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Measurement of Mitochondrial DNA Release in Response to ER Stress

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Abstract

Mitochondria house the metabolic machinery for cellular ATP production. The mitochondrial network is sensitive to perturbations (*e.g.*, oxidative stress and pathogen invasion) that can alter membrane potential, thereby compromising function. Healthy mitochondria maintain high membrane potential due to oxidative phosphorylation (Ly *et al.*, 2003). Changes in mitochondrial function or calcium levels can cause depolarization, or a sharp decrease in mitochondrial membrane potential (Bernardi, 2013). Mitochondrial depolarization induces opening of the mitochondrial permeability transition pore (MPTP), which allows release of mitochondrial components like reactive oxygen species (mtROS), mitochondrial DNA (mtDNA) or intermembrane space proteins into the cytosol (Martinou and Green, 2001; Tait and Green, 2010; Bronner and O'Riordan, 2014). These contents trigger inflammation, and can lead to cell death (West *et al.*, 2011). Both mtROS and cytosolic mtDNA contribute to the activation of inflammasomes, multiprotein complexes that process the proinflammatory cytokines, IL-18 and IL-1β. Studies indicate that cytosolic mtDNA in particular can bind two different inflammasome sensors, AIM2 and NLRP3, leading to inflammasome activation (Burckstummer *et al.*, 2009; Hornung and Latz, 2010). In this protocol, you will be able to specifically extract cytosolic mtDNA and quantify the amount using a qPCR assay.

Part I. Extraction and purification of cytosolic mtDNA

Materials and Reagents

A. For extraction (see Figure 1)

1. 1.5 ml microcentrifuge tubes (Denville Scientific Inc., catalog number: C2170)
2. Gloves
3. 6 well-plates, tissue culture-treated (Corning, catalog number: 3506)
4. Cell lifter (Biologix Group Limited, catalog number: 70-2180)
5. Murine immortalized bone marrow derived macrophages (Bernardi, 2013)

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6. Thapsigargin, 97%, ACROS Organics™ (Thermo Fisher Scientific, Fisher Scientific™, catalog number: AC328570010) (Bronner *et al.*, 2015)
7. 1% NP-40 (Igepal CA-630) (Sigma-Aldrich, catalog number: I8896) (Bronner and O'Riordan, 2014)
8. DPBS (Thermo Fisher Scientific, Gibco™, catalog number: 14040-133) (Burckstummer, 2009)
9. DMEM (Thermo Fisher Scientific, Gibco™, catalog number: 11965-092)
10. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10437-028)
11. Medium (see Recipes)
12. NP-40 (Igepal CA-630) solution (see Recipes)
13. Thapsigargin stock solution (see Recipes)

B. For purification

1. Gloves
2. DNeasy blood & tissue kit, 50 samples (QIAGEN, catalog number: 69504) or 250 samples (QIAGEN, catalog number: 69506)
3. Ethanol 200 proof (Decon Labs, catalog number: 2716)

Equipment

1. Centrifuge (Thermo Fisher Scientific, Fisher Scientific™, model: accuSpin™ Micro 17)

Procedure

1. Seed 1×10^6 cells in 2 ml per well into 6-well plate in the appropriate medium.
2. The following day (~16 h later), aspirate medium and add medium containing thapsigargin (10 μM in 2 ml medium for 4–6 h) (Hornung and Latz, 2010).
3. At relevant times post treatment (*e.g.*, 2 h, 4 h and 8 h) wash cells with 1× DPBS once then aspirate 1× DPBS.
4. Add 1% NP-40 (100 μl) to each well and scrape cells.
5. Place lysates into prelabeled microcentrifuge tubes and incubate on ice for 15 min (Livak and Schmittgen, 2001).
6. Spin lysates at 13,000 rpm (16,000 × *g*) for 15 min at 4 °C to pellet the insoluble fraction.
7. Transfer supernatant (the cytosolic fraction) to a new tube and discard the pellet.

a. Use supernatant in the next step to extract cytosolic mitochondrial DNA.

8. Use DNeasy Blood & Tissue Kit to purify mitochondrial DNA from the cytosolic fraction according to the manufacturer's instructions (Ly *et al.*, 2003).

   a. Add 100 µl ethanol (96–100%) to the cytosolic fraction and continue to step 4 in the DNeasy Blood & Tissue Kit protocol.

