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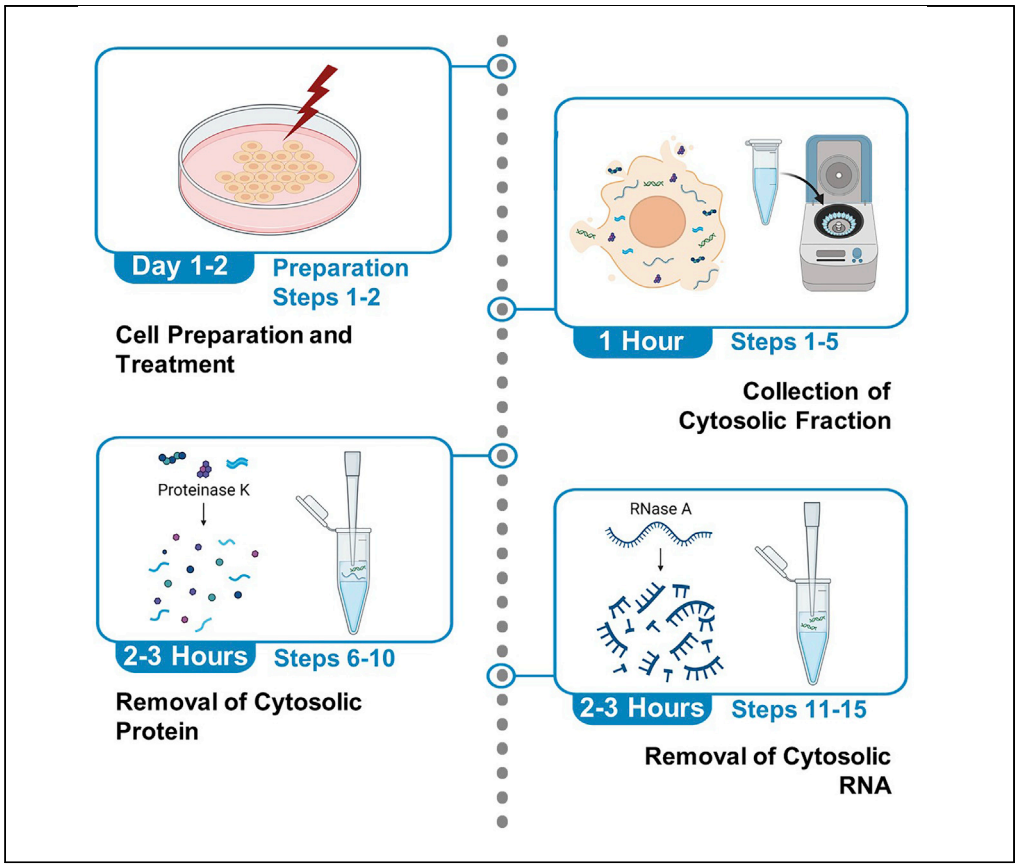
Isolation of endogenous cytosolic DNA from cultured cells



DNA damage caused by genetic instability or extrinsic treatment can induce DNA leakage from the nucleus or mitochondria into the cytosol and activate innate and adaptive immunity. To enable characterization of these endogenous cytosolic DNAs and the mechanisms that produce them, we developed an approach for isolation of cytosolic DNA with no detectable mitochondrial contamination. Here we describe cytosolic compartment separation followed by DNA purification from colorectal cancer cells and illustrate how this may be expanded to other cell types.

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Highlights

Protocol for isolation of cytosolic DNA produced by DNA damage or instability

Isolated cytosolic DNA is free from detectable mitochondrial contamination

Protocol is optimized for colon cancer cells but easily adapted to other cell types

Enables sequencing and structural characterization of cytosolic DNA

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Isolation of endogenous cytosolic DNA from cultured cells

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SUMMARY

DNA damage caused by genetic instability or extrinsic treatment can induce DNA leakage from the nucleus or mitochondria into the cytosol and activate innate and adaptive immunity. To enable characterization of these endogenous cytosolic DNAs and the mechanisms that produce them, we developed an approach for isolation of cytosolic DNA with no detectable mitochondrial contamination. Here we describe cytosolic compartment separation followed by DNA purification from colorectal cancer cells and illustrate how this may be expanded to other cell types.

