Purification of cytosolic fraction and quantification of mtDNA by qPCR

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Abstract

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.

Keywords

Mitochondria DNA (mtDNA), cytosol fractionation, qPCR

Solutions to prepare

DMEM containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (all from Gibco)

Cytoplasmic buffer (**CB**, 150 mM NaCl, 50 mM HEPES, 1 mg/mL digitonin, pH 7.4). Prepare **Lysis buffer** (CB + 1% SDS supplemented with Protease Inhibitor Cocktail (Roche)).

A. Cell culture and purification of cytosolic fraction

1. Plate HeLa cells in DMEM 15 cm plates (3.5 x 10^6 cells per plate).
2. The following day, prepare cytosolic buffer with fresh digitonin.
3. Prepare lysis buffer.
4. Trypsinize cells and centrifuge at 1500 rpm for 5 min at 22 ˚C.
5. Resuspend cells in PBS and count cells.
6. Collect the same number of cells from each genotype (5 x 10^6) and centrifuge at 1500 rpm for 5 min at 22 ˚C.
7. Resuspend cells in 1 mL PBS and transfer 50 µL to a prechilled Eppendorf another (WCE). Keep on ice.
8. Transfer the remaining 950 µL to a prechilled Eppendorf and centrifuge at 4500 RPM for 5 min at 22 ˚C.
9. Remove supernatant and resuspend pellet in 500 µL cytosolic buffer.
10. Rotate for 10 minutes at 4 ˚C.
11. Centrifuge extract at 980 g for 3 min at 4 ˚C. Transfer supernatant to new Eppendorf tube and save pellet for analysis (Pel)
12. Centrifuge supernatant at 17,000 g for 10 min 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.

B. qPCR

1. Combine 10 uL SYBR Green Master Mix (BioRad) with 6.78 uL Sterile Water (American Bio) per sample.
2. Combine 16.78 µL diluted SYBR Green Master Mix with 0.61 µL each of 10 µM forward and reverse primers per sample. Pipette this mixture into wells of 96-well qPCR plate. Perform at least two technical replicates for each sample.
3. Pipette 2 uL of diluted DNA from step A15 in well with SYBR Green Master Mix.
4. Cover plate with Optical Adhesive Covers (Applied Biosystems).
5. Spin down plate in table top centrifuge
6. Run qPCR in CFX96 Real-Time System (BioRad) using the following protocol:

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	

C. Data analysis

1. 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.
2. Subtract the ΔCt of the control sample from each sample ΔCt to calculate the ΔΔCt value.
3. Calculate relative expression using the $2^{-\Delta\Delta Ct}$ method.
4. WT mtDNA abundance was given a value of 1.