Notes

1. This protocol is optimized for using immortalized bone marrow derived macrophages (iBMDM) (Bronner *et al.*, 2015). Protocol can be optimized for chosen experimental cell type.
2. Thapsigargin serves as a positive control for triggering mitochondrial DNA release. Thapsigargin prevents the uptake of calcium into the endoplasmic reticulum (ER) by blocking SERCA channels. Under these conditions, the ER leaks calcium without being able to replenish its calcium stores, and consequently calcium accumulates in the cytosol or mitochondria. Mitochondrial calcium overload triggers depolarization, leading to the release mitochondrial contents into the cytosol.
3. Make a 10% NP-40 stock solution and dilute to 1% NP-40.
4. Use DPBS that contains calcium and magnesium to ensure that cells will remain attached during the wash step.
5. Prelabeled microcentrifuge tubes do not need to be prechilled.
6. 1×10^6 to 1×10^7 (seeded in 100 mm tissue culture dishes) cells will yield 1–20 µg of mtDNA.

Recipes

1. Medium

   DMEM

   10% Fetal Bovine Serum (FBS)

   Add 50 ml heat-inactivated FBS to 450 ml of DMEM.

   Notes:

   a. FBS is heat inactivated for 30 min at 55 °C.

   b. This medium has been optimized for iBMDM. Use medium optimized for experimental cell type.

2. NP-40 (Igepal CA-630) solution

   a. For 10% NP-40, add 1 ml of NP-40 to 9 ml of dH_2O.

   b. For 1% NP-40, add 1 ml of 10% NP-40 to 9 ml of dH_2O.

3. Thapsigargin stock solution
    a. The concentration stated above has been optimized for iBMDM. The concentration used on different cell types must be optimized.
    b. Stock solution of thapsigargin remains usable up to one year after reconstitution and can be aliquoted and stored at −20 °C
    c. Stock solution of thapsigargin is 5 mM (1 mg in 307 μl DMSO). Dilute thapsigargin (4 μl into 2 ml of medium) into medium that will be added to the wells.

Part II. Amplification of mtDNA via qPCR

After extracting DNA from the cytosolic fraction, quantitative PCR is employed to measure cytosolic mitochondrial DNA.

Materials and Reagents

1. 96 well qPCR plate (Denville Scientific Inc., catalog number: C18096) (Bernardi, 2013)
2. 1.5 ml microcentrifuge tubes (Denville Scientific Inc., catalog number: C2170)
3. Gloves
4. Brilliant II SYBR® green with low ROX (Agilent Technologies, catalog number: 600830)
5. Sterilized double distilled water
6. Primers (see sequences below)

Equipment

1. qPCR machine (Stratagene MX3000P)

Procedure

1. Prepare the SYBR qPCR master reaction mix as follows in Table 1 for mitochondrial genes of interest and internal control (housekeeping gene for used here is 18S rDNA) in 1.5 ml microcentrifuge tubes:

    The following primers were used:

    Cytochrome *c* oxidase I (mt gene)

    Forward: 5'-GCCCCAGATATAGCATTCCC-3'

    Reverse: 5'-GTTCATCCTGTTCCTGCTCC-3'

    18S rDNA (internal control)

    Forward: 5'-TAGAGGGACAAGTGGCGTTC-3'

    Reverse: 5'-CGCTGAGCCAGTCAGTGT-3'

2. Mix gently but thoroughly by pipetting.
3. Incubate reaction mix on ice until ready to use.
4. Aliquot DNA samples (1 µl) into wells (run in triplicates) (Bronner *et al.*, 2015).
5. Aliquot corresponding Master Mix (19 µl) into the corresponding wells (Bronner and O'Riordan, 2014).
6. Use the thermal cycler program as seen below in Table 2:
7. Once qPCR is complete, calculate relative fold change in cytochrome *c* oxidase I from the Ct values.

Representative data

Adapted method for calculating relative fold change (Livak and Schmittgen, 2001) between thapsigargin-treated and untreated Ct values that are represented in Table 3:

1. ΔCt Treatment = Target gene - Reference gene

   ΔCt Treatment (thapsigargin) = 19.15 – 20.54

   ΔCt Control = Target gene - Reference gene

   ΔCt Control (untreated) = 26.16 – 26.18
2. ΔΔCt = ΔCt Treatment - ΔCt Control

   ΔΔCt = −1.39 – (−0.02)

   ΔΔCt = −1.37
3. Relative Fold Change = $2^{-(\Delta\Delta Ct)}$

   Relative Fold Change = $2^{-(-1.37)}$

   Relative Fold Change = 2.6

This result indicates that thapsigargin treatment results in a 2.6 fold increase in cytosolic mtDNA compared to untreated (Figure 2).

Notes

1. Use plates that are optimized for qPCR machine.
2. The total reaction volume is 20 µl. When adding DNA ensure the amount is consistent between samples.
   a. 10 ng and 2 µg represent the minimum and maximum amount of DNA to use for qRT-PCR analysis.
3. Pipette as accurately as possible during steps 4 and 5, since small variations in volumes can increase variability in the qPCR results.

