Please cite this article as: Jae-Min *et. al.*, (2016). Assessment of Mitochondrial DNA Content and Mass in Macrophages, *Bio-protocol* 6 (9): e1796. DOI: 10.21769/BioProtoc.1796.

bio-protocol http://www.bio-protocol.org/e1796 Vol 6, Iss 9, May 5, 2016

Assessment of Mitochondrial DNA Content and Mass in Macrophages

Jae-Min Yuk[1], Tae Sung Kim[2, 3] and Eun-Kyeong Jo[2, 3*]

[1]Department of Infection Biology, Chungnam National University School of Medicine, Daejeon, S. Korea; [2]Department of Microbiology, Chungnam National University School of Medicine, Daejeon, S. Korea; [3]Infection Signaling Network Research Center, Chungnam National University School of Medicine, Daejeon, S. Korea

[*]For correspondence: hayoungj@cnu.ac.kr

[Abstract] Mitochondria are essential regulators in not only ATP generation and metabolic reprogramming but also the generation of reactive oxygen species (ROS) in response to pathogenic stimuli. During exposure to environmental stresses including oxidative stress, exercise, cell division and caloric restriction, mitochondria can be divided to increase mitochondrial number, size, and mass. Moreover, mitochondrial biogenesis has a crucial role in the resolution of inflammation through preserving metabolic function. Recently, diverse biochemical methods have been utilized to evaluate activity of mitochondrial biogenesis. In this protocol, we will describe an in vitro assay to measure mitochondrial DNA content and mass. Quantitative real-time PCR analysis for determination of mitochondrial DNA content is a powerful tool with the addition of flow cytometry or confocal microscopy for evaluating mitochondrial mass. Together, these protocols may provide the significant information for mitochondria studies.

Materials and Reagents

1. Microcentrifuge tube (1.5 ml or 2 ml)
2. Lipopolysaccharide (InvivoGen, catalog number: tlrl-3pelps)
3. G-DEX™ IIc Genomic DNA Extraction Kit (iNtRON Biotechnology, catalog number: 17231)
4. Absolute or 70% ethanol (Merck Millipore Corporation, catalog number: 100983)
5. Isopropanol (Sigma-Aldrich, catalog number: I9030)
6. DEPC water (Merck Millipore Corporation, Calbiochem, catalog number: 9062-500 ML_CN)
7. Primer (EC21, Solgent Co)
   Stock concentration: 100 μM; Working concentration: 10 μM
   a. mND-1 (mitochondrially encoded NADH dehydrogenase 1)
      forward: 5'-GGCTACATACAATTACGCAAAG-3'
      reverse: 5'-TAGAATGGAGTAGACCGAAAGG-3'
   b. mPyruvate kinase
      forward: 5'-ACTGGCCGGTGTCATAGTGA-3'

Please cite this article as: Jae-Min *et. al.*, (2016). Assessment of Mitochondrial DNA Content and Mass in Macrophages, *Bio-protocol* 6 (9): e1796. DOI: 10.21769/BioProtoc.1796.

reverse: 5'-TGTTGACCAGCCGTATGGATA-3'

8. SYBR green PCR master mix (QIAGEN, catalog number: 330520)
9. MitoTracker Green FM (Thermo Fisher Scientific, Molecular Probes™, catalog number: M-7514)
10. Accutase (Sigma-Aldrich, catalog number: A6964)
11. Phosphate Buffered Saline (PBS) (Thermo Fisher Scientific, Gibco™, catalog number: 70011044)
12. Formaldehyde for cell fixation (Sigma-Aldrich, catalog number: F8755)
13. Triton® X-100 (Sigma-Aldrich, catalog number: T8787)
14. DMSO (Sigma-Aldrich, catalog number: D8418)
15. DMEM (Thermo Fisher Scientific, catalog number: 11965)
16. Macrophage Colony-Stimulating Factor (M-CSF) from mouse (Sigma-Aldrich, catalog number: M9170)
17. Tris-HCl (Sigma-Aldrich, catalog number: T5941)
18. EDTA solution (Sigma-Aldrich, catalog number: 03690)
19. Heat-inactivated FBS (Thermo Fisher Scientific, Gibco™, catalog number: 1600044)
20. Penicillin (50 U/ml)/Streptomycin (50 mg/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15140-122)
21. TE buffer (see Recipes)
22. M-CSF-containing medium (see Recipes)

Equipment

1. Tabletop microcentrifuge (KITA, Hanil, model: Micro12)
2. DNA electrophoresis chamber (Takara Bio Company, catalog number: AD140)
3. Qiagen Rotor gene 6000 instrument for quantitative real-time PCR analysis (Takara Bio Company, catalog number: AD140)
4. BD FACSCanto™ II flow cytometer (BD Biosciences)
5. 37 °C, 5% CO_2 incubator (Thermo Fisher Scientific, catalog number: 3111)
6. Centrifuge (LaboGene, catalog number: i730R)
7. Water bath or heat block for heating at 65 °C
8. Vortex mixer (PRONEER, model: MX-S)

Software

1. FlowJo (Tree Star Inc)

Procedure

A. Quantification of mitochondrial DNA content

Please cite this article as: Jae-Min *et. al.*, (2016). Assessment of Mitochondrial DNA Content and Mass in Macrophages, *Bio-protocol* 6 (9): e1796. DOI: 10.21769/BioProtoc.1796.

