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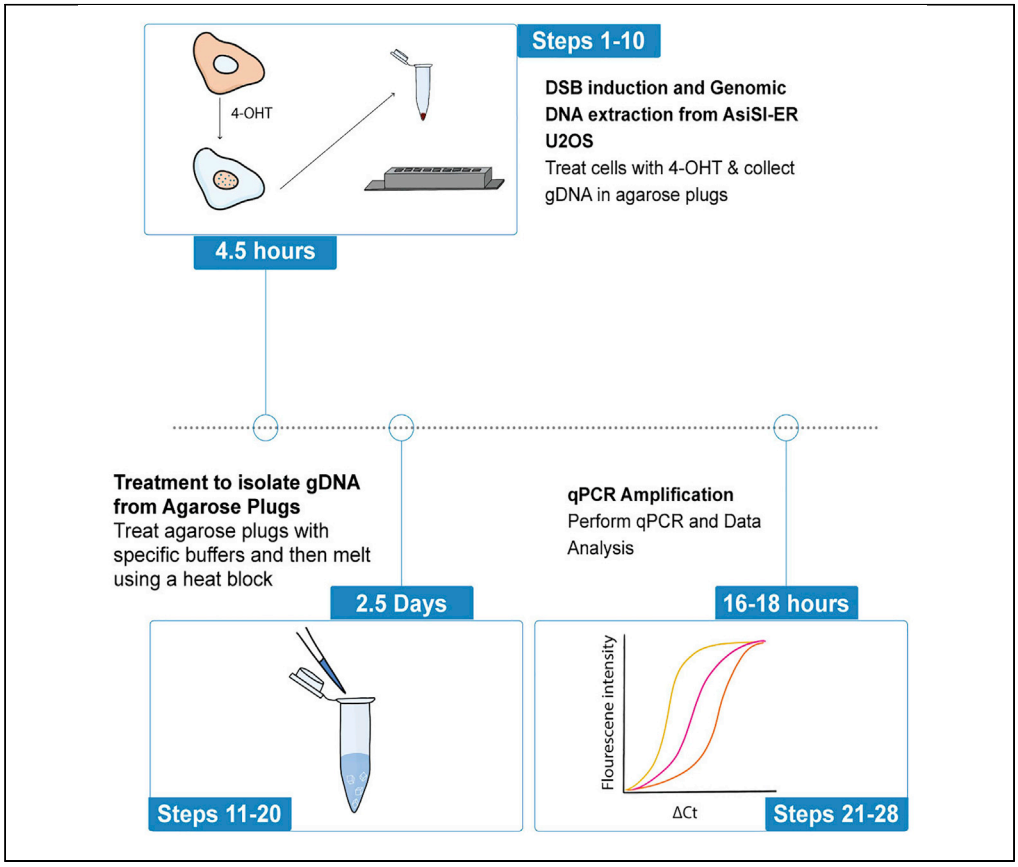

DNA end resection is a critical step in the homologous recombination pathway of repairing DNA double-strand breaks (DSBs) that can be visualized in cells by detecting the generation of single-stranded DNA (ssDNA) intermediates formed during the resection of the DSBs. Here, we describe quantitative polymerase-chain-reaction-based procedures to quantitatively measure ssDNA intermediates formed during the DNA end resection. Using the ER-A*si*SI system, we use differential digestion patterns by restriction endonucleases that digest unresected double-stranded DNA at DSB sites.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Detailed steps for genomic DNA extraction from "ER-*Asi*SI" U2OS cells

The protocol involves the isolation of gDNA from agarose plugs

Measure DNA end resection by qPCR amplification and quantifies data

Probes the extent of DNA end resection at site-specific double-strand break sites

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Protocol to measure end resection intermediates at sequence-specific DNA double-strand breaks by quantitative polymerase chain reaction using ER-*Asi*SI U2OS cells

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SUMMARY

DNA end resection is a critical step in the homologous recombination pathway of repairing DNA double-strand breaks (DSBs) that can be visualized in cells by detecting the generation of single-stranded DNA (ssDNA) intermediates formed during the resection of the DSBs. Here, we describe quantitative polymerase-chain-reaction-based procedures to quantitatively measure ssDNA intermediates formed during the DNA end resection. Using the ER-A*si*SI system, we use differential digestion patterns by restriction endonucleases that digest unresected double-stranded DNA at DSB sites.