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We acknowledge that this protocol was adapted (Nakahira *et al.*, 2011) and modified for use with immortalized bone marrow macrophages infected by a bacterial pathogen.

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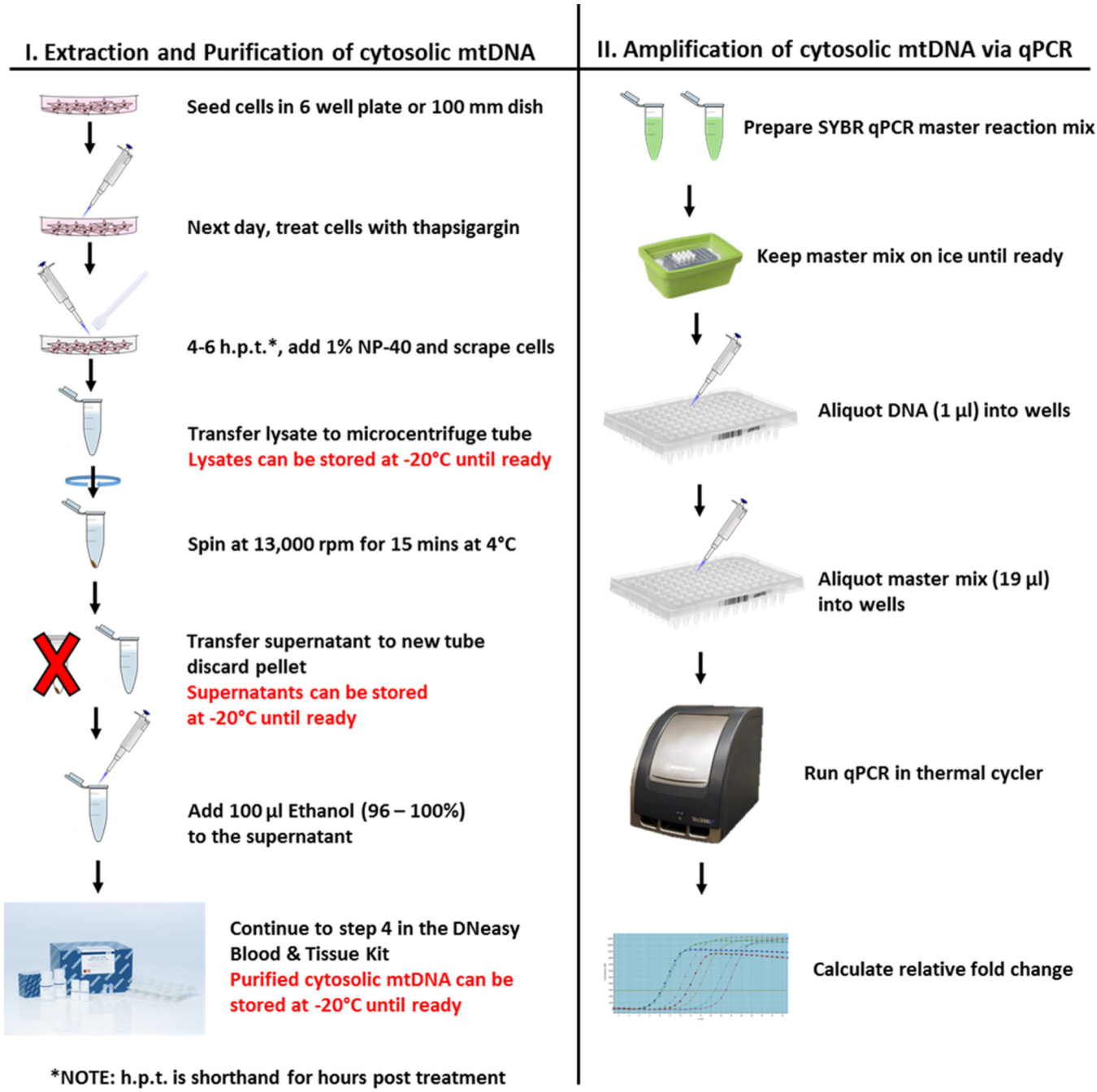

