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Human Absolute Mitochondrial DNA Copy Number Quantification Kit

Catalog #: MTH-CNQ

User Manual
Last Revised: September 18th, 2024

Introduction

The mitochondrial genome (mtDNA) is a circular, intron-free, double-stranded, haploid DNA strand 16.5 kb in length that encodes 37 genes. mtDNA copy number (mtDNA-CN) is a biomarker for mitochondrial dysfunction associated with overall mortality and a number of age-related diseases, including cardiovascular disease, chronic kidney disease, and cancer. The importance of mtDNA-CN measurement to disease suggests the potential for mtDNA-CN to be a useful biomarker in the clinic.

The RayBio® Human Absolute Mitochondrial DNA Copy Number Quantification Kit (MTH-CNQ) is a Taqman™ probe-based qPCR assay for the specific quantitative measurement of human mtDNA-CN. Both the primer and probe of mtDNA specifically target one of the most conserved regions on human mtDNA, with no amplification of any off-target sequence on nuclear genomic DNA. Meanwhile, both primer and probe of the novel single copy gene (IFNB1) specifically recognizes and amplifies a 110 bp-long nuclear genomic DNA and serves as a reference for data normalization. The optimized PCR formulation provides superior specificity and efficiency with a wide linear dynamic range. This kit allows for the determination of human mitochondrial DNA copy number in one reaction, by the comparison of mitochondrial (mt) and nuclear (n) DNA using various genomic DNA from cultured cells, tissue, saliva, urine, spinal fluid, lavage fluid, blood, etc.

Product Use

MTH-CNQ 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-CNQ-MIX	1 mL / 1 tube	dNTPs, Hot Start *Taq* DNA Polymerase	-20°C
Primer and Probe Mix	MTH-CNQ-PP	1 mL / 1 tube	Human Long and Short mtDNA Primers and Probes	-20°C
Reference DNA (1000 copies/cell)	MTH-CNQ-ReDNA	20 µL / 1 tube	Reference Human DNA	-20°C
Nuclease-free H_2O	MTH-CNQ-H_2O	1 mL / 1 tube	Nuclease-free H_2O	-20°C

Note: Do not mix reagents from different lots.

Additional Materials Required

- Fluorescence qPCR instrument capable of reading FAM (494 nm maximum absorption, 518 nm maximum emission) and VIC (532 nm maximum absorption, 552 nm maximum excitation) channels
- 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 human 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 2 µL/reaction. We recommend the same amount of DNA loading across all reactions.

3. We highly recommend running duplicates or triplicates for each sample, reference DNA and nuclease-free H_2O.

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) + Reference DNA x 2 + 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 (Reference DNA) x 2 + 1 (Nuclease-free H_2O) x 2 + 2 = 46.

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	Reference DNA	Nuclease-free H_2O	Sample
2X Probe qPCR Master Mix	10 µL	10 µL	10 µL
Primers and Probe Mix	8 µL	8 µL	8 µL
Reference DNA	2 µL	-	-
Nuclease-free H_2O	-	2 µL	-
Sample DNA	-	-	2 µL
Total Volume	**20 µL**	**20 µL**	**20 µL**

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

Reference DNA-1	Reference DNA-2	Sample 7-1	Sample 7-2	Sample 15-1	Sample 15-2
H_2O-1	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		
Sample 6-1	Sample 6-2	Sample 14-1	Sample 14-2		

3. **Load Samples.** Add 2 µL of appropriate DNA sample (Reference DNA, Nuclease-free H_2O, Sample DNA) 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 at low speed for 15 seconds 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, Non-Template Control, and Amplification Control accordingly to your 96-well plate setup. Select FAM and VIC 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 copy number 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	35
	Anneal, extend, and detect fluorescence	60	40 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 Copy Number Quantification.** Use the comparative ΔΔCq (Quantification Cycle Value) method and multiply the Reference DNA copy number x $2^{-\Delta\Delta Cq}$ for mtDNA copy number 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 mtDNA copy number

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Typical Data

Calculate the mean Cq value for each set of duplicate Reference DNA and samples. The nuclease-free H_2O should be "Undetermined". Then, use the comparative ΔΔCq (Quantification Cycle Value) method and multiply the Reference DNA copy number x $2^{-\Delta\Delta Cq}$ for mtDNA copy number quantification.

