Measuring mtDNA turnover, synthesis, and supercoiling via selective bromodeoxyuridine incorporation

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Abstract

Increasing work on mitochondria is focused on the dynamic aspects of this organelle, a key part of which is regulation of the mitochondrial genome (mtDNA). Here we describe a protocol that selectively incorporates bromodeoxyuridine into mtDNA for the measurement of mtDNA turnover, synthesis or supercoiling via an adapted immunoblot and Southern blot protocol. For complete use and execution of this protocol see Al Khatib et al.[1,2] and Lei et al.[3]

Introduction

While many studies report changes in mtDNA copy number[4], they seldom show what is driving this change, whether it be from changes to rates of synthesis, turnover or supercoiling. Here we present a set of protocols to selectively label mtDNA with BrdU, followed by immunoblotting of BrdU to measure synthesis, turnover and supercoiling of mtDNA. While others have used this approach to label mtDNA with BrdU[5-12], experimental details are sparse. Thus, we present a concise and systematic set of steps to label mtDNA, transfer DNA onto a PVDF membrane, and blot for BrdU with minimal specialized chemicals and equipment. In addition, our method is robust enough to not only measure total levels of mtDNA for synthesis and turnover, but also separate linear, supercoiled and relaxed forms of mtDNA for subsequent quantification and analysis. As a result, our protocols allow for pinpointing the molecular mechanisms underlying changes in mtDNA copy number, as we have shown in models of mitochondrial dysfunction such as loss of TOP1MT[1,2], or reduced TFAM expression[3].

Methods

Before you begin

This protocol outlines the steps required to selectively incorporate bromodeoxyuridine (BrdU) into mtDNA allowing analysis via a modified Southern and immuno blot protocol (a.k.a Southwestern), which can be used to measure changes in synthesis, turnover and supercoiling of the mtDNA genome (Figures 1 & 2). As this protocol includes cell culture experiments should be performed in appropriate biosafety conditions.

Drug preparation

Timing: [3h]

1. Prepare stock solutions of BrdU (50 mM), aphidicolin (10 mM), and uridine (50 mg/mL) in sterile DMSO.

a. Stock solutions should be stored at -20˚C until needed.

Cell culture

Timing: [3 days]

2. For synthesis or turnover assays, cells should be seeded at 50,000 per well of a 6-well plate in 2 mL of DMEM containing 10% FBS. For the supercoiling assay cells should be seeded at 1 x 10^6 cells of a 10 cm-dish in 10 mL of DMEM with 10% FBS.

Note: Use of DMEM with 10% FBS is suggested as a placeholder, use appropriate cell culture media based on the cell line being cultured.

3. Cells should be incubated at 37˚C at 5% CO_2 for approximately three days or until nearly confluent (95% confluent)

Note: Time to confluency varies depending upon speed of growth of cells. Cells should be monitored for confluency using a brightfield microscope as necessary.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BD Pharmingen™ Purified Mouse Anti-BrdU	BD Biosciences	Cat#555627
Goat anti-Mouse IgG Secondary Antibody, HRP	Invitrogen	Cat#31430
Chemicals		
Tris base	ChemCruz	Cat#sc-3715B
TWEEN-20	Sigma-Aldrich	Cat#P1379-1L
Skim milk powder	CRITERION	Cat#C6962
Sodium hydroxide	EMD Millipore	Cat#1064621000
Sodium chloride	Fisher BioReagents	Cat# BP358-1
Sodium citrate dihydrate	Ward's Science	Cat# 470302-528
Agarose (molecular biology grade)	Wisent	Cat# 800-015 CG
Uridine	Sigma-Aldrich	Cat#U3003-5G
Aphidicolin	Hello Bio	Cat# HB3690
Bromodeoxyuridine (BrdU)	Invitrogen	Cat# B23151
Enzymes		
BamHI-HF	NEB	Cat# R3136S
Topoisomerase I	NEB	Cat# M0301S
Critical commercial assays		
E.Z.N.A Tissue DNA Kit	Omega Biotek	Cat#NC0721312

SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Scientific	Cat#34095
Experimental models: Cell lines		
Human: U2OS	ATCC	Cat# HTB-96
Human: HCT116	ATCC	Cat#CCL-247
Human: Fibroblasts	Laboratory of Timothy Shutt	N/A
Mouse: mouse embryonic fibroblasts (wild-type and TFAM$^{+/-}$)	Laboratory of Gerald Shadel	N/A

Materials and equipment

Materials availability
All reagents are commercially available.