BEFORE YOU BEGIN

Recently, there is increasing recognition that cytosolic DNA (cyDNA) sensing contributes to many homeostatic and pathogenic immune responses in the body, such as tolerance and antitumor immunity, respectively. Better understanding how such sensing is regulated requires in-depth characterization of the nature of the cyDNA and how this can be altered by infection or DNA damaging treatments. Current cyDNA isolation methods are highly variable across studies and often fail to confirm the absence of mitochondrial contamination. Contamination from mitochondrial DNA may bias results since it would not normally be accessible to Stimulator of Interferon Genes (STING). It is also particularly problematic since even though some mitochondrial DNA is released naturally into the cytosol, contamination of cyDNA preparations by DNA from intact mitochondria may not represent the structures and sequences of mitochondrial DNA that are naturally released. Here, we describe the isolation of cyDNA free from mitochondrial contamination by collection of the cytosolic compartment of the cell, followed by Proteinase K, RNase A, and phenol-chloroform extractions to remove cytosolic protein and RNA. Isolated cyDNA can then be used for downstream applications such as stimulation of other cells to test cyDNA sensing capacity.

While the protocol shown here has been optimized for the mouse colorectal cancer cell line MC38 (Corbett et al., 1975), it can be extended to other cell lines by adjusting the digitonin concentration in the cytosolic extraction buffer and determining the optimal concentration by examining the distribution of cell compartment markers by western blotting the cytosolic and pelleted fractions following step 5 (Figure 1). The optimal digitonin concentration will yield a cytosolic fraction free of contamination by proteins from the mitochondria (COX-IV), plasma membrane (EGFR) and nucleus (Lamin B1).

Cell preparation

Timing: 1 day

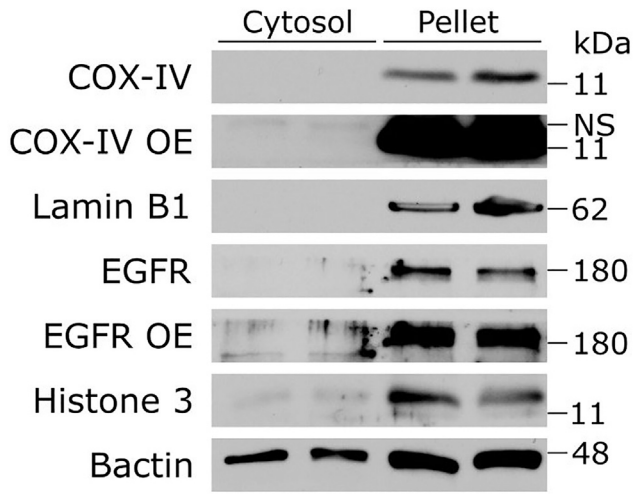

Figure 1. Confirmation of cytosolic fraction purity

Protein was isolated from cytosolic and pelleted fractions of MC38 cells collected in step 5 and analyzed via western blot for mitochondrial (COX-IV), nuclear (Lamin B1 and Histone 3), and cell membrane (EGFR) markers. OE indicates blot overexposure, NS indicates a non-specific band.

When seeding cells, consider that they should be approximately 80% confluent at day 0 following treatment. One plate of MC38 cells can be plated at 5.0 × 10^6 cells per 10 cm plate for radiation treatment and cyDNA isolation the following day. This leads to an approximate yield of 15–35 μg of cyDNA, as measured on the Nanodrop spectrophotometer.

1. Seed cell line of interest into desired number of 10 cm tissue culture treated plates at 1.0 to 5.0 × 10^6 cells per plate 24 h before treatment. MC38 cells can be seeded in High Glucose DMEM with 10% FBS, 1% HEPES, and 1% Penicillin/Streptomycin.

Treatment of cells

Timing: 30 min to 2 days

2. If desired, treat the seeded cells with a DNA damaging treatment of interest to induce cyDNA release. In MC38 cells, increased cyDNA quantities are observed following 10 Gy of radiation. Genetically instable and cancer cells will likely also contain cyDNA in the absence of treatment.