Example Calculations:

Samples	**Cq** (IFNB1, VIC)	**Cq** (mtDNA, FAM)
Sample 1-1	25.286	17.257
Sample 1-2	25.185	17.535
Reference DNA-1	25.078	18.252
Reference DNA-2	24.881	18.249
Nuclease-free H_2O-1	Undetermined	Undetermined
Nuclease-free H_2O-2	Undetermined	Undetermined
Average Sample 1	**25.236**	**17.396**
Average Reference DNA	**24.979**	**18.251**

ΔΔCq = ΔCq (mtDNA) - ΔCq (IFNB1)

= [mtDNA (Cq, Sample 1) - (Cq, Reference DNA)] - [IFNB1 (Cq, Sample 1) - (Cq, Reference DNA)]

= [17.396-18.251] - [25.236-24.979]

= -1.112

The mtDNA copy number per diploid cell of Sample 1

= Reference sample mtDNA copy number x $2^{-\Delta\Delta Cq}$

= 1000 x $2^{-(-1.112)}$

= 1000 x 2.161

= 2161

Result: The average mtDNA copy number of Sample 1 is 2161 per diploid cell

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A. Sensitivity

The minimum detectable concentration of DNA is 30 pg/reaction. 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, which means there is high specificity for mtDNA and IFNB1 amplification.

Species	Input DNA 1 ng	IFNB1-VIC		mtDNA-FAM		Average	
		Rep-1	Rep-2	Rep-1	Rep-2	IFNB1	MIT1
Human DNA	A549	27.40	19.39	26.58	19.38	26.99	19.39
	293T	27.70	19.65	27.35	19.42	27.53	19.54
	Hela	26.99	18.96	26.71	18.82	26.85	18.89
Non-Human DNA	*E.coli*	32.39	32.16	29.35	29.86	32.28	29.60
	BHK	32.09	33.96	29.42	28.89	33.03	29.15
	VERO	32.35	31.86	30.45	30.06	32.11	30.25
	SF9	32.92	32.37	29.86	29.90	32.64	29.88
	CHO	32.37	32.50	29.89	30.51	32.43	30.20
Non-amplification control	Nuclease-free H_2O	Undetermined					

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C. Reproducibility

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

293T (ng)	Channel	Cq value					Copies/Cell				
		Rep-1	Rep-2	Rep-3	Rep-4	CV%	Rep-1	Rep-2	Rep-3	Rep-4	CV%
100	IFNB1-VIC	21.31	21.94	21.71	21.86	1.11	2512.1	2344.9	2939.3	2213.1	10.9
	mtDNA-FAM	15.38	15.08	15.17	15.08	0.80					
30	IFNB1-VIC	23.08	23.03	22.73	22.95	0.59	2505.5	2240.0	2495.0	2219.2	5.7
	mtDNA-FAM	17.15	16.24	16.42	16.18	2.36					
10	IFNB1-VIC	24.03	24.35	24.49	24.28	0.69	2689.2	2523.8	2453.6	2634.1	3.6
	mtDNA-FAM	18.00	17.39	18.21	17.26	2.25					
3	IFNB1-VIC	25.79	25.98	25.63	25.75	0.48	2820.7	2467.8	2622.0	2052.0	11.3
	mtDNA-FAM	19.69	19.05	19.26	19.08	1.32					
1	IFNB1-VIC	27.17	27.81	27.21	27.91	1.22	2423.6	2049.8	2359.9	2440.7	6.8
	mtDNA-FAM	21.29	21.14	20.99	21.00	0.58					
0.3	IFNB1-VIC	28.12	28.91	28.73	28.76	1.06	2521.6	2296.3	2406.5	2001.1	8.4
	mtDNA-FAM	22.18	22.08	22.48	22.13	0.70					
0.1	IFNB1-VIC	30.76	30.91	30.62	30.35	0.67	2660.3	2075.4	2884.0	1769.0	19.0
	mtDNA-FAM	24.74	24.23	24.10	23.90	1.28					
0.03	IFNB1-VIC	32.35	31.86	31.38	31.69	1.11	3277.8	1293.9	1279.6	1541.2	45.0
	mtDNA-FAM	26.04	25.86	26.03	25.44	0.94					
Reference DNA	IFNB1-VIC	26.44	26.88	26.19	26.59	0.94					
	mtDNA-FAM	21.84	21.25	21.21	20.97	1.50					
Nuclease-free H_2O	IFNB1-VIC	Undetermined									
	mtDNA-FAM	Undetermined									

