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

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Abstract

An often-overlooked aspect of mitochondrial biology is the mitochondrial DNA (mtDNA). The multi-copy mtDNA is highly dynamic, with changes in supercoiling, synthesis rates, and turnover rates that are tightly associated with mitochondrial and cellular functions. To better understand the state of the mtDNA, here we describe a protocol that selectively incorporates bromodeoxyuridine into the mtDNA for subsequent measurement via an adapted Southern blot followed by immunoblotting (a.k.a. Southwestern blot). This basic protocol can be applied with slight modifications for the measurement of mtDNA synthesis, turnover or supercoiling to understand mtDNA changes.

Key words: mtDNA, immunoblot, BrdU

1. Introduction

Mitochondria harbour their own genome (mtDNA), a remnant of their bacterial ancestry. In humans, the circular 16 569 bp mtDNA genome encodes 37 canonical genes, which include 13 proteins, two ribosomal RNAs and 22 tRNAs [1,2]. Unlike the nuclear genome, mtDNA is present in hundreds of copies per cell and replicates independently of the cell cycle in a manner that is tightly regulated. The importance of properly maintaining mtDNA is evidenced by changes to mtDNA copy number associated with disease [3]. Given the circular nature of mtDNA, supercoiling is another important aspect that can affect the genome function [4]. Understanding how mtDNA is regulated is crucial for understanding mitochondrial function [5,6]. As a result, there is a need for methods that monitor changes to mtDNA topology and replication. Incorporation of the thymine analog 5'-bromo-2'-deoxyuridine (BrdU) into nuclear DNA, is a frequently applied technique to measure cell proliferation and cell cycle progression [7-10]. BrdU incorporation during DNA synthesis can be measured when coupled with antibodies against BrdU via immunohistochemistry, flow cytometry or other antibody-based methods [11,12]. While BrdU incorporation is typically used for monitoring nuclear DNA synthesis, this approach can also be used to monitor mitochondrial DNA (mtDNA) synthesis and turnover [13-15,6,16-23], as well as changes in mtDNA topology (*i.e.*, supercoiling) [24-26]. However, there are no detailed protocols available for using BrdU incorporation to measure these dynamic aspects of mtDNA. Here we describe how to use BrdU to label mtDNA, which can then be visualized following agarose gel electrophoresis and an adapted Southern transfer and immunoblot protocol. As mtDNA is far less abundant than nuclear DNA, approaches to minimize nuclear DNA labeling, such as the nuclear DNA synthesis inhibitor aphidicolin [27], or enrich mtDNA may be required. Overall, this BrdU incorporation approach, using common instruments and reagents, can be applied with slight modifications to measure multiple aspects of

mtDNA dynamics including rates of mtDNA replication (Figure 1), mtDNA turnover (Figure 2), and mtDNA supercoiling (Figure 3).

2. Materials

Prepare all solutions using ultrapure water that is DNAse free, or sterile DMSO as noted. Ensure all solutions that are used on cultured cells are sterilized using a 0.22 µM filter, and all buffers are autoclaved. Storage conditions are listed for each solution. Sodium azide is not added to any of the reagents.

2.1 Sterile drugs for cell treatment

1. Aphidicolin (1000X): 10 mM in DMSO. Aliquot and store at -20˚C (see note 1).

2. 5'-Bromo-2'-Deoxyuridine (BrdU) (1000X): 50 mM in DMSO (1000X). Aliquot and store at -20˚C (see note 2).

3. Uridine (1000X): 50 mg/mL in water. Store and aliquot at -20˚C.

2.2 Agarose gel electrophoresis and Southern transfer

1. Tris-acetate-EDTA (TAE; 50X): 2M Tris, 1M acetic acid, 10 mM EDTA in water. Store at room temperature.

2. Tris-borate-EDTA (TBE; 10X): 1M Tris, 0.9M boric acid, 10 mM EDTA in water. Store at room temperature.

3. Saline-sodium citrate (SSC; 20X): 3M sodium chloride, 0.3M sodium citrate in water. Store at room temperature.

4. Denaturing buffer: 0.5M sodium hydroxide, 1.5 M sodium chloride. Store at room temperature.

2.3 BrDU Immunoblotting

1. PVDF membranes (0.2 µM pore size)

2. Tris-buffered saline (TBS; 10X): 250 mM Tris, 27 mM KCl, 1.37M NaCl, pH 7.4.

2. TBS containing 1% Tween-20 (TBST).

3. TBST containing 5% milk (TBSTM).

4. Mouse anti-BrdU antibody (BD Biosciences, cat# 555627)

5. Anti-mouse HRP-linked secondary antibody (Invitrogen, cat# 31430)

6. SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific™)

7. Filter paper (Whatman™ grade 3MM cellulose paper)

8. Glass container (*e.g.*, square 9 in x 9 in Pyrex glass dish).

9. Casting tray for agarose gel

10. 96-tube microcentrifuge racks (weighing approximately 250g each)

3. Methods

3.1 Cell culture and drug treatment

1. Seed cells at a high density in a 6-well plate (35 mm well diameter) (see note 3) and allow cells to grow until 95% confluent, approximately 2-3 days (see note 4).

2. To block nuclear DNA replication prior to BrdU treatment, remove media and replace with fresh media containing 10 µM aphidicolin for at least four hours (see note 5).

3. To initiate specific incorporation of BrdU into mtDNA, add BrdU (1000X stock) to a final concentration of 50 µM.

3.2 DNA isolation

1. First harvest cells via trypsinization. Remove media and add a sufficient volume of PBS to cover the well (approximately 1-2 mL). Aspirate PBS before adding a sufficient volume of 0.25% trypsin-EDTA to cover the cells (approximately 300 µL for a 35mm well) and incubate at 37˚C until cells detach from the plate, about 1-2 minutes. Add approximately 1 mL of complete cell culture media and transfer media containing trypsinized cells to a centrifuge tube. Pellet cells by centrifuging at 1250 rpm for 5 minutes.

2. Rinse the cell pellet by re-suspending the cells in 2-5 mL of PBS before centrifuging at 1250 rpm for 5 minutes to re-pellet the cells.

3. Remove PBS and immediately isolate total DNA from the cell pellet using a commercial kit such as E.Z.N.A Tissue DNA kit per manufacturer's directions. Elute DNA in the minimum elution volume (note 6), and store at -20˚C if necessary.

4. Measure DNA concentration, A260/280, and A260/230 using a Nanodrop spectrophotometer or similar. A260/280 and A260/230 should be approximately 1.8 and 2.0 respectively. Typical yield for concentrations is approximately 250-500 ng/µL.

3.3 Restriction enzyme digest – if necessary (see step 3.9.3)

1. Prepare 0.5 µg of total DNA for restriction digestion to linearize mtDNA and reduce nuclear background. For human mtDNA, BamHI is recommended (see note 7).