⚠ CRITICAL: DNA damaging treatments should be given at sub-lethal doses or time points to prevent the isolation of nuclear DNA released during apoptosis. See troubleshooting 5

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
COX-IV	Abcam	Cat# ab16056
Lamin B1	Abcam	Cat# ab133741
EGFR	Santa Cruz	Cat# sc-373746
Histone H3	Abcam	Cat# ab1791
Beta-actin	Cell Signaling Technology	Cat# 8457S

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
NaCl	Fisher Chemical	Cat# S271-3
HEPES	Fisher Scientific	Cat# SH30237.01
Digitonin	Sigma-Aldrich	Cat# 11024-24-1
Hexylene Glycol	Fisher Scientific	Cat# AAL03187AP
Protease Inhibitor Cocktail	Sigma-Aldrich	Cat# P8340-5mL
HyPure Molecular Biology Grade Water	Fisher Scientific	Cat# SH30538.02
Penicillin/Streptomycin	Fisher Scientific	Cat# SV30010
FBS	Thermo Fisher Scientific	Cat# 12483-020
Trypsin (Diluted to 0.125% with PBS)	Thermo Fisher Scientific	Cat# 25200-072
Proteinase K (Suspended at 20 mg/mL in 2 mM $CaCl_2$, 10 mM Tris)	Fisher Scientific	Cat# BP1700-500
CaCl	Fisher Scientific	Cat# BP510-500
Phenol-chloroform isoamyl alcohol	Fisher Scientific	Cat# BP1752I-400
Isopropanol	Fisher Scientific	Cat# A416P-4
Glyco-Blue	Thermo Fisher Scientific	Cat# AM9515
Ethanol	Sigma-Aldrich	Cat# P016EAAN
10mM Tris (pH 8.0 in Nuclease Free Water)	Fisher Scientific	Cat# BP152-500
RNase A	Thermo Fisher Scientific	Cat# EN0531
40% Acrylamide/Bis Solution 29:1	BIO-RAD	Cat# 1610146
Ammonium persulfate	Fisher Scientific	Cat# BP179-100
TEMED	Fisher Scientific	Cat# BP150-100
SYBR Gold	Thermo Fisher Scientific	Cat# S11494
Critical commercial assays		
Pierce BCA Protein Assay	Thermo Scientific	Cat# 23225
Experimental models: Cell lines		
MC38 Cells (C57BL/6J Mouse, Colorectal Cancer, Female)	Kerafast	Cat# ENH204-FP
Other		
High Glucose DMEM (10% FBS, 1% HEPES, 1% Penicillin/Streptomycin)	NA	NA
Corning CytoSmart Cell Counter	Corning	Cat# 6749
10cm Tissue Culture Dish	Fisher Scientific	Cat# 130182
0.2 µm Syringe Filter	Fisher Scientific	Cat# 13-1001-06
Calcium and Magnesium Free PBS	NA	NA
1.5 mL Microcentrifuge Tubes	Fisher Scientific	Cat# 02682002
ThermoIEC Micromax RF Microcentrifuge	Thermo Fisher Scientific	NA
RIPA Buffer (50 mM Tris-HCl, 150 mM NaCl, 50 mM sodium pyrophosphate, 1 mM EDTA, 0.5% NP40, 1% Triton X-100)	NA	NA
Nanodrop 1000	Thermo Fisher Scientific	ND-1000 UV/Vis
10X TBE (0.89 M Tris, 0.89 M boric acid, 25 mM EDTA)	NA	NA

MATERIALS AND EQUIPMENT

Cytosolic Extraction Buffer

Reagent	Stock concentration	Final concentration	Amount
NaCl	5M	150mM	1.5 mL
HEPES	1M	50mM	2.5 mL
Digitonin	20mg/mL	200ug/mL	0.5 mL
Hexylene Glycol	98%	1M	6.41 mL
ddH_2O	N/A	N/A	To 50 mL

Cytosolic Extraction Buffer modified from (Baghirova et al., 2015).

Protease inhibitor cocktail should be added at 1:100 immediately before buffer use when confirming proper cell fractionation by western blot.

Note: Buffer can be stored at 4°C for at least one month after filter sterilization using a 0.2 μm filter.

STEP-BY-STEP METHOD DETAILS

Collection of cytosolic fraction

Timing: 45–60 min

The following steps describe collection of cells and cytosolic cellular fraction.