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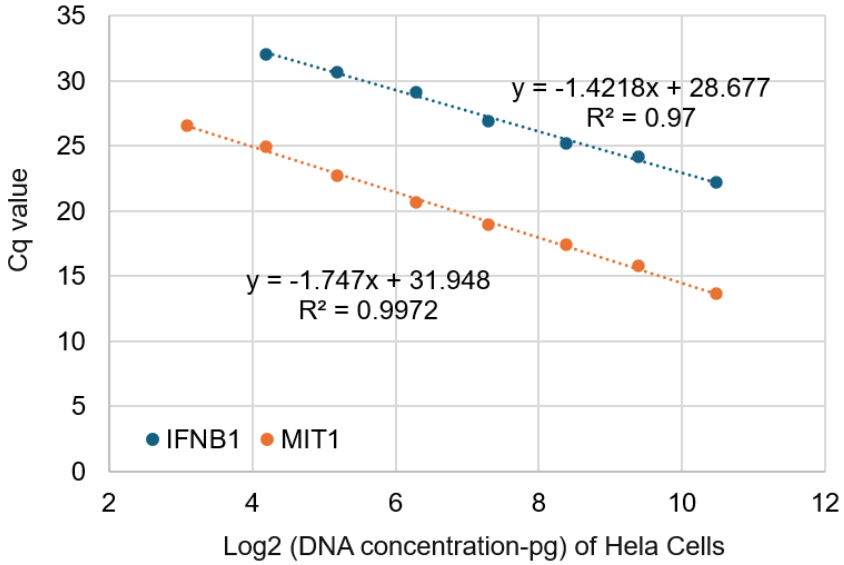
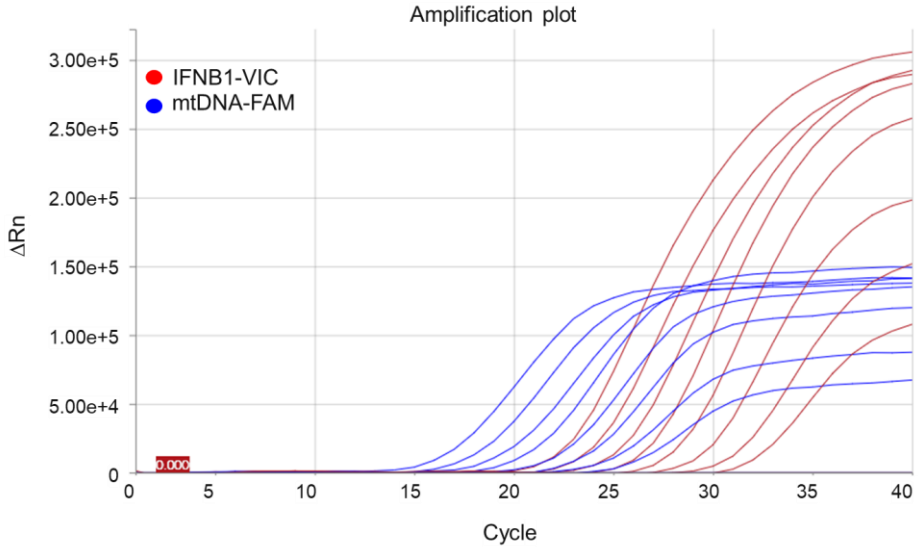
Example standard curves of FAM and VIC amplification



Standard curves of mtDNA-FAM and IFNB1-VIC amplification for different DNA concentrations (From 30 pg - 100,000 pg/reaction) of Hela cells detected by this kit.

Example amplification curves of FAM and VIC



Amplification curves of mtDNA-FAM and IFNB1-VIC 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 color developing time. Please perform the experiment exactly according to the instructions.

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.