Figure 1. Flowchart for extracting, purifying, and amplifying cytosolic mtDNA

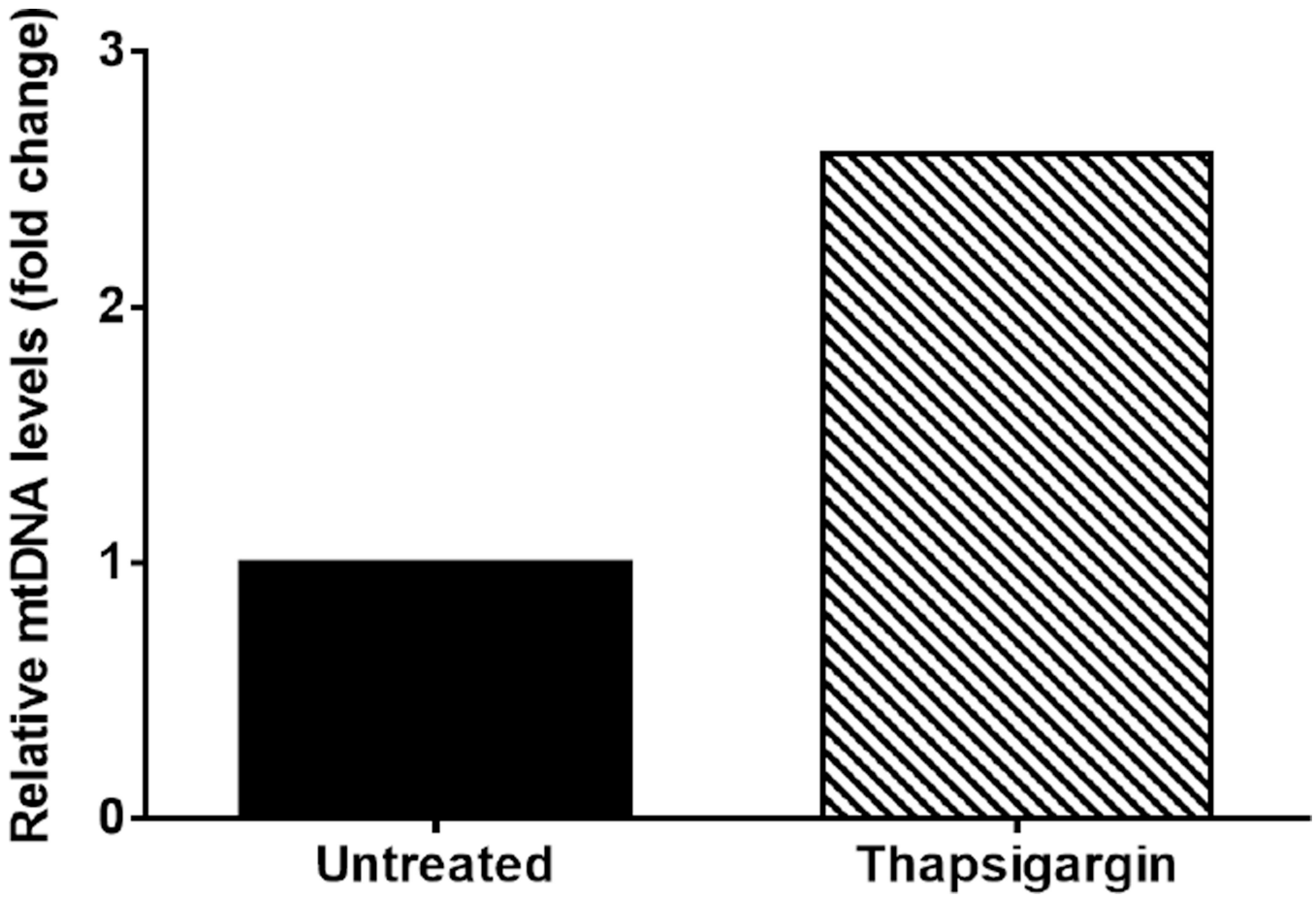

Figure 2. Graph of representative data from Table 3

Graph depicts cytosolic mtDNA relative fold change seen in Thapsigargin treated iBMDM when compared to untreated iBMDM.

Table 1

Master Mix recipe for quantifying mtDNA release into cytosol via qPCR.

Reagents	Amount (μl)
SYBR Green with Low Rox Master Mix	10
Forward primer	1
Reverse primer	1
Sterile dH_2O	7
Total	19

Table 2

Thermal cycler program for mtDNA release qPCR

Procedure	# of Cycles	Temperature	Time
Hot start activation	1	95 °C	10 min
Denaturation	40	95 °C	30 sec
Annealing/Extension		53 °C	30 sec
Dissociation	1	72 °C	30 sec
		95 °C	1 min
		55 °C	30 sec
		95 °C	30 sec

Table 3

Representative values from mtDNA release qPCR

	Untreated	Thapsigargin
Cytochrome *c* oxidase I	26.16	19.15
18S rDNA	26.18	20.54