2. Perform restriction enzyme digestion per manufacturer's directions with appropriate buffers. For enzymes from New England Biolabs, follow the protocol found using NEBcloner.

3.4 Agarose gel electrophoresis

1. Prepare a 9 cm x 11 cm 0.45% agarose gel in TAE buffer with 1.5 mm thick wells. This will require about 100 mL of agarose solution and yield wells with approximately 50 µL of loading volume and a 1 cm thick gel.

2. Prepare and load DNA samples (at least 0.5 µg) (note 8).

3. Run agarose gel at 55V for 4h at room temperature.

4. Image agarose gel using an imaging system such as a ChemiDoc imaging system (note 9).

3.5 Transfer to PVDF membrane

5. Rinse gel briefly with water (approximately 40 mL) to remove buffer.

6. Soak gel in a sufficient volume of denaturing buffer to submerge the gel (approximately 40 mL) for 1 hour with gentle shaking.

7. Rinse gel briefly with water to remove buffer.

8. Soak gel in 10X SSC (approximately 40 mL) for 30 minutes.

9. While the gel is soaking, prepare the transfer platform. Fill a shallow container larger than the agarose gel (*e.g.*, a 9 in x 9 in Pyrex dish for a 9 cm x 11 cm gel) approximately halfway with 10X SSC (requires approximately 500 mL of buffer). Place a plastic platform large enough to support the agarose gel which elevates it sufficiently above the 10X SSC buffer (*e.g.*, the inverted casting tray for the agarose gel) (note 10). Cut and place a piece filter paper on top of the plastic platform large enough to overhang, touching the 10X SSC buffer (Figure 4).

10. Once the gel has been soaked, place it on top of the filter paper on the transfer platform which has been pre-wetted with 10X SSC.

11. Layer a PVDF membrane, previously activated by rinsing in 50 mL of methanol for 10 seconds, filter paper, pre-wetted with 10X SSC, and a stack of dry paper towels at least 8 cm tall. Since the mtDNA bands are in the top half of the agarose gel when run using the above suggested runtimes, the gel and/or the PVDF membrane can be cut to only transfer in the top half.

12. Weigh down the paper towels with a light weight (*e.g.* two 96 microcentrifuge tube racks, weighing a total of approximately 500g) on top of a plastic sheet protector and allow the gel to transfer via capillary action overnight at least 16h. For the weight, any object heavy enough to compress the paper towels such that they are all in direct contact with each other will work.

3.6 BrdU immunoblotting

1. Disassemble the transfer platform and soak the PVDF membrane in approximately 40 mL of 6X SSC for 5 minutes.

2. UV cross-link the PVDF membrane using “auto-cross link” on a UV Stratalinker™ 1800 or equivalent function on other machines. When UV cross-linking, place the PVDF membrane on top of the soaked filter paper from the transfer to ensure the membrane does not dry out.

3. Block membrane with TBSTM (approximately 15 mL) for 1 hour at room temperature.

4. Incubate membrane for 2 hours with anti-BrdU antibody (1:2000 in TBSTM, approximately 15 mL) at room temperature.

5. Wash membrane 3x for 5 minutes with TBST (approximately 15 mL each wash).

6. Incubate membrane with HRP-conjugated anti-mouse IgG (1:5000 in TBSTM, approximately 15 mL) for 1 hour.

7. Wash membrane 3x for 5 minutes for TBST (approximately 15 mL each wash).

8. Develop blots using ECL solution and imager of choice. It is recommended to use SuperSignal™ West Femto enhanced chemiluminescence substrate. Image using a chemiluminescence imager (see note 11).

3.7 mtDNA synthesis assay (Figure 1)

1. Follow steps 1 and 2 in section 3.1, until the BrdU incorporation.

2. To observe the rate of mtDNA synthesis, perform BrdU incorporation over a range of timepoints. After treating with aphidicolin for at least 4 hours, add BrdU (1000X stock) to a final concentration of 50 µM. Incubate cells for desired timepoints (*e.g.*, 4, 8, 16, 24 hours).

3. Proceed immediately to isolate DNA per step 3.2 (see note 12).

4. Linearize mtDNA by performing a restriction enzyme digest per step 3.3.

5. Proceed with steps 3.4-3.6 and image the final BrdU immunoblot (Figure 1b).

3.8 mtDNA turnover assay (Figure 2)

1. Follow steps 1 and 2 in section 3.1, until BrdU incorporation.

2. For pulse labelling of mtDNA while minimizing nuclear DNA incorporation, replace aphidicolin-only media with media containing BrdU (50 µM) and aphidicolin (10 µM). Incubate cells for 24h.

3. To measure mtDNA turnover, aspirate media and replace with fresh media containing 10 µM aphidicolin and 2 mM uridine. The high concentration of uridine is required to outcompete incorporation of residual BrdU into mtDNA that is newly replicated during the chase period. Incubate cells for the desired timepoints (*e.g.*, 24, 48, 72, 96 hours) to allow previously BrdU-labeled mtDNA to be turned over.

4. Isolate DNA per step 3.2. For step 3 of the DNA isolation, we recommend approaches to reduce nuclear DNA (see note 5), Given the ratio of nuclear DNA to mtDNA, even low levels of BrdU incorporation into the nuclear genome can mask the mtDNA signal, as undigested nuclear DNA runs as a band of ~20 kb that can overlap with the region of interest for the mtDNA genome (Figure 5). In our hands, the following adaptations to the E.Z.N.A Tissue DNA kit greatly reduce the abundance of nuclear DNA. 1) start with $>2X10^7$ cells resuspend in 200 PBS (it is essential to use more than the recommended number of cells). After addition of the BL lysis buffer, the solution should be viscous (this is critical for removal of nuclear DNA). 2) Before continuing with the DNA isolation procedure, run the viscous solution through a homogenizer column (*e.g.*, Omega Biotek, HCR 003) to reduce the viscosity (in this step, larger nuclear DNA is fragmented resulting in a homogeneous smear that does not interfere with the mtDNA signal). 3) Collect the flow through containing mtDNA, and proceed with DNA isolation.

5. Linearize mtDNA by performing a restriction enzyme digest per step 3.3.

6. Proceed with through sections 3.4-3.6 and image the final BrdU immunoblot (Figure 2b).

<u>3.9 mtDNA supercoiling assay (Figure 3)</u>

1. For step 1 of 3.1, seed cells in a 10 cm cell culture plate (see note 3).

2. Proceed with steps 2 and 3 of section 3.1 to allow cells to become near-confluent, block nuclear DNA synthesis, and initiate BrdU incorporation. In step 4, allow BrdU incorporation for 24h.

