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Purification of cytosolic fraction and quantification of mtDNA by qPCR

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This protocol describes the purification of a cytosolic fraction depleted of membrane from cultured cells, and the quantification of mitochondrial DNA (mtDNA) in this fraction by qPCR.

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Mitochondria DNA (mtDNA), Cytosol fractionation, qPCR, ASAPCRN

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Solutions to prepare:

DMEM solution:

A	B
FBS	10%
Penicillin	100 U/ml
Streptomycin	100 mg/ml
L-glutamine	2 mM

Cytoplasmic buffer:

A	B
NaCl	150 mM
HEPES	50 mM
Digitonin, pH 7.4	1 mg/mL

Lysis buffer:

A	B
NaCl	150 mM
HEPES	50 mM
Digitonin, pH 7.4	1 mg/mL
SDS supplemented with Protease Inhibitor Cocktail (Roche)	1%

Cell culture and purification of cytosolic fraction

1 Plate HeLa cells in DMEM **15 cm** plates (3.5×10^6 cells per plate).

2 The following day, prepare cytosolic buffer with fresh digitonin.

3 Prepare lysis buffer.

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4 5m

Trypsinize cells and centrifuge at **1500 rpm** for **00:05:00** at **22 °C** .

5 Resuspend cells in PBS and count cells.

6 5m

Collect the same number of cells from each genotype (5 x 10^6) and centrifuge at **1500 rpm** for **00:05:00** at **22 °C** .

7 Resuspend cells in **1 mL** PBS and transfer **50 µL** to a prechilled Eppendorf another (WCE). Keep **On ice** .

8 5m

Transfer the remaining **950 µL** to a prechilled Eppendorf and centrifuge at **4500 rpm** for **00:05:00** at **22 °C** .

9 Remove supernatant and resuspend pellet in **500 µL** cytosolic buffer.

10 10m

Rotate for **00:10:00** at **4 °C** .

11 3m

Centrifuge extract at **980 x g** for **00:03:00** at **4 °C** . Transfer supernatant to new Eppendorf tube and save pellet for analysis (Pel).

10m

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12 

Centrifuge supernatant at **17000 x g** for **00:10:00** at **4 °C** .

13 Collect supernatant (Cyt).

14 Purify DNA from WCE and Cyt fractions using DNeasy Kit (Qiagen).

15 Measure DNA concentration and dilute samples 1:10.

qPCR

16 Combine **10 µL** SYBR Green Master Mix (BioRad) with **6.78 µL** Sterile Water (American Bio) per sample.

17 

Combine **16.78 µL** diluted SYBR Green Master Mix with **0.61 µL** each of **10 Micromolar (µM)** forward and reverse primers per sample. Pipette this mixture into wells of 96-well qPCR plate. Perform at at least two technical replicates for each sample.

18 

Pipette **2 µL** of diluted DNA from step 15 in well with SYBR Green Master Mix.

19 Cover plate with Optical Adhesive Covers (Applied Biosystems).

20 

Spin down plate in table top centrifuge.

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Run qPCR in CFX96 Real-Time System (BioRad) using the following protocol:

A	B	C
95 ˚C	3 min	
95 ˚C	10 sec	Repeat 39x
55 ˚C	10 sec	
72˚C	30 sec	
95 ˚C	10 sec	
65 ˚C	5 sec	
95 ˚C	5 sec	

Data analysis

22 Subtract the nuclear gene (hB2M) mean threshhold cycle (Ct) values from WCE samples from mtDNA amplicon of interest mean Ct values from Cyt samples to calculate ΔCt.

23 Subtract the ΔCt of the control sample from each sample ΔCt to calculate the ΔΔCt value.

24 Calculate relative expression using the $2^{-\Delta\Delta Ct}$ method.

25 WT mtDNA abundance was given a value of 1.

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