# Determination of Mitochondrial DNA Upon Drug Treatment

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**[Abstract]** Drug-induced mitochondrial injury can be caused by many different mechanisms including inhibition of mitochondrial DNA replication, transcription, translation, and altered protein function. Determination of the level of mitochondrial DNA relative to the nuclear DNA levels provides important information on potential mitochondrial toxicity.

## Materials and Reagents

1. HepG2 cells (ATCC, catalog number: HB 8065)
2. DMSO (cell culture grade) (Sigma-Aldrich, catalog number: D2650)
3. Phosphor-buffered saline (PBS) (Life Sciences, catalog number: 10010049)
4. QIAamp DNA mini kit (QIAGEN, catalog number: 51304)
5. TaqMan universal mastermix (Life Technologies, Applied Biosystems®, catalog number: 4352042)
6. β-actin Assay-on-Demand kit (Life Technologies, Applied Biosystems®, catalog number: 4331182)
7. Eagle's minimum essential medium (Life Technologies, Gibco®, catalog number: 41090)
8. GlutaMAX™
9. Fetal bovine serum (FBS) (HyClone, catalog number: SH30071.03)
10. 100 units/ml penicillin, 100 units/ml streptomycin (Life Technologies, Gibco®, catalog number: 15140)
11. Sodium pyruvate (Life Technologies, Gibco®, catalog number: 11360)
12. Cells were cultured in Eagle's minimum essential medium (see Recipes)
    *Note: Cells were cultured in Eagle's minimum essential medium.*

## Equipment

1. 12-well plates (Corning, catalog number: 3513)
2. ABI Prism 7900HT Fast Real-Time PCR system (Life Technologies, Applied Biosystems®)

 

**Procedure**

1. HepG2 cells were seeded into 12-well plates at a density of 2 x $10^5$ cells per well and allowed to attach overnight. The volume of medium was 1.0 ml in each well.
2. After the overnight incubation, the media in each well was replaced with 1.0 ml of fresh media containing tested compounds and controls, and incubated for 10 more days. The media was replaced with fresh media and compounds every 3 to 4 days. The DMSO concentration was kept at 1.0% for all treatments including control samples (no drug, DMSO only).
3. Following the incubation, the cells were washed once with PBS and the total DNA was extracted from the cells using the QIAamp DNA Mini Kit according to the manufacturer's protocol.
4. Real-time PCR reactions were performed using TaqMan universal mastermix in an ABI Prism 7900HT Fast Real-Time PCR System.
5. Quantification of mtDNA was achieved by amplification of a fragment of the mitochondrial specific cytochrome b gene using the primers and probe described in table 1. Chromosomal DNA was quantified by the amplification of a fragment of the β-actin gene using a β-actin Assay-on-Demand kit.
6. Amplification reactions for the quantification of mitochondrial and chromosomal DNA were performed independently using approximately 25 ng of total DNA in a volume of 20 µl.

**Table 1. Real-Time PCR primers and probe used in the quantification of the cytochrome b gene from HepG2 cells**

| Primer/probe name | Oligonucleotide sequence | Concentration in qPCR |
|---|---|---|
| Cytochrome b forward | CCTTCCACCCTTACTACACAATCAA | 0.9 µM |
| Cytochrome b reverse | GGTCTGGTGAGAATAGTGTTAATGTCA | 0.9 µM |
| Cytochrome b probe | FAM-ACGCCCTCGGCTTAC-BHQ1 | 0.2 µM |

7. Data analysis
   a. The relative amount of mtDNA in treated samples was determined using a relative quantification method based upon the $2^{-\Delta\Delta C_T}$ formula (Livak and Schmittgen, 2001).
   b. The amount of mtDNA (% mtDNA) in compound treated samples relative to the DMSO treated controls was calculated based upon the following formula:

% mtDNA = 100 x $2^{-\Delta\Delta C_T}$

$\Delta\Delta C_T = \Delta C_{T,\ treated} - \Delta C_{T,\ control}$

$\Delta C_{T,\ treated} = (C_T,\ cyt\ b - C_T,\ \beta\text{-actin})_{\ treated}$

$\Delta C_{T,\ control} = (C_T,\ cyt\ b - C_T,\ \beta\text{-actin})_{\ control}$

$C_T$, cyt b and $C_T$, β-actin represent the cycle threshold values for the amplification of cytochrome b and β-actin, respectively, as determined by the computational analysis of amplification curves using the ABI Prism software. The final results are presented as the mean % mtDNA ± SD from 3 independent experiments, each performed in triplicate.

c. The $2^{-\Delta\Delta CT}$ method was validated for cytochrome b and β-actin genes by determining the $\Delta C_T$ values for amplification reactions containing various amounts of total cellular DNA. Minimal differences were observed in the $\Delta C_T$ values in samples containing 5 to 40 ng of total cellular DNA; indicating that neither the amplification nor detection efficiencies of cytochrome b and β-actin were affected by the amount of DNA template within the dilution range relevant for the quantitative analysis performed in this study table 2.

d. The effect of a positive control compound ddC (dideoxy cytidine) is shown in Table 3.

**Table 2. Validation of the $2^{-\Delta\Delta C_T}$ method for cytochome b and β-actin target genes**

| Amount of total cellular DNA (ng/reaction) | $C_T$ Value[a] | | $\Delta C_T$ Value |
|---|---|---|---|
| | Cytochome b | β-actin | |
| 5 | 15.7 ± 0.4 | 22.2 ± 0.3 | -6.6 ± 0.1 |
| 10 | 17.3 ± 0.4 | 23.9 ± 0.3 | -6.7 ± 0.1 |
| 20 | 18.8 ± 0.3 | 25.7 ± 0.3 | -6.9 ± 0.1 |
| 40 | 20.3 ± 0.4 | 27.3 ± 0.3 | -7.0 ± 0.1 |

[a]The data represent the mean ± SD of 3 independent experiments performed in triplicate

**Table 3. Effect of positive control ddC on the levels of mtDNA in HepG2 cells**

| Compound | Concentration (μM) | Relative amount of mtDNA (% mtDNA)[a] | p-value compared to DMSO (control)[b] |
|---|---|---|---|
| DMSO (control) | - | 100.0 ± 8.8 | - |
| ddC | 0.2 | 57.0 ± 10.4 | < 0.0001 |
| | 2.0 | 25.1 ± 7.8 | < 0.0001 |
| | 20 | 6.9 ± 2.9 | < 0.0001 |

[a]The data represent the mean ± SD of 3 independent experiments performed in triplicate

[b]Paired, two-tailed Student's t-test

## Recipes

1. Eagle's minimum essential medium
   GlutaMAX™
   10% fetal bovine serum
   100 units/ml penicillin
   100 units/ml streptomycin
   1 mM sodium pyruvate

## Acknowledgments

All of the work was sponsored by Gilead Sciences, Inc. This protocol was adapted from Feng *et al.* (2014).

## References

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