1. Harvest the cultured cells.
   a. Wash the cells once with 5 mL ice cold PBS per plate.
   b. Trypsinize the cells as per typical cell splitting protocol. For MC38 cells:
      i. Add 0.5 mL 0.125% Trypsin/EDTA to each cell culture plate.
      ii. Incubate the plates at 37°C for 5–10 min.
   c. Collect the cells from the plate using 1 mL ice cold PBS and transfer to a 1.5 mL microcentrifuge tube. Keep samples on ice moving forward.
   d. Spin down the cells at 500×*g* for 10 min at 4°C. Discard supernatant.

⚠ CRITICAL: Steps 2–4 comprise the subcellular fractionation of the cells for collection of the cytosol. Proper sample incubation time and spin conditions are critical for this step to prevent contamination from other cell compartments.

2. Gently resuspend the cell pellet in 550 μL ice cold Cytosolic Extraction Buffer (see materials and equipment).
3. Incubate on ice for 10 min.
4. Spin the samples at 2000×*g* for 10 min at 4°C.
5. Transfer all supernatant containing the cytosolic fraction to a fresh 1.5 mL microcentrifuge tube.

Note: To confirm proper cytosolic fractionation of your specific cell line, use the supernatant and pellet (which can be lysed via resuspension in 1X RIPA buffer) from step 5 to perform western blots for cell compartment markers that should be absent from a pure cytosolic fraction (Figure 1). All buffers should contain 1:100 protease inhibitor cocktail when using fractions for western blotting. COX-IV confirms the absence of mitochondria (and therefore unreleased mitochondrial DNA) in the cytosolic fraction. Lack of nuclear markers, such as Lamin B1, in the cytosolic fraction indicate a lack of nuclear contamination. Note that the use of a histone as a nuclear marker may lead to a false indication of nuclear contamination as histones may be present on cyDNA (MacKenzie et al., 2017). Markers of other cell components, such as EGFR for the cell membrane, can also be evaluated if desired. See troubleshooting 2 and 3.

Removal of cytosolic protein

Timing: 2 h to 1 day

The following steps describe in detail treatment of the cytosolic fraction with Proteinase K and phenol-chloroform extraction for Proteinase K removal (Härtlova et al., 2015).

6. Take a small aliquot of the cytosolic fraction. Use a protein quantification method (such as the Pierce BCA Protein Assay) to determine the protein concentration. Normalize input of the fraction into step 7 using this value. For an 80% confluent 10 cm plate of MC38 cells in step 1, approximately 1 mg of protein is observed in the cytosolic fraction. See troubleshooting 4.

Note: Once the average amount of protein is determined from several replicates of the cytosolic fraction from step 5, step 6 may be omitted in subsequent experiments that are set up with identical conditions and the pre-titrated amount of Proteinase K can be used for consistency.

7. Add Proteinase K to cytosolic fraction isolated in step 5 at a ratio of 0.55 mg:1 mg of total protein in 550 μL of sample. See troubleshooting 1.
8. Incubate the samples at 55°C for 1 h.
9. Perform phenol-chloroform isoamyl alcohol extraction.

Note: Use of commercial columns for nucleic acid purification should be avoided to prevent loss of small cyDNA fragments.

a. Add 500 μL of phenol-chloroform isoamyl alcohol.

Note: A final 1:1 ratio of sample to phenol-chloroform isoamyl alcohol is ideal after step 9a. Transfer of all supernatant in step 5 ensures the correct ratio with the amount of phenol-chloroform isoamyl alcohol indicated above.

b. Shake the samples vigorously for 30 s.
c. Spin the samples at 18300×*g* for 15 min at 4°C.
d. Add 500 μL of 100% isopropanol to a fresh 1.5 mL microcentrifuge tube.
e. Transfer 400 μL of the top (aqueous) layer of the samples from step 9c to tubes prepared in step 9d. See troubleshooting 1.
f. Add 1.0 μL of Glyco-Blue. This will aid in precipitating the DNA (Green and Sambrook, 2016).

Note: Low concentrations of glycogen will be present in the final product due to this step. While this concentration is unlikely to affect downstream analyses, Glyco-Blue may also be added to later negative controls. Omission of this step would likely decrease the efficacy of the extraction process and lead to lower final DNA yield.

g. Shake the samples vigorously for 30 s.

