep-1	Rep-2	Average	Rep-1	Rep-2	Average	Rep-1	Rep-2	Average
JUN	16.28	16.37	16.325	15.22	15.06	15.14	Undetermined	Undetermined	None
FAM	18.01	18.42	18.215	17.41	17.53	17.47	18.29	18.75	18.52

$$\begin{aligned} \Delta\Delta Cq &= \Delta Cq (\text{JUN}) - \Delta Cq (\text{FAM}) \\ &= [\text{JUN (Cq, Sample 1)} - (\text{Cq, Non-damaged DNA})] - [\text{FAM (Cq, Sample 1)} - (\text{Cq, Non-damaged DNA})] \\ &= [16.325 - 15.14] - [18.215 - 17.47] \\ &= 0.44 \end{aligned}$$

The mtDNA damage of Sample 1:

$$\begin{aligned} &= (1 - 2^{-\Delta\Delta Cq}) \times 100\% \\ &= (1 - 2^{-0.44}) \times 100\% \\ &= 0.26287 \times 100\% \\ &= 26.287\% \end{aligned}$$

Result: The average mtDNA damage of Sample 1 is 26.287%

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 μ L/reaction.

B. Specificity

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

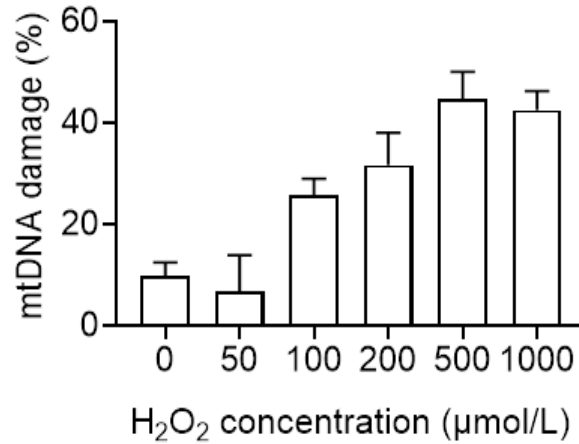
Species	Input DNA (1ng)	FAM			JUN		
		Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
Mouse DNA	NIH/3T3	17.065	17.111	17.147	20.583	20.973	20.524
Non-Mouse DNA	A549	34.226	33.762	33.102	Undetermined		
	293T	32.909	31.689	31.928	Undetermined		
	Hela	33.748	34.618	34.968	Undetermined		
	<i>E.coli</i>	32.451	32.015	31.026	Undetermined		
	BHK	31.633	30.552	30.965	Undetermined		
	VERO	32.146	30.251	30.148	Undetermined		
	SF9	33.626	33.288	33.515	Undetermined		
Non-amplification control	Nuclease-free H ₂ O	Undetermined			Undetermined		

C. Reproducibility

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

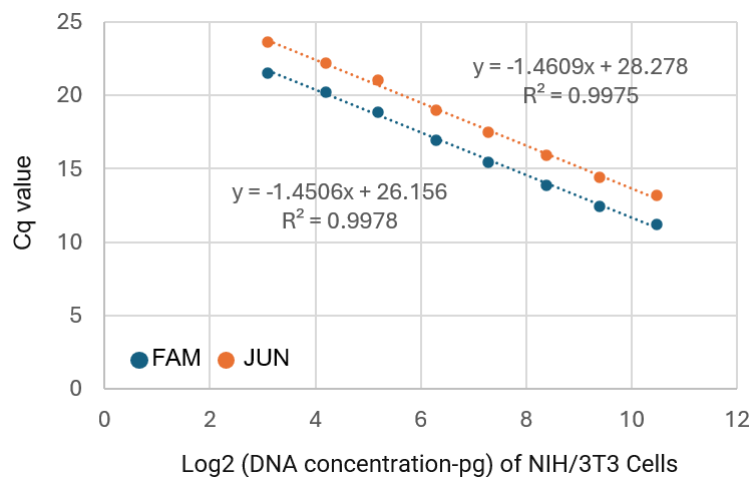
NIH/3T3 (ng)	Channel	Cq value					mtDNA damage (%)				
		Rep-1	Rep-2	Rep-3	Rep-4	CV%	Rep-1	Rep-2	Rep-3	Rep-4	CV%
100	FAM	11.31	10.95	11.52	10.93	2.57	16.71	16.72	21.41	22.79	16.28
	JUN	13.62	13.15	13.44	12.57	3.49					
30	FAM	12.25	12.64	12.71	12.15	2.24	20.43	23.26	18.50	23.63	11.34
	JUN	14.49	14.72	14.68	13.77	3.07					
10	FAM	13.88	13.77	13.98	13.83	0.64	18.98	15.04	19.43	18.26	11.07
	JUN	16.15	16.00	15.94	15.55	1.60					
3	FAM	15.54	15.49	15.48	15.27	0.79	17.34	13.60	20.30	17.30	16.01
	JUN	17.84	17.75	17.43	17.00	2.16					
1	FAM	17.06	16.51	17.09	16.99	1.60	13.63	17.84	11.87	17.41	19.97
	JUN	19.43	18.67	19.17	18.73	1.91					
0.3	FAM	18.93	18.92	19.14	18.47	1.49	10.04	8.11	10.52	11.21	13.33
	JUN	21.35	21.26	21.25	20.31	2.33					
0.1	FAM	20.27	20.31	20.17	20.18	0.34	-1.38	30.74	34.03	24.43	73.13
	JUN	22.87	22.24	21.84	21.79	2.25					
0.03	FAM	21.89	21.86	21.48	20.93	2.08	9.78	5.69	20.12	20.86	53.55
	JUN	24.32	24.24	23.43	22.60	3.40					
Non-damaged DNA	FAM	13.68	13.43	13.56	13.85	1.30					
	JUN	16.25	15.90	15.82	15.86	1.25					
Damage DNA	FAM	15.89	15.17	15.22	15.39						
	JUN	Undetermined									
Non-amplification control	FAM	Undetermined									
	JUN	Undetermined									

Example of H₂O₂-induced mtDNA damage



H₂O₂-induced mtDNA lesions. Quantification of mtDNA damage (%) by PCR amplification of total DNA isolated from NIH/3T3 cells exposed to 0 – 1000 µM hydrogen peroxide (H₂O₂) for 60 minutes showing a steadily increasing mtDNA damage over H₂O₂ 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 NIH/3T3 cells detected by this kit.

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 μ L/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.