1. Prepare bone marrow-derived macrophages (BMDM) in 24 well plates (1 x 10^6 cells per each well, medium volume: 1 ml). Please refer to Chen (2012).
2. Briefly, primary BMDM are differentiated for 5 days in M-CSF-containing medium.
3. Remove the M-CSF-containing medium and then wash the adherent cells with 500 µl PBS three times.
4. Add a fresh culture medium to exclude diverse alternative effects by M-CSF and FBS.
5. After 24 h, BMDM were stimulated with lipopolysaccharide (LPS, 100 ng/ml) for the various time points in the incubator at 37 °C (Figure 1).
6. Remove the supernatant and then wash the adherent cells in 500 µl PBS.
7. Remove PBS leaving behind the adherent cells.
8. Add 300 µl cell lysis buffers, pipette three times to lyse the adherent cells, and then transfer into a 1.5 ml microcentrifuge tube.
9. Add 1.5 µl RNase A solution to the 1.5 ml tube containing the cell lysate and then incubate for 30 min in a heat block at 37 °C.
10. Chill samples on ice for 5 min, add 100 µl protein precipitation (PPT) buffer into each sample, and vigorously mix by a vortex mixer for 30 sec.
11. Centrifuge at 16,000 *x g* for 5 min and transfer 300 µl of the supernatants into a new 1.5 ml tube.
12. Add 300 µl of 100% isopropanol and then gently mix the samples containing the DNA and protein pellet by inverting several times.
13. Centrifuge at 16,000 *x g* for 1 min, remove the supernatants, and then add 1 ml 70% ethanol to wash DNA (white pellet) in each tube.
14. Centrifuge at 16,000 *x g* for 1 min, remove the ethanol, and dry the DNA pellet by leaving the cap open for 10 min.
15. Incubate the DNA samples into 100 µl DNA rehydration buffer at 65 °C for 1 h using a water bath or heat block. In this case, we generally obtained 10-20 µg DNA from 1 x 10^6 cells.
16. Prepare DNA mixture for Real-Time PCR analysis as follows:
    a. 10 µl 2x SYBR green PCR master mix
    b. 2 µl DNA samples (50 ng/µl)
    c. 1 µl Forward primer (10 µM)
    d. 1 µl Reverse primer (10 µM)
    e. 6 µl DEPC water
17. Perform quantitative Real-Time PCR cycling as follows:
    a. 95 °C for 10 min
    b. 40 cycles
       95 °C for 10 sec
       60 °C for 30 sec
       72 °C for 30 sec
       72 °C for 10 min

Please cite this article as: Jae-Min *et. al.*, (2016). Assessment of Mitochondrial DNA Content and Mass in Macrophages, *Bio-protocol* 6 (9): e1796. DOI: 10.21769/BioProtoc.1796.

c. 4 °C hold

18. The quantification of mitochondrial DNA contents was calculated by the mitochondrial (mND-1) to nuclear DNA (mPyruvate kinase) ratio (Figure 1).

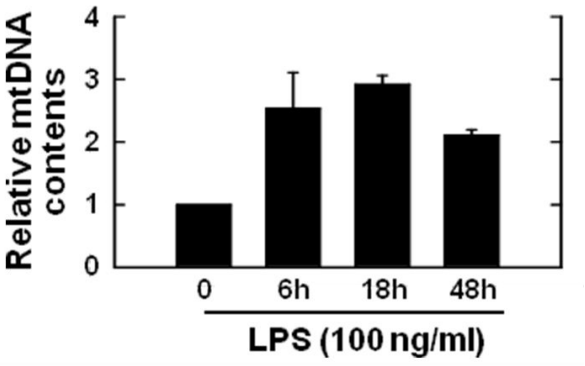

Figure 1. TLR4 stimulation resulted in enhanced mitochondrial DNA content in macrophages. BMDMs were stimulated with LPS (100 ng/ml) for the indicated durations. Cells were collected and then analyzed by quantitative real-time PCR for measuring mitochondrial DNA content in BMDMs. The mitochondrial DNA content (mND-1) was normalized to the nuclear DNA (mPyruvate kinase).

B. Mitochondrial DNA mass

1. Preparation of bone marrow-derived macrophages (BMDM) in 24 well plate (1 x 10^6 cells per each well, medium volume: 1 ml).
2. Remove the supernatant and then add 1 ml fresh culture medium in each well.
3. After 24 h, BMDM cells are stimulated with LPS (100 ng/ml) for 6 h.
4. Remove the supernatant and wash the adherent cells with 500 µl fresh PBS 3 times.
5. Add 500 µl culture medium containing MitoTracker Green FM (Working concentration: 200 nM).
6. Incubate the samples at 37 °C for 45~60 min.
7. Remove the culture medium and wash the cells with 500 µl fresh PBS.
8. Add 500 µl accutase into each sample.
9. Transfer each sample to a FACS tube and centrifuge at 16,000 *x g* for 5 min.
10. Remove the supernatants and wash the cells with 500 µl fresh PBS.
11. Carefully replace the PBS with pre-warmed growth medium containing 3.7% formaldehyde at 37 °C for 15 min or at 4 °C overnight.
12. Total fluorescence of MitoTracker Green FM is measured by flow cytometry (Figure 2).