Optional: After step 9g, samples can be incubated at least 16 h at −20°C to increase final yield by up to 40%.

h. Spin the samples at 18300×*g* for 10 min at 4°C.
i. Remove the supernatant.
j. Add 500 μL of 70% ethanol. Gently flick the tube to dislodge the pellet.
k. Spin the samples at 18300×*g* for 10 min at 4°C.
l. Remove the supernatant.
m. Allow the pellet to dry at 24°C.

⚠ CRITICAL: The pellet in step 9m should be allowed to dry completely as carryover of remaining ethanol may influence the efficiency of the rest of the protocol or downstream cyDNA analysis.

10. Resuspend the pellet in 200 μL of 10 mM Tris-HCl buffer (pH 8.0).

Removal of cytosolic RNA

Timing: 2 h to 1 day

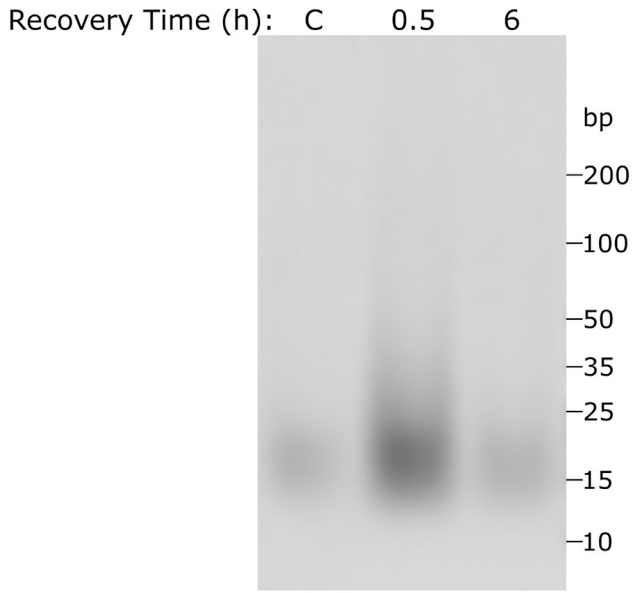

Figure 2. Visualization of cyDNA isolated after radiation of MC38 cells

Cells were treated with 10 Gy of radiation and allowed to recover for the indicated times before cyDNA isolation. Samples were analyzed by electrophoresis on an 8% PAGE gel (20% of 40% acrylamide/bis solution 29:1, 1X TBE, 1% ammonium persulfate, 0.1% TEMED). Gel was stained with 1/10000 SYBR Gold in 1X TBE for 40 min. C indicates cyDNA from unirradiated control cells, size shown in base pairs.

The following steps describe treatment with RNase A to remove RNA contaminants followed by a phenol-chloroform extraction for RNase A removal (Härtlova et al., 2015).

11. Add RNase A to samples at a final concentration of 500 μg/mL.
12. Incubate samples for 1 h at 37°C.

> ***Note:*** An excess dose and long treatment time with RNase A in steps 11–12 ensures full removal of contaminating cytosolic RNA from the final isolation that could greatly bias downstream analyses. This contaminating RNA may show up in downstream DNA characterization methods, and act as a false positive during subsequent stimulations by activating cytosolic RNA sensors that give similar readouts to their DNA sensing counterparts, such as STING.

13. Add 300 μL of 10 mM Tris-HCl (pH 8.0) for easy extraction and to reach the correct ratio of sample to phenol-chloroform isoamyl alcohol (see step 9a note) in step 14.
14. Perform phenol-chloroform isoamyl alcohol extraction as described in step 9.
15. Resuspend the pellet in 50 μL 10 mM Tris-HCl (pH 8.0).

EXPECTED OUTCOMES

Cytosolic DNA isolation should yield 200–700 ng/uL (as quantified by a Nanodrop spectrophotometer) of cyDNA per 10 cm plate of starting material and will vary depending on the cell number, cell type, and treatments used. From MC38 cells, cyDNA size ranges from 10–150 bp (Figure 2).

QUANTIFICATION AND STATISTICAL ANALYSIS

Cytosolic DNA can be quantified by a Nanodrop spectrophotometer (or another spectrophotometer with similar spectral capabilities) or Qubit and visualized on an 8% DNA PAGE gel (Minoo et al., 2006) (Figure 2). If desired, more precise analysis of size can be determined using an Agilent Bioanalyzer High Sensitivity DNA kit.