3. Isolate DNA as per section 3.2. For samples where mtDNA topology is to be investigated, avoid the restriction digest in section 3.3, as linearizing mtDNA will remove its topology.

4. Optional. Before proceeding to section 3.4, control samples for relaxed and linear mtDNA bands can be generated. To generate a positive control for relaxed mtDNA (Figure 3-lane 4), incubate 1 µg of DNA with topoisomerase I as recommended by the manufacturer. To generate a control for linear mtDNA fragments (Figure 3-lane 3), a restriction enzyme digest that cuts the mtDNA once can be used as described in 3.3.

5. During the agarose gel run in section 3.4, load 1 µg of DNA and use TBE buffer rather than TAE buffer and run the gel for 16-18h at 30V at 4˚C (see note 13)

6. Proceed through sections 3.4-3.6 and image the final BrdU immunoblot (Figure 3b)

4. Notes

1. Although the supplier recommends use of fresh aphidicolin solutions, we have stored aliquots for up to 6 months at -80°C without noticeable loss in function.

2. 5-ethynyl-2′-deoxyuridine (EdU) can be used as an alternative to BrdU. However, EdU requires different methods to visualize (*e.g.*, EdU antibody or Click-chemistry [28])

3. Cell seeding numbers depend on cell type and desired outcomes, and may need to be adjusted. In general, for assays that require imaging of a single linear mtDNA, a near confluent well of a 6-well (35 mm well diameter) is sufficient to yield the recommended 0.5 µg of DNA. To examine mtDNA topology, more mtDNA is required (at least 1 µg), and it is recommended to use a confluent 10 cm dish. This protocol has been successfully applied to HCT116 cells, human fibroblasts cells, and U2OS cells which are seeded at 400,000 cells, 450,000 cells, and 400,000 cells per well of a 6-well plate (35 mm well diameter) or 2,000,000 cells, 2,500,000 cells, and 2,000,000 cells of a 10 cm dish respectively.

4. Depending on the cell type, allowing the cells to grow to near confluence may help reduce nuclear DNA replication, which can overwhelm the mtDNA signal when cells are pulsed with BrdU.

5. Aphidicolin is added to reduce nuclear background (Figure 6). It must be added for at least 4h prior to BrdU, but can be added for longer periods (up to 24 hours depending on assay timing). In cases where aphidicolin treatment is undesirable, alternative methods can be used to reduce nuclear background or enrich for mtDNA. For example, prior to DNA isolation, mitochondria can be enriched via subcellular fractionation [29,30] or isolation using a commercial kit [31]. Alternatively, mtDNA can be enriched during DNA isolation by using a miniprep kit [32,33].

6. A minimum elution volume helps to ensure DNA concentration is sufficiently high (at least for subsequent steps, as the yield of DNA may be low for certain cell types).

7. The restriction enzyme choice will depend on the species of the cells being examined (Figure 7). BamHI can be used for human DNA to resolve a single linear band for synthesis or turnover assays. However other restriction enzymes can be used to generate mtDNA fragments of various sizes. Note that the observed restriction digest pattern may vary based on cell type and mtDNA haplotype.

8. 0.5 µg of DNA per reaction is recommended when aiming to visualize a single linear band of mtDNA when using a 9 cm x 11 cm agarose gel with 10 1.5 mm wells

(approximate volume 53 µL). However, for visualization of mtDNA topology, or other times when multiple bands are expected, increasing DNA to 1 µg increases the signal sufficiently to visualize multiple bands.

9. Many different loading dyes/DNA dyes can be used for the agarose gel electrophoresis. However, there are important considerations for staining and imaging the agarose gel that are relevant to the supercoiling assay (Step 3.9). First, it is recommended to stain the gel with ethidium bromide or similar DNA stain after the gel has been run, as DNA dye added the to the loading buffer or the gel can intercalate with mtDNA and affect its topology and migration through the gel. It is also important that UV imaging of the gel is done only once at the end of the assay, as UV imaging of the agarose gel mid-way through the runtime to check separation alters the conformation of mtDNA during the supercoiling assay (Figure 8).

10. Any appropriately sized object can work here, as long as it is large enough to support the agarose gel and elevates at least 3 cm above the 10X SSC buffer.

11. Depending on the imaging system, there may be an option to optimize contrast for a region of interest. This may be helpful to enhance the signal from the mtDNA bands compared to the nuclear background.

12. Although it is possible to freeze cell pellets and store them prior to DNA isolation, it is recommended to isolate DNA from fresh cells to have the best yield and preserve mtDNA topology, especially when looking at mtDNA supercoiling.

13. The supercoiling assay requires greater separation of the similarly sized relaxed and supercoiled mtDNA topoisomers. As a result, it is recommended to running the gel overnight at a lower voltage with TBE buffer rather than TAE due to TBE’s greater buffering capacity.

Figure captions

Figure 1: Representative results from mtDNA synthesis assay. 0.5 µg of total DNA isolated from U2OS cells treated as indicated was separated on agarose gels (**a**) and BrdU visualized after transfer and immunoblotting (**b**). For the mtDNA synthesis assay, BrdU was incorporated for 4h, 8h, 16h and 24h prior to DNA isolation. Total DNA was digested using BamHI. BrdU signal from the synthesis assay was quantified via imageJ densiometry (**c**). Raw intensity values were plotted as percentage values normalized to 24h post-BrdU incorporation.

Figure 2: Representative results from mtDNA turnover assay. 0.5 µg of total DNA isolated from U2OS cells treated as indicated was separated on agarose gels (**a**) and BrdU visualized after transfer and immunoblotting (**b**). For the mtDNA synthesis assay, BrdU was incorporated for 24h prior to DNA isolation immediately (0h) or 24h, 48h, 72h post-chase. Total DNA was digested using BamHI. BrdU signal from the synthesis assay was quantified via imageJ densiometry (**c**). Raw intensity values were plotted as percentage values normalized to 0h post-chase.

Figure 3: Representative results from the mtDNA supercoiling assay. 1µg of total DNA isolated from HCT116 cells treated as indicated was separated on an agarose gel (**a**) and BrdU visualized after transfer and immunoblotting (**b**). The use of controls reveals the location of possible mtDNA topologies, as BamHI digestion shows the location of the linear band, while treatment with topoisomerase removed the supercoiled band illustrating its location.

Figure 4: Diagram for assembly of the platform to transfer DNA from the agarose gel to the PVDF membrane. The agarose gel and PVDF membrane are sandwiched between a filter paper in contact with buffer and a dry filter paper, paper towels, and a weight system to allow the buffer to transfer DNA fragments by capillary action. The transfer is done at room temperature overnight.