Denaturing buffer

Reagent	**Final concentration**	**Amount**
Sodium hydroxide	0.5 M	20 g
Sodium chloride	1.5 M	87.7 g
ddH_2O	n/a	1 L
Total	**n/a**	1 L

10X Tris buffered saline (TBS)

Reagent	**Final concentration**	**Amount**
Tris base	20 mM	24.2 g
Sodium chloride	137 mM	80 g
ddH_2O	n/a	1 L
Total	**n/a**	1 L

20X saline sodium citrate (SSC) buffer

Reagent	**Final concentration**	**Amount**
Sodium chloride	3 M	175.3 g
Sodium citrate	0.3 M	77.4 g
ddH_2O	n/a	1 L
Total	**n/a**	1 L

- 10X saline sodium citrate: add 500 mL of 20X SSC buffer to 500 mL of ddH_2O.
- 6X saline sodium citrate: add 8.3 mL of 10X SSC buffer to 41.7 mL ddH_2O
- Blocking solution: add 1 g of milk powder to 10 mL of TBST
- 1X Tris buffered saline with Tween 20 (TBST): add 100 mL of 10X TBS, and 1 mL of Tween-20 to 900 mL ddH_2O

Step-by-step method details

Inhibition of nuclear DNA synthesis and BrdU incorporation

Timing: [4 days]

Aphidicolin inhibition of nuclear DNA synthesis, in conjunction with BrdU treatment allows selective incorporation of BrdU into mtDNA. Subsequent steps, where BrdU is probed via immunoblotting, allow visualization of a signal that is specific for mtDNA rather than the nuclear DNA. For the mtDNA turnover assay, uridine is added following the BrdU pulse labelling to outcompete any residual BrdU and reduce BrdU incorporation into newly synthesized mtDNA. Note that specific drug concentrations and treatment times may need to be optimized for different cell types.

1. Add aphidicolin to cell culture media to a final concentration of 10 µM, and incubate for at least 4 hours or up to overnight.
2. Following aphidicolin treatment, add BrdU to the cell culture media at a final concentration of 50 µM.
3. Treat with BrdU for appropriate timepoint(s) Ex: For synthesis assay, BrdU treatment is done for 4h, 8h, 16h and 24h. For the turnover assay, use a pulse of 24h. For analyzing mtDNA topology, label cells for 24h.
4. If performing the mtDNA turnover assay, replace pulse labelling medium with fresh medium containing 2 mM uridine for appropriate timepoints.

DNA isolation and restriction digestion

Timing: 1 day

Total DNA, including selectively labelled mtDNA, can be collected by collected by a variety of methods. We recommend the use of a commercial DNA isolation kit such as the E.Z.N.A Tissue DNA Kit (Omega bio-tek). For mtDNA synthesis and turnover assays, restriction digestion by BamHI will linearize mtDNA creating a single band and digest nuclear DNA to reduce background. Different restriction enzymes can be used, and this may be preferable if looking at mtDNA from other organisms. However, other restriction enzymes or mtDNA from other organisms may give different digestion patterns. Relaxation of mtDNA supercoiling by treatment with topoisomerase I is optional, and can be useful to ensure the position of the supercoiled mtDNA signal after electrophoresis and immunoblotting. (Figure 2)

5. Remove plates from the incubator, aspirate media and wash cells twice with an appropriate volume) of PBS, approximately 2 mL per well of a 6-well plate or 10 mL for a 10 cm dish.

6. Add 0.5 mL to 1 mL of trypsin to each well/plate and return the plate to the incubator until cells detach (approximately 5 mins).
7. Resuspend cells in 5-10 mL of standard cell culture media.
8. Pellet cells via centrifugation at 1250 rpm for 5 minutes.
9. Discard media and resuspend cell pellet in 1 mL of PBS.
10. Transfer cell suspension to a 1.5 mL microcentrifuge tube, pellet cells again, and resuspend once more in PBS.
11. Remove PBS and proceed to DNA isolation of the cell pellet immediately.
12. Perform DNA isolation using E.Z.N.A Tissue DNA kit, as per manufacturer's protocol, eluting DNA in the minimum elution volume.
13. Measure DNA concentration, A260/280, and A260/230 ratio.

Pause point: Store eluted DNA at -20˚C if not proceeding to next step immediately.

14. Perform enzymatic treatment using BamHI (required for synthesis and turnover assays) or topoisomerase I (optional for supercoiling assay)
    a. Dilute 1 µg of DNA in appropriate buffer solution and add 20U of BamHI or 10U of topoisomerase I.
    b. Incubate digest at 37˚C for 1 hour.