To examine the origin of cyDNA fragments, such as cellular compartment or gene, qPCR of regions less than 100 bp in length can be performed and normalized to total cyDNA concentration or concentration of genomic DNA isolated from the pellet in step 5 (West et al., 2015). Isolated cyDNA can also be used to stimulate other cells to determine either the sensitivity of their cyDNA-sensing pathways, such as that of STING, or the stimulatory capacity of cyDNA from the source cells (Mowat et al., 2021).

LIMITATIONS

Previous literature indicates that DNA binding proteins may be present on cyDNA (Andreeva et al., 2017) that would be removed by this protocol. Additionally, the cytosolic extraction buffer used in this protocol is optimized for MC38 mouse colorectal carcinoma cells and will require confirmation and possibly titration of digitonin concentration for other cell lines (troubleshooting 2, 3, and 4).

TROUBLESHOOTING

Problem 1

Nanodrop spectrophotometer quantification of isolated cytosolic DNA indicates that the 260/280 ratios are very low (1.6–1.7) and the cyDNA yield is lower than expected.

Potential solution

It is possible that the Proteinase K step did not remove all protein. Increase the concentration of Proteinase K. Additionally, optimal performance of Proteinase K depends on the inclusion of 2 mM $CaCl_2$ in the suspended Proteinase K stock.

Alternatively, low 260/280 values may indicate contamination from accidentally touching the interface of the protein (bottom) layer during the phenol-chloroform isoamyl alcohol extraction in step 9e. To avoid this, sample should be collected from the top of the aqueous (top) layer. If necessary, smaller volumes may be collected from the aqueous layer to decrease the possibility of this contamination. This will, however, lead to decreased final yields.

Problem 2

Mitochondrial or nuclear proteins are observed in the cytosolic fraction when performing western blotting to test purity of the cytosolic fraction isolated in step 5.

Potential solution

This suggests the cytosolic extraction buffer is too strong and leads to lysis of the internal organelles and/or the nucleus. Lower concentrations of digitonin in the buffer should be tested to determine the optimal concentration for the cells of interest.

> *Note:* Using histones as a nuclear marker can give a false impression of nuclear contamination since cyDNA can be bound by histones. An alternate marker to test for nuclear contamination is recommended.

Problem 3

Very low or absent yield of cyDNA is observed using a Nanodrop spectrophotometer after isolation despite all nuclear and mitochondrial protein markers being properly observed in the pelleted fraction from step 5.

Potential solution

This could suggest that the cytosolic extraction buffer is too gentle and failing to lyse the plasma membrane. Higher concentrations of digitonin in the buffer should be tested to determine the optimal concentration for the cells of interest.

Alternatively, while we have observed baseline cyDNA in immortalized and cancer cell cultures, a low yield could indicate the cell type of interest carries low levels of cyDNA. To investigate this, immunofluorescence microscopy with DAPI staining or an anti-double stranded DNA antibody can be performed (Mowat et al., 2021). Use of mitochondrial and nuclear localization co-stains will allow for confirmation that non-organelle associated cyDNA is present.

Problem 4

Little to no protein is detected within the cytosolic fraction in step 6.

Potential solution

Similarly to Troubleshooting 3, this would suggest that the cytosolic extraction buffer has failed to lyse the plasma membrane. Increased concentrations of digitonin in the buffer should be tested.

Problem 5

Unusually high DNA concentrations (over 1 μg/μL) are observed using a Nanodrop spectrophotometer after cyDNA isolation.

Potential solution

If this outcome has been observed upon treatment with a DNA damaging agent, this may indicate that the cells have begun the process of apoptotic cell death. During this process, fragmented nuclear and mitochondrial DNA would be released into the cytosol, leading to unusually high cyDNA yields. However, as the STING pathway is inhibited during apoptosis (White et al., 2014), cyDNA harvested under such conditions may not be relevant in a physiological context. As such, DNA damaging treatments used for cyDNA collection should be pre-titrated and utilized at sub-lethal doses.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kristi Baker (kbaker2@ualberta.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

S.R.M. optimized the protocol and wrote and edited the manuscript. K.B. supervised the project, edited the manuscript, and secured funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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