Figure 5: Background signal from undigested nuclear DNA. 1 µg of total DNA was isolated from HCT116 cells, treated as indicated, and separated on an agarose gel (**a**) with BrdU visualized after transfer and immunoblotting (**b**). DNA isolation was completed per manufacturer's recommendations (S1) or with the suggested modifications (S2).

Figure 6: Aphidicolin reduces nuclear DNA background. 0.5 µg of total undigested DNA isolated from two primary human fibroblast lines (F1 and F2) was treated with aphidicolin as indicated (10 µM for 4 hours prior to BrdU incorporation and 10 µM during BrdU incorporation for 4 hours) and separated on an agarose gel (**a**). BrdU labelled DNA was visualized after transfer and immunoblotting (**b**).

Figure 7: Alternative restriction enzyme digests for human mtDNA. 0.5 µg of total DNA isolated from U2OS cells or human fibroblasts was treated as indicated, separated on an agarose gel (**a, b**) and BrdU visualized after transfer and immunoblotting (**c, d**) illustrating undigested mtDNA, double digest with BamHI and NheI, or double digest with XhoI and PvuII. mtDNA from U2OS cells shows an extra band following BamHI and NheI double digest around 2 kB, likely due to differences in mtDNA haplotype

Figure 8: Multiple UV exposures compromise mtDNA supercoiling assay. mtDNA supercoiling assay was performed on three different HCT116 samples. 0.5 µg of total DNA isolated from HCT116 cells was treated as indicated, separated on an agarose gel and BrdU visualized after transfer and immunoblotting. BrdU immunoblots are shown from agarose gels that were imaged once at the end of running (**a**) or imaged once mid-way through running, then resumed (**b**). Extra UV exposure leads to the creation of an extra band, and alteration of mtDNA topology.

References

1. Greaves LC, Reeve AK, Taylor RW, Turnbull DM (2012) Mitochondrial DNA and disease. J Pathol 226 (2):274-286. doi:10.1002/path.3028
2. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290 (5806):457-465. doi:10.1038/290457a0
3. Bogenhagen D, Clayton DA (1977) Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. Cell 11 (4):719-727. doi:10.1016/0092-8674(77)90286-0
4. Menger KE, Rodriguez-Luis A, Chapman J, Nicholls TJ (2021) Controlling the topology of mammalian mitochondrial DNA. Open Biol 11 (9):210168. doi:10.1098/rsob.210168
5. Clyde D (2022) Mitochondrial DNA copy number and disease. Nat Rev Genet 23 (3):136. doi:10.1038/s41576-022-00451-2
6. Ylikallio E, Tyynismaa H, Tsutsui H, Ide T, Suomalainen A (2010) High mitochondrial DNA copy number has detrimental effects in mice. Hum Mol Genet 19 (13):2695-2705. doi:10.1093/hmg/ddq163
7. Mead TJ, Lefebvre V (2014) Proliferation assays (BrdU and EdU) on skeletal tissue sections. Methods Mol Biol 1130:233-243. doi:10.1007/978-1-62703-989-5_17
8. Salic A, Mitchison TJ (2008) A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proc Natl Acad Sci U S A 105 (7):2415-2420. doi:10.1073/pnas.0712168105
9. Waldman FM, Chew K, Ljung BM, Goodson W, Hom J, Duarte LA, Smith HS, Mayall B (1991) A comparison between bromodeoxyuridine and 3H thymidine labeling in human breast tumors. Mod Pathol 4 (6):718-722
10. Matatall KA, Kadmon CS, King KY (2018) Detecting Hematopoietic Stem Cell Proliferation Using BrdU Incorporation. Methods Mol Biol 1686:91-103. doi:10.1007/978-1-4939-7371-2_7
11. Bick MD, Davidson RL (1974) Total substitution of bromodeoxyuridine for thymidine in the DNA of a bromodeoxyuridine-dependent cell line. Proc Natl Acad Sci U S A 71 (5):2082-2086. doi:10.1073/pnas.71.5.2082
12. Gratzner HG (1982) Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science (New York, NY) 218 (4571):474-475. doi:10.1126/science.7123245
13. Wu H, Zhang W, Xu F, Peng K, Liu X, Ding W, Ma Q, Cheng H, Wang X (2023) C17orf80 binds the mitochondrial genome to promote its replication. J Cell Biol 222 (10). doi:10.1083/jcb.202302037
14. Magnusson J, Orth M, Lestienne P, Taanman JW (2003) Replication of mitochondrial DNA occurs throughout the mitochondria of cultured human cells. Exp Cell Res 289 (1):133-142. doi:10.1016/s0014-4827(03)00249-0
15. Chatre L, Ricchetti M (2013) Prevalent coordination of mitochondrial DNA transcription and initiation of replication with the cell cycle. Nucleic Acids Res 41 (5):3068-3078. doi:10.1093/nar/gkt015
16. De S, Kumari J, Mudgal R, Modi P, Gupta S, Futami K, Goto H, Lindor NM, Furuichi Y, Mohanty D, 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 (Pt 10):2509-2522. doi:10.1242/jcs.101501
17. Hussain M, Mohammed A, Saifi S, Khan A, Kaur E, Priya S, Agarwal H, Sengupta S (2021) MITOL-dependent ubiquitylation negatively regulates the entry of PolgammaA into mitochondria. PLoS Biol 19 (3):e3001139. doi:10.1371/journal.pbio.3001139
18. Noack H, Bednarek T, Heidler J, Ladig R, Holtz J, Szibor M (2006) TFAM-dependent and independent dynamics of mtDNA levels in C2C12 myoblasts caused by redox stress. Biochim Biophys Acta 1760 (2):141-150. doi:10.1016/j.bbagen.2005.12.007
19. Reyes A, Melchionda L, Nasca A, Carrara F, Lamantea E, Zanolini A, Lamperti C, Fang M, Zhang J, Ronchi D, Bonato S, Fagiolari G, Moggio M, Ghezzi D, Zeviani M (2015) RNASEH1 Mutations Impair mtDNA Replication and Cause Adult-Onset Mitochondrial Encephalomyopathy. Am J Hum Genet 97 (1):186-193. doi:10.1016/j.ajhg.2015.05.013
20. Itsara LS, Kennedy SR, Fox EJ, Yu S, Hewitt JJ, Sanchez-Contreras M, Cardozo-Pelaez F, Pallanck LJ (2014) Oxidative stress is not a major contributor to somatic mitochondrial DNA mutations. PLoS Genet 10 (2):e1003974. doi:10.1371/journal.pgen.1003974
21. Battersby BJ, Shoubridge EA (2001) Selection of a mtDNA sequence variant in hepatocytes of heteroplasmic mice is not due to differences in respiratory chain function or efficiency of replication. Hum Mol Genet 10 (22):2469-2479. doi:10.1093/hmg/10.22.2469
22. Ruhanen H, Borrie S, Szabadkai G, Tyynismaa H, Jones AW, Kang D, Taanman JW, Yasukawa T (2010) Mitochondrial single-stranded DNA binding protein is required for maintenance of mitochondrial DNA and 7S DNA but is not required for mitochondrial nucleoid organisation. Biochim Biophys Acta 1803 (8):931-939. doi:10.1016/j.bbamcr.2010.04.008
23. Kai Y, Takamatsu C, Tokuda K, Okamoto M, Irita K, Takahashi S (2006) Rapid and random turnover of mitochondrial DNA in rat hepatocytes of primary culture. Mitochondrion 6 (6):299-304. doi:10.1016/j.mito.2006.10.002
24. Al Khatib I, Deng J, Lei Y, Torres-Odio S, Rojas GR, Newman LE, Chung BK, Symes A, Zhang H, Huang SN, Pommier Y, Khan A, Shadel GS, West AP, Gibson WT, Shutt TE (2023) Activation of the cGAS-STING innate immune response in cells with deficient mitochondrial topoisomerase TOP1MT. Hum Mol Genet 32 (15):2422-2440. doi:10.1093/hmg/ddad062
25. Al Khatib I, Deng J, Symes A, Kerr M, Zhang H, Huang S-yN, Pommier Y, Khan A, Shutt TE (2022) Functional characterization of two variants of mitochondrial topoisomerase TOP1MT that impact regulation of the mitochondrial genome. Journal of Biological Chemistry 298 (10):102420. doi:https://doi.org/10.1016/j.jbc.2022.102420
26. Lei Y, VanPortfliet JJ, Chen Y-F, Bryant JD, Li Y, Fails D, Torres-Odio S, Ragan KB, Deng J, Mohan A, Wang B, Brahms ON, Yates SD, Spencer M, Tong CW, Bosenberg MW, West LC, Shadel GS, Shutt TE, Upton JW, Li P, West AP (2023) Cooperative sensing of mitochondrial DNA by ZBP1 and cGAS promotes cardiotoxicity. Cell 186 (14):3013-3032.e3022. doi:https://doi.org/10.1016/j.cell.2023.05.039
27. Krokan H, Wist E, Krokan RH (1981) Aphidicolin inhibits DNA synthesis by DNA polymerase alpha and isolated nuclei by a similar mechanism. Nucleic Acids Res 9 (18):4709-4719. doi:10.1093/nar/9.18.4709