Please cite this article as: Jae-Min *et. al.*, (2016). Assessment of Mitochondrial DNA Content and Mass in Macrophages, *Bio-protocol* 6 (9): e1796. DOI: 10.21769/BioProtoc.1796.

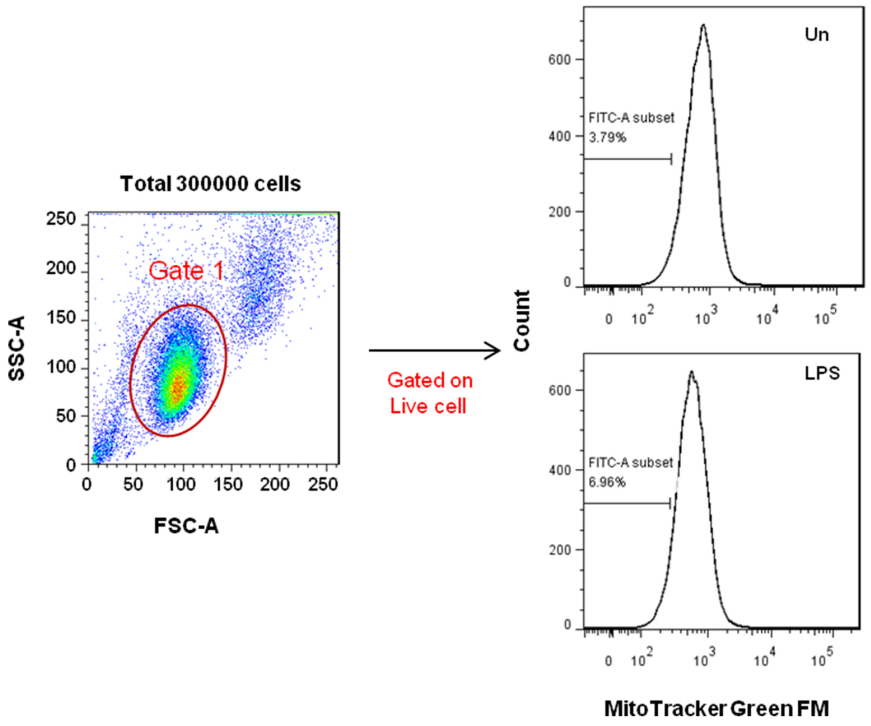

Figure 2. TLR4 stimulation resulted in enhanced mitochondrial DNA content in macrophages. BMDMs were stimulated with LPS (100 ng/ml) for 6 h. Cells were collected and then assessed by a flow cytometry analysis to measure Mitotracker fluorescence signals (Left). Live cells were assigned according to size and granularity (Right). Histogram of MitoTracker Green FM signal indicates the cell counts that correspond to the fluorescence intensity of mitochondrial mass.

Recipes

1. TE buffer
   10 mM Tris-HCl
   0.1 mM EDTA
2. M-CSF-containing medium
   DMEM
   10% heat-inactivated FBS
   Penicillin (50 U/ml)/Streptomycin (50 mg/ml)

Acknowledgments

The protocol was adapted from Experimental Procedures of previously published paper in Yuk *et al*. (2015). This work was supported by research fund of Chungnam National University.

Please cite this article as: Jae-Min *et. al.*, (2016). Assessment of Mitochondrial DNA Content and Mass in Macrophages, *Bio-protocol* 6 (9): e1796. DOI: 10.21769/BioProtoc.1796.

References

1. Chen, R. (2012). Isolation and culture of mouse bone marrow-derived macrophages (BMM'phi'). *Bio-protocol* 2(3): e68.
2. Yang, C. S., Kim, J. J., Kim, T. S., Lee, P. Y., Kim, S. Y., Lee, H. M., Shin, D. M., Nguyen, L. T., Lee, M. S., Jin, H. S., Kim, K. K., Lee, C. H., Kim, M. H., Park, S. G., Kim, J. M., Choi, H. S. and Jo, E. K. (2015). Small heterodimer partner interacts with NLRP3 and negatively regulates activation of the NLRP3 inflammasome. *Nat Commun* 6: 6115.
3. Yuk, J. M., Kim, T. S., Kim, S. Y., Lee, H. M., Han, J., Dufour, C. R., Kim, J. K., Jin, H. S., Yang, C. S., Park, K. S., Lee, C. H., Kim, J. M., Kweon, G. R., Choi, H. S., Vanacker, J. M., Moore, D. D., Giguere, V. and Jo, E. K. (2015). Orphan nuclear receptor ERRalpha controls macrophage metabolic signaling and A20 expression to negatively regulate TLR-Induced inflammation. *Immunity* 43(1): 80-91.