Note: Cleanup of DNA is not required following enzymatic treatment, DNA can be used immediately for agarose gel electrophoresis.

Agarose gel electrophoresis and immunoblotting of BrdU-labelled DNA

The final step applies the concept of BrdU-labelling of mtDNA to give a semi-quantitative readout for use of monitoring mtDNA synthesis, turnover, or supercoiling. Briefly, this protocol uses agarose gel electrophoresis to separate the mtDNA based on size or topology, followed by transfer to a PVDF membrane for immunoblotting of BrdU. As a result, based on the treatment times and conditions of protocol 1, mtDNA synthesis can be monitored by probing for BrdU at increasing treatment timepoints, mtDNA turnover can be monitored by probing for BrdU in cells pulsed for 24 hours chased by removal of BrdU for various timepoints, and mtDNA topology can be monitored based on the migration of the mtDNA in the agarose gel. (Figure 1)

Timing: 4-5 days

14. Prepare a 0.45% agarose gel in TAE buffer (for synthesis or turnover assays) or TBE buffer (supercoiling assays).
15. Prepare and load DNA for each sample (500 ng for synthesis or turnover assays or 1 µg for supercoiling assay).
16. Run gel for 4 hours at room temperature at 55V (synthesis or turnover) or at 30V for 18 hours at 4˚C.

17. Following electrophoresis, image agarose gel using an imaging system such as a ChemiDoc imaging system (Bio-Rad).
    a. A faint smear should appear representing the nuclear DNA background. For synthesis and turnover assays, the linear mtDNA band may or may not be visible depending on total amount of mtDNA.
18. Wash gel briefly with water.
19. Soak gel in denaturing buffer for 1 hour with gentle shaking.
20. Rinse gel briefly with water.
21. Soak gel in 10X SSC for 30 minutes.
22. While the gel is soaking, prepare the transfer platform.
    a. Fill a shallow container larger than the agarose gel approximately halfway with 10X SSC.
    b. Place a plastic platform of an appropriate size for the agarose gel into the container.
    c. Cut and place a piece filter paper on top of the plastic platform large enough to overhang, touching the 10X SSC buffer.
23. Once the gel has been soaked place it on top of the filter paper on the transfer platform.
24. Layer a PVDF membrane, filter paper, and a stack of paper towels at least 8 cm tall.
25. Weigh down the paper towels and allow gel to transfer via capillary action overnight.
26. Disassemble the transfer platform and soak the PVDF membrane in 6X SSC.
27. UV cross-link PVDF membrane using UV Stratalinker 1800 (Stratagene) using the "auto cross-link" setting.
28. Block membrane using blocking solution for 1 hour at room temperature.
29. Incubate for 2 hours with anti-BrDU antibody (1:2000 in 1% milk in TBST).
30. Wash with TBST 3 times for 5 minutes each using an appropriate volume to cover the membrane.
31. Incubate with HRP-conjugated anti-mouse IgG (1:5000) in 1% milk in TBST for 1 hour and wash with TBST 3 times for 5 minutes each.
32. Develop blots using Supersignal West Femto enhanced chemiluminescence substrate and image using a chemiluminescence imager.

Note: Appropriate volumes for washing, blotting and blocking of membrane depends on the size of the membrane and the container it is in. For a 9 cm x 6 cm membrane in a 11 cm x 8 cm container 10 mL of solution is sufficient.

Results & Discussion

The presented protocol uses several approaches to minimize BrdU-labelled nuclear DNA background throughout the protocol, first using a nearly confluent plate to minimize further growth of cells while permitting mtDNA synthesis and turnover, and second using aphidicolin to inhibit nuclear DNA synthesis. Some cell lines may have too high a ratio of genomic DNA to mtDNA, and resulting blotting may suffer from high nuclear background.

This is a particular issue with the supercoiling assay, as restriction digestion to reduce the background nuclear DNA smear can cause nicking of DNA resulting in loss of supercoiled mtDNA and increased relaxed mtDNA. As an alternative, mitochondria can be isolated by differential centrifugation to remove nuclei and enrich mitochondria prior to the DNA isolation step[13]. It may also be possible to enrich for mtDNA using DNA isolation methods that favor the purification of plasmid DNA over total genomic DNA[14,15].

Troubleshooting

Problem 1:

Nuclear DNA background is too high/there is no observable mtDNA signal.

Potential solution:

- Increase concentration of aphidicolin, in our experience 10 µM is effective with U2OS cells, mouse embryonic fibroblasts, and HCT116 cells.
- Ensure that aphidicolin is still present when BrdU is added to cell culture media.