28. Simeonov A (2008) EdU click. ASSAY and Drug Development Technologies 6:157+
29. Walsh DJ, Bernard DJ, Pangilinan F, Esposito M, Harold D, Parle-McDermott A, Brody LC (2022) Mito-SiPE is a sequence-independent and PCR-free mtDNA enrichment method for accurate ultra-deep mitochondrial sequencing. Commun Biol 5 (1):1269. doi:10.1038/s42003-022-04182-2
30. Gould MP, Bosworth CM, McMahon S, Grandhi S, Grimberg BT, LaFramboise T (2015) PCR-Free Enrichment of Mitochondrial DNA from Human Blood and Cell Lines for High Quality Next-Generation DNA Sequencing. PloS one 10 (10):e0139253. doi:10.1371/journal.pone.0139253
31. Devall M, Burrage J, Caswell R, Johnson M, Troakes C, Al-Sarraj S, Jeffries AR, Mill J, Lunnon K (2015) A comparison of mitochondrial DNA isolation methods in frozen post-mortem human brain tissue--applications for studies of mitochondrial genetics in brain disorders. Biotechniques 59 (4):241-242, 244-246. doi:10.2144/000114343
32. Quispe-Tintaya W, White RR, Popov VN, Vijg J, Maslov AY (2013) Fast mitochondrial DNA isolation from mammalian cells for next-generation sequencing. Biotechniques 55 (3):133-136. doi:10.2144/000114077
33. Quispe-Tintaya W, White RR, Popov VN, Vijg J, Maslov AY (2015) Rapid mitochondrial DNA isolation method for direct sequencing. Methods Mol Biol 1264:89-95. doi:10.1007/978-1-4939-2257-4_9

Figure 1

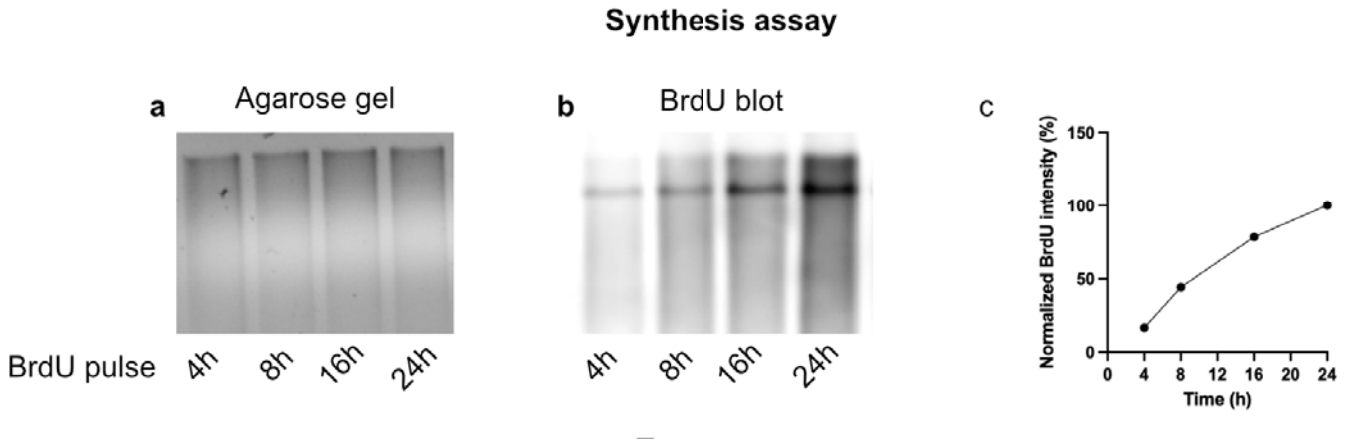

Synthesis assay
a
Agarose gel
BrdU pulse
4h
8h
16h
24h
b
BrdU blot
4h
8h
16h
24h
c
Normalized BrdU intensity (%)
150
100
50
0
0
4
8
12
16
20
24
Time (h)