For complete details on the use and execution of this protocol, please refer to Fitieh et al. (2022).[1]

BEFORE YOU BEGIN

DSB repair by homologous recombination (HR) is initiated by 5′–3′ nucleolytic degradation of DNA ends in a process termed DNA end resection.[2] DSB resection generates long 3′-ssDNA overhangs that are essential for HR repair and promote the activation of the DNA damage checkpoint.[3] DSB resection has been monitored indirectly by detecting immunofluorescence of nuclear protein foci like Replication protein A (RPA) foci or Bromodeoxyuridine (BrdU) foci formation in mammalian cells. The formation of RPA foci and BrdU depends on ssDNA products of resection, and measurement of the number or intensity of these foci is semi-quantitative. In addition, the Single Molecule Analysis of Resection Tracks (SMART) assay can measure the length of DNA resection tracks in cells exposed to DNA damage.[4] While the smart assay provides a quantitative readout of DNA end resection, it is a relatively time-consuming and labour-intensive method. Researchers also used different sequence-specific DSB-inducible systems such as Fok1, A*si*SI, I-PpoI, I-SceI-based and HO-dependent systems to induce with a high degree of precision DSBs at specific genomic loci in human and yeast cells.[5–9] These systems allowed them to study DSB response while ensuring no break occurs elsewhere in the genome.[5–9] Another tool is Zinc-finger nucleases (ZFNs), which were built by attaching zinc-finger DNA-binding domains to the catalytic domain of the *Fok* I endonuclease.[10] While ZFNs were used for targeted gene editing in the eukaryotes,[11] they were toxic and had a high frequency of off-target mutations.[12] Another way to induce DSBs in cells is CRISPR/Cas9 system.[13,14] However, the repairing kinetics of Cas9-induced DSBs is generally slow and often last for more than twenty hours in mammalian cells.[15,16] This is likely due to the Cas9- single guide RNA (sgRNA) complex