Problem 2:

DNA extraction yields low concentration of DNA.

Potential solution:

- Use the minimum elution volume recommended by the manufacturer.
- Ensure that DNA extraction is performed immediately following cell pelleting and use fresh not frozen cells.

Problem 3:

Lack of signal on PVDF membrane

Potential solution:

- Ensure that PVDF membrane does not dry out at any point.
- See above troubleshooting steps to ensure that DNA extraction is performed correctly, and nuclear DNA signal is minimized.
- As an alternative, mitochondria can be isolated by differential centrifugation[13] to remove nuclei and DNA isolation can be performed on the “mitochondria-enriched” fraction. It may also be possible to enrich for mtDNA using DNA isolation methods that favor the purification of plasmid DNA over total genomic DNA[14,15].

Problem 4:

Unexpected results with supercoiling assay

Potential solution:

- Ensure that agarose gel electrophoresis is done without DNA intercalators (*e.g.*, ethidium bromide)

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Author contributions

JD and AM designed and optimized methods, performed experiments, and analyzed data; TS provided overall supervision to the study.

Declaration of interests

None

References

1. Al Khatib, I., Deng, J., Symes, A., Kerr, M., Zhang, H., Huang, S.-y.N., Pommier, Y., Khan, A., and Shutt, T.E. (2022). Functional characterization of two variants of mitochondrial topoisomerase TOP1MT that impact regulation of the mitochondrial genome. Journal of Biological Chemistry *298*, 102420. https://doi.org/10.1016/j.jbc.2022.102420.
2. Al Khatib, I., Deng, J., Lei, Y., Torres-Odio, S., Rojas, G.R., Newman, L.E., Chung, B.K., Symes, A., Zhang, H., Huang, S.N., et al. (2023). Activation of the cGAS-STING innate immune response in cells with deficient mitochondrial topoisomerase TOP1MT. Hum Mol Genet *32*, 2422-2440. 10.1093/hmg/ddad062.
3. Lei, Y., VanPortfliet, J.J., Chen, Y.-F., Bryant, J.D., Li, Y., Fails, D., Torres-Odio, S., Ragan, K.B., Deng, J., Mohan, A., et al. (2023). Cooperative sensing of mitochondrial DNA by ZBP1 and cGAS promotes cardiotoxicity. Cell *186*, 3013-3032.e3022. https://doi.org/10.1016/j.cell.2023.05.039.
4. Clyde, D. (2022). Mitochondrial DNA copy number and disease. Nat Rev Genet *23*, 136. 10.1038/s41576-022-00451-2.
5. De, S., Kumari, J., Mudgal, R., Modi, P., Gupta, S., Futami, K., Goto, H., Lindor, N.M., Furuichi, Y., Mohanty, D., and Sengupta, S. (2012). RECQL4 is essential for the

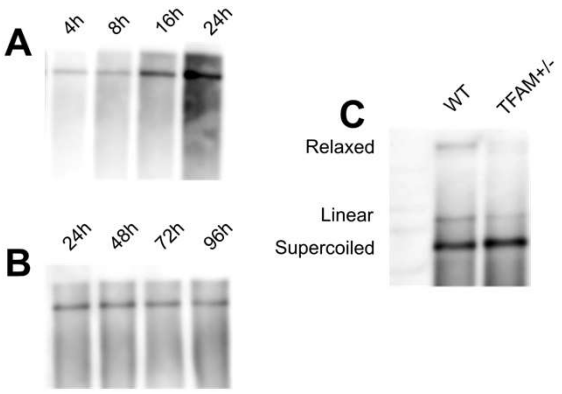
transport of p53 to mitochondria in normal human cells in the absence of exogenous stress. J Cell Sci *125*, 2509-2522. 10.1242/jcs.101501.