Figure 2

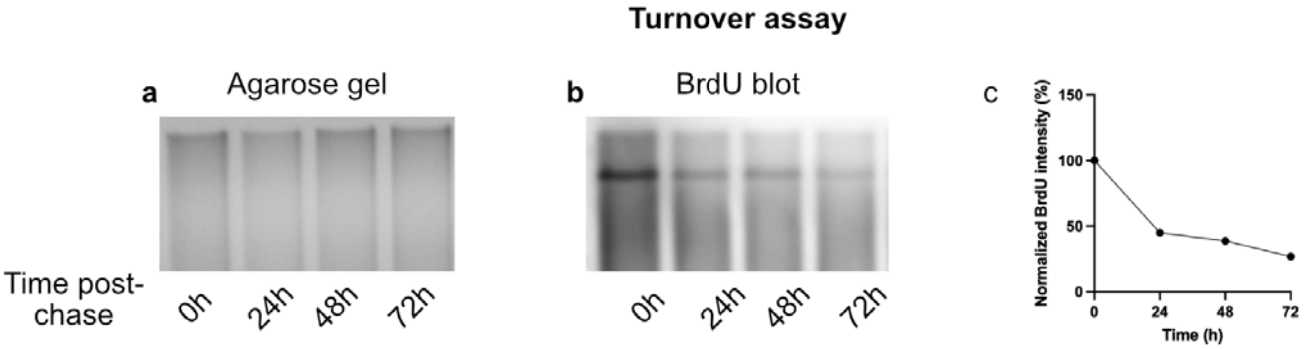

Turnover assay
a
Agarose gel
b
BrdU blot
c
Time post-chase
0h
24h
48h
72h
0h
24h
48h
72h
Normalized BrdU intensity (%)
150
100
50
0
0
24
48
72
Time (h)

Figure 3

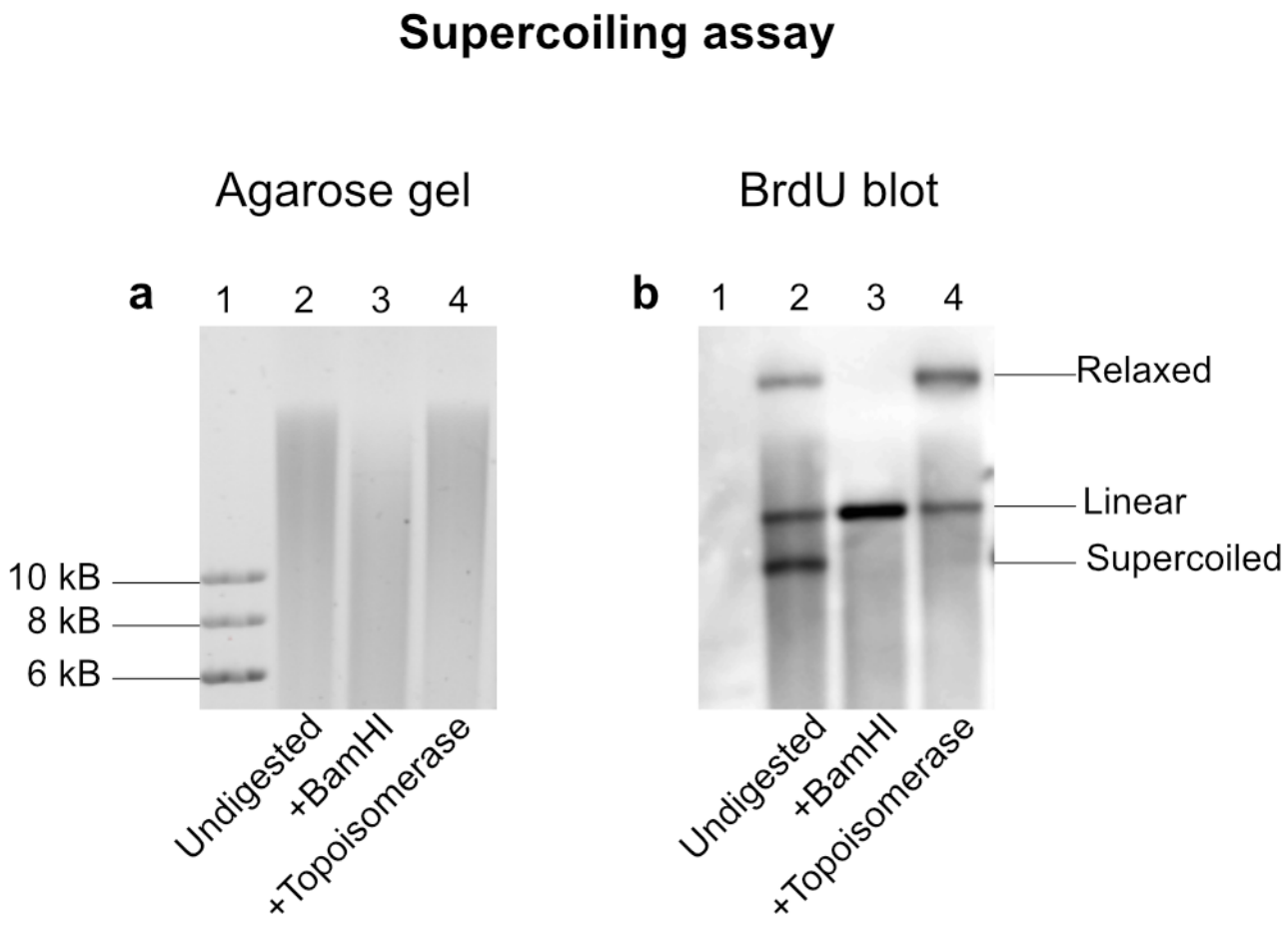

Supercoiling assay
Agarose gel
BrdU blot
a
1
2
3
4
10 kB
8 kB
6 kB
Undigested
+BamHI
+Topoisomerase
b
1
2
3
4
Relaxed
Linear
Supercoiled
Undigested
+BamHI
+Topoisomerase