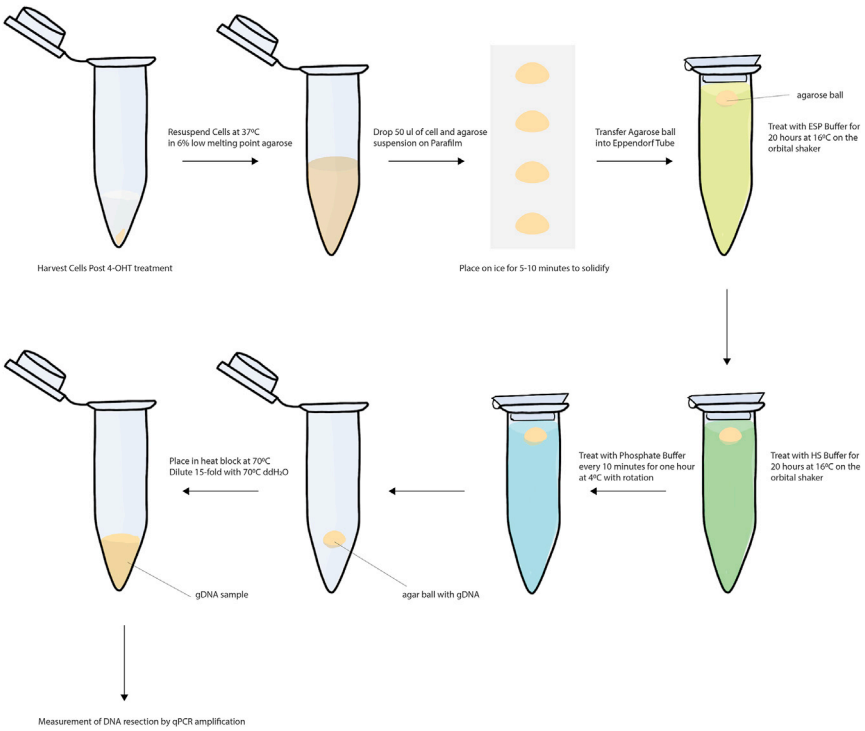

Figure 1. Schematic representation of the key steps of the protocol

Flow chart of the isolation of genomic DNA for Quantifying DNA end resection intermediates by qPCR (Modified from Zhou et al.).

being retained longer at the cleaved DNA sites.[15,16] This might affect the DSB pathway choice by influencing the binding/function of factors that regulate the selection of the DNA repair pathway. Off-target effects are a severe problem of CRISPR/Cas9 genome editing and have limited the widespread use of this technology in DSB response studies.[17] These off-target effects stem from sgRNAs can tolerate a few mismatches with unwanted target sites,[18] and CRISPR has been shown to associate with many off-target sites in the genome.[19]

Here we describe a detailed Polymerase Chain Reaction (qPCR)-based protocol, which the Paull lab previously established.[2] The method directly measures the levels of ssDNA intermediates generated by resection at specific DSB sites in the ER-*Asi*SI U2OS cells. It is more quantitative and precise with respect to the extent and efficiency of resection as compared to previously described methods. The advantage of the qPCR method is that it directly measures the ssDNA intermediates. The ER-*Asi*SI U2OS cells stably express a restriction endonuclease, A*si*SI, fused to an estrogen receptor hormone-binding domain.[20] Upon treatment of cells with hydroxytamoxifen (4-OHT), the A*si*SI enzyme travels to the nucleus to generate sequence-specific (5′-GCGATCGC-3′) DSBs. By embedding cells in low-gelling point agarose, we extracted genomic DNA (gDNA) without shearing DNA during extraction (Figure 1). Previous studies done in budding yeast have established a feature of resection enzymes (Figure 2A) that allows it to distinguish original double-stranded DNA (dsDNA) from ssDNA.[21]

Ahead of time

Timing: 4 h

1. Please note that the protocol does not need preparation except for growing ER-A*si*SI U2OS cells in culture. The next day, cells are treated with 4-OHT to induce DSBs. This protocol is based on the method developed by Zhou et al.[2]