6. Hussain, M., Mohammed, A., Saifi, S., Khan, A., Kaur, E., Priya, S., Agarwal, H., and Sengupta, S. (2021). MITOL-dependent ubiquitylation negatively regulates the entry of PolgammaA into mitochondria. PLoS Biol *19*, e3001139. 10.1371/journal.pbio.3001139.
7. Kai, Y., Takamatsu, C., Tokuda, K., Okamoto, M., Irita, K., and Takahashi, S. (2006). Rapid and random turnover of mitochondrial DNA in rat hepatocytes of primary culture. Mitochondrion *6*, 299-304. 10.1016/j.mito.2006.10.002.
8. Lentz, S.I., Edwards, J.L., Backus, C., McLean, L.L., Haines, K.M., and Feldman, E.L. (2010). Mitochondrial DNA (mtDNA) biogenesis: visualization and duel incorporation of BrdU and EdU into newly synthesized mtDNA in vitro. J Histochem Cytochem *58*, 207-218. 10.1369/jhc.2009.954701.
9. Magnusson, J., Orth, M., Lestienne, P., and Taanman, J.W. (2003). Replication of mitochondrial DNA occurs throughout the mitochondria of cultured human cells. Exp Cell Res *289*, 133-142. 10.1016/s0014-4827(03)00249-0.
10. Noack, H., Bednarek, T., Heidler, J., Ladig, R., Holtz, J., and Szibor, M. (2006). TFAM-dependent and independent dynamics of mtDNA levels in C2C12 myoblasts caused by redox stress. Biochim Biophys Acta *1760*, 141-150. 10.1016/j.bbagen.2005.12.007.
11. Reyes, A., Melchionda, L., Nasca, A., Carrara, F., Lamantea, E., Zanolini, A., Lamperti, C., Fang, M., Zhang, J., Ronchi, D., et al. (2015). RNASEH1 Mutations Impair mtDNA Replication and Cause Adult-Onset Mitochondrial Encephalomyopathy. Am J Hum Genet *97*, 186-193. 10.1016/j.ajhg.2015.05.013.
12. Ylikallio, E., Tyynismaa, H., Tsutsui, H., Ide, T., and Suomalainen, A. (2010). High mitochondrial DNA copy number has detrimental effects in mice. Hum Mol Genet *19*, 2695-2705. 10.1093/hmg/ddq163.
13. Djafarzadeh, S., and Jakob, S.M. (2017). Isolation of Intact Mitochondria from Skeletal Muscle by Differential Centrifugation for High-resolution Respirometry Measurements. J Vis Exp. 10.3791/55251.
14. Quispe-Tintaya, W., White, R.R., Popov, V.N., Vijg, J., and Maslov, A.Y. (2015). Rapid mitochondrial DNA isolation method for direct sequencing. Methods Mol Biol *1264*, 89-95. 10.1007/978-1-4939-2257-4_9.
15. Quispe-Tintaya, W., White, R.R., Popov, V.N., Vijg, J., and Maslov, A.Y. (2013). Fast mitochondrial DNA isolation from mammalian cells for next-generation sequencing. Biotechniques *55*, 133-136. 10.2144/000114077.

Figure legends

Figure 1. BrdU immunoblotting to visualize mtDNA. A) Assay to measure mtDNA synthesis illustrates increasing BrdU-labelled DNA following 4h, 8h, 16h, and 24h of BrdU-labelling in U2OS cells. B) Assay to measure turnover showing BrdU-labelled mtDNA 24h, 48h, 72h, and 96h following a 24h BrdU-pulse in U2OS cells. C) Assay to measure mtDNA supercoiling, adapted from Lei et al.[3], shows DNA isolated from wild-type and TFAM$^{+/-}$ MEFs and demonstrates changes in the relative amounts of linear, supercoiled, and relaxed mtDNA.

Figure 2: BrdU immunoblotting to visualize mtDNA topology. The mtDNA is a circular molecule that migrates through agarose differently depending on its topology. Untreated BrdU-labelled mtDNA appears in three distinct bands represented supercoiled, linear, and relaxed fractions (lane 1). Restriction digestion of human mtDNA with BamH1 creates a single linear band (lane 2). Relaxation of mtDNA with Topoisomerase I eliminates supercoiled mtDNA (lane 3).



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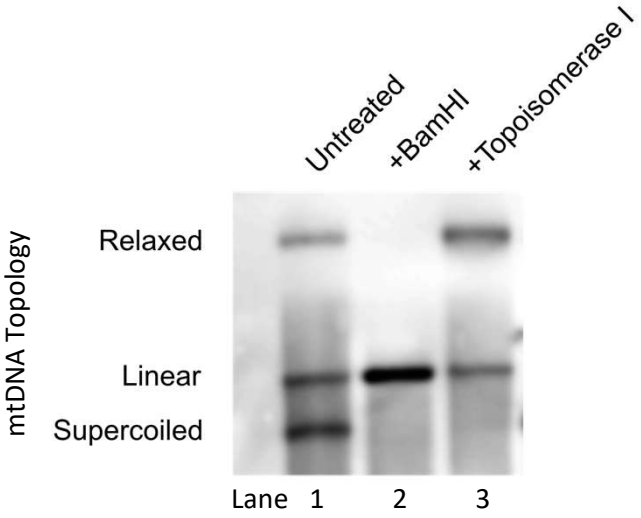

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