Figure 4

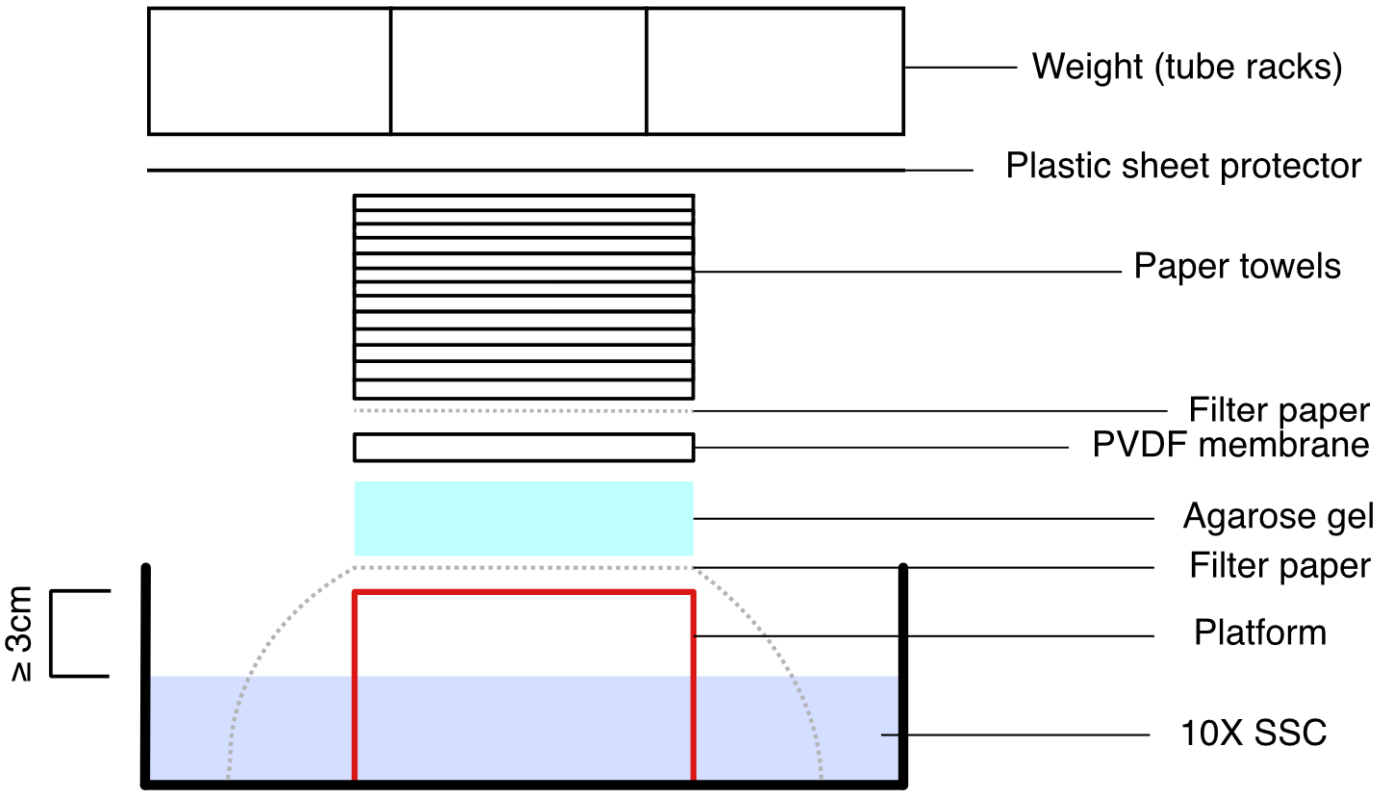

Weight (tube racks)
Plastic sheet protector
Paper towels
Filter paper
PVDF membrane
Agarose gel
Filter paper
Platform
10X SSC
≥ 3cm