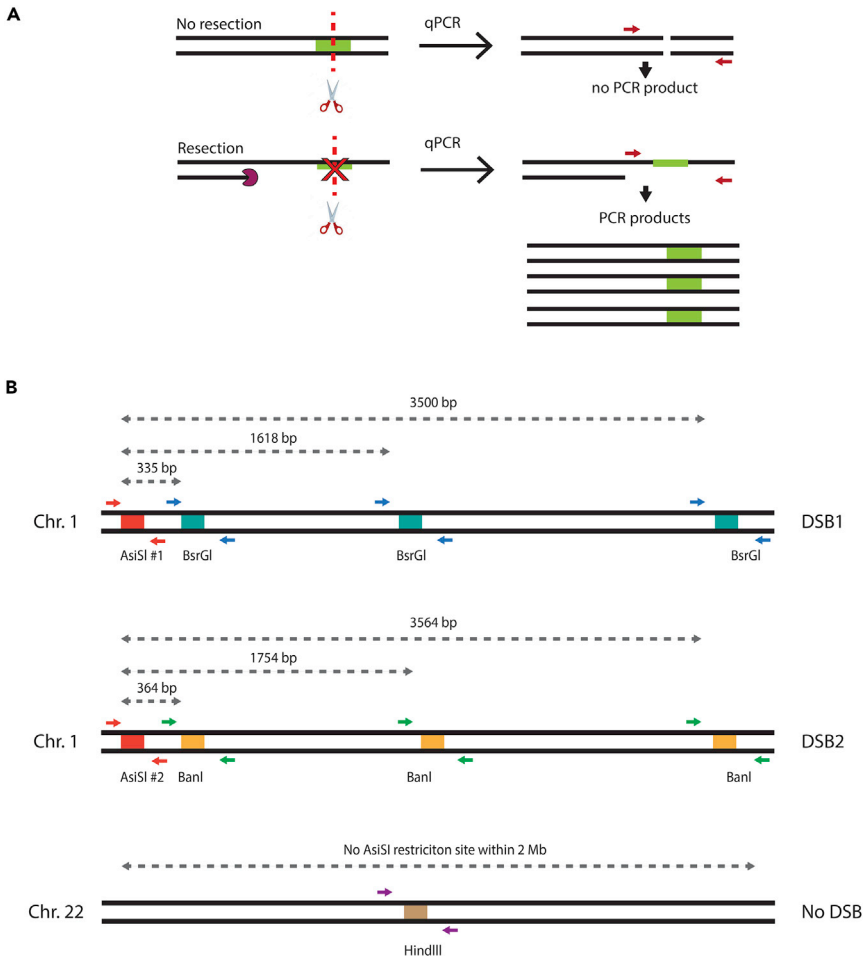

Figure 2. Schematic Representation for Quantifying DNA end resection intermediates by qPCR

(A) Expected Outcomes for qPCR when quantifying DNA end resection. A restriction enzyme (denoted by scissors) creates a DSB at a sequence-specific region. If the DSB is resected, the ssDNA will not be cut by the restriction enzyme, as denoted by the "X" on the restriction site, and therefore, PCR products will be produced.

(B) Schematic of qPCR primers and Probes designed to measure DSB % at two selected *Asi*SI cut sites on Chromosome 1. The primer pair for each restriction cut site is denoted with the same colour as the region. Chromosome 22 includes a No DSB as a negative control (Modified from Zhou et al.[2]).

Note: Most of the listed primer pairs are adjacent to known cleavage sites in the U2OS genome by *Asi*SI[20] and have been validated by the Legube group who generated the *Asi*SI expressing U2OS stable cell line.

Note: Primer sequences to *Asi*SI sites other than the ones cited here may be sourced from publications by the Legube group referencing *ER-Asi*SI U2OS.

⚠ CRITICAL: a suitable negative control for qPCR is the "no template control", such that all components for the PCR reaction are provided except the template DNA (PCR master mix, forward and reverse primers, water) should be included as a control.

2. Primers can be custom-synthesized and dissolved according to guidelines from the manufacturer and should be stored at −20°C.

Note: The sequence of the different DNA primers is referenced in **Materials and Resources** and the key resources table.

⚠ CRITICAL: Primers can be aliquoted at −20°C to avoid freeze-thaw cycles.

3. According to previous research, we used two specific A*si*SI restriction sites cleaved with high efficiency in the human ER-A*si*SI cell line, Double-Strand Break 1 (DSB1), Chr 1: 89231183) and Double-Strand Break 2 (DSB2), Chr 1: 109838221.[20,22]

⚠ CRITICAL: Across varying distances from each A*si*SI site, three pairs of qPCR primers across *Bsr*GI or BanI cut sites were designed using free software such as Primer3 web-based software, as depicted in Figure 2B.

Note: The extent of DNA end resection (short range Vs long range) can be measured by designing very close or distal primers from the cut sites.

Note: To monitor the percentage of DSB (DSB %) present at the two sites, two pairs of primers were designed across DSB1 and DSB2 and another pair was designed to act as a negative control on Chromosome 22 where there is no A*si*SI sequence (Figure 2B).

4. Locate the required reagents and equipment.

Tissue culture

Timing