Figure 5

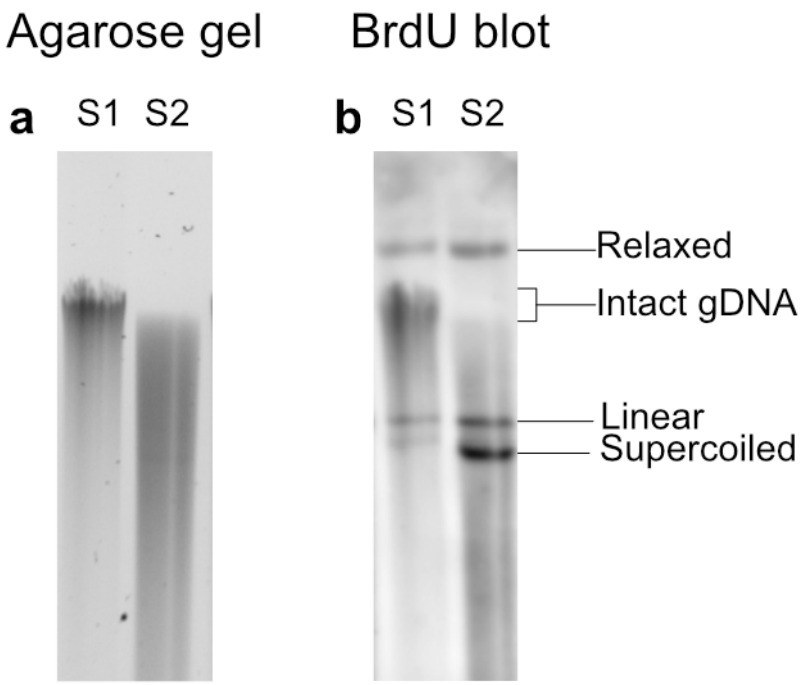

Agarose gel
BrdU blot
a
S1
S2
b
S1
S2
Relaxed
Intact gDNA
Linear
Supercoiled

Figure 6

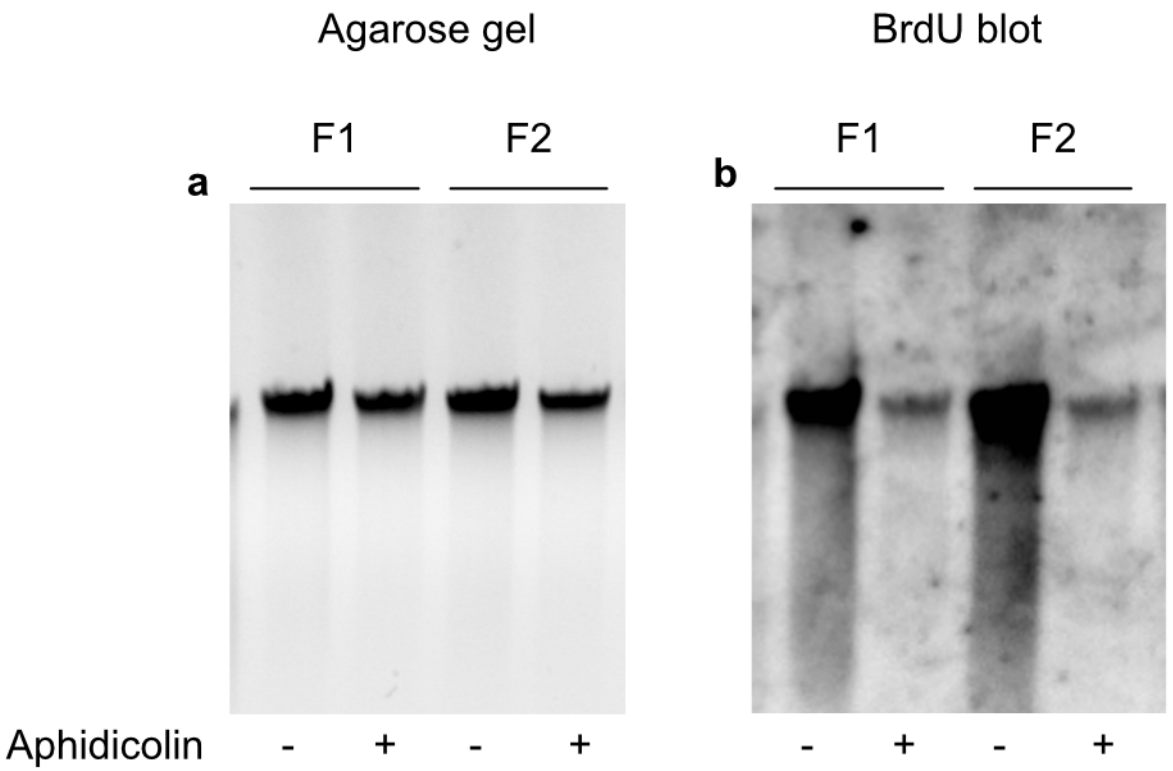


Figure 7

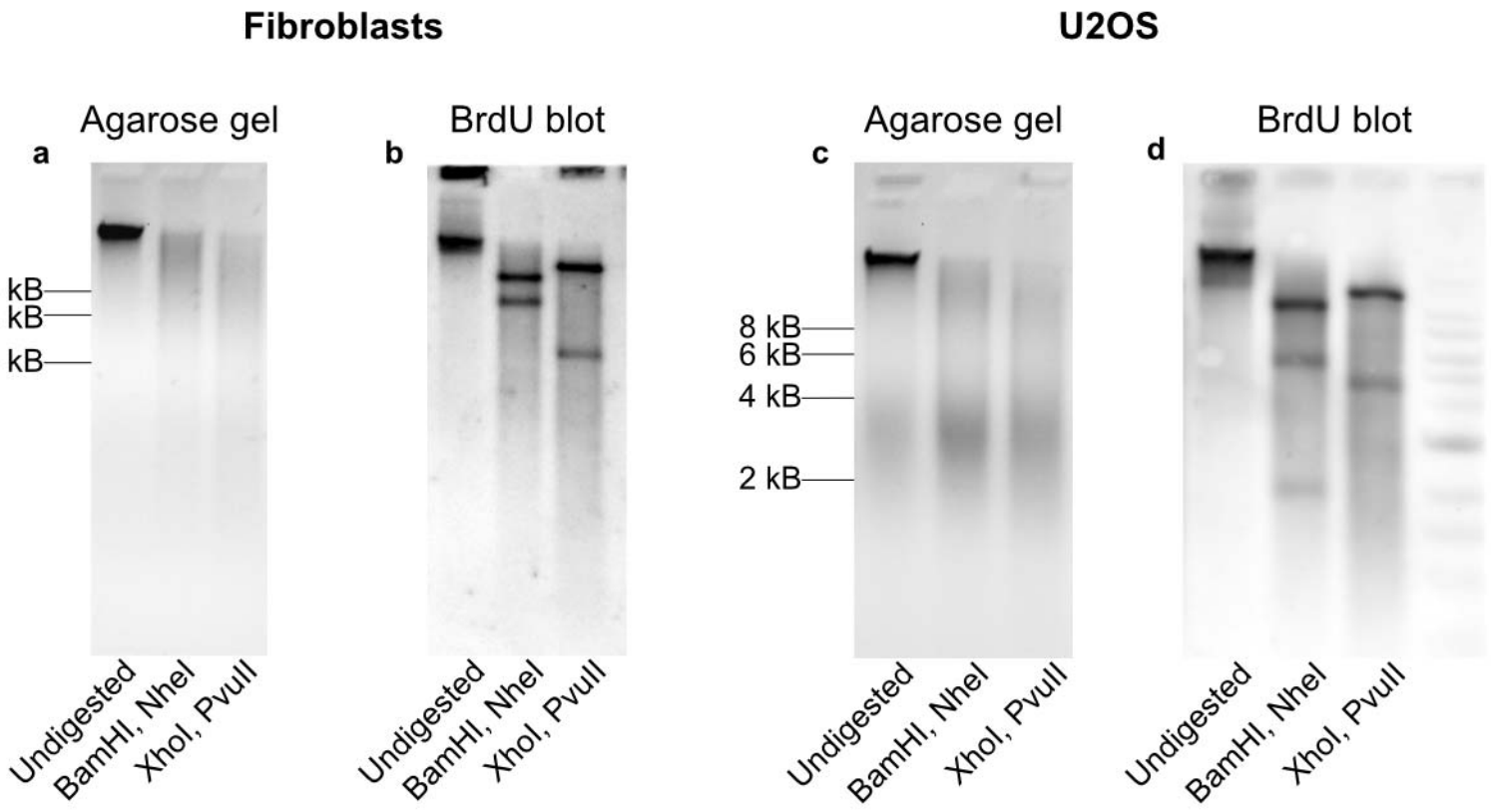
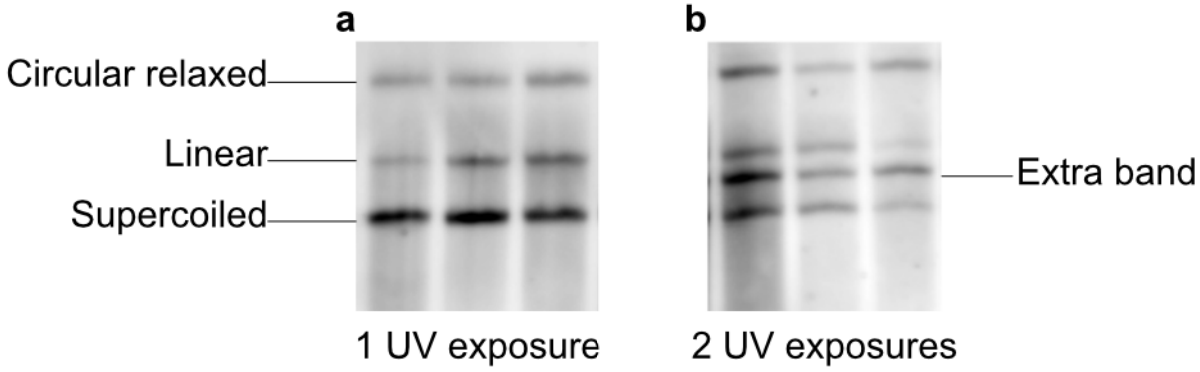

Fibroblasts
U2OS
Agarose gel
BrdU blot
Agarose gel
BrdU blot
a
b
c
d
kB
kB
kB
8 kB
6 kB
4 kB
2 kB
Undigested
BamHI, NheI
XhoI, PvuII
Undigested
BamHI, NheI
XhoI, PvuII
Undigested
BamHI, NheI
XhoI, PvuII
Undigested
BamHI, NheI
XhoI, PvuII

Figure 8


a
b
Circular relaxed
Linear
Supercoiled
Extra band
1 UV exposure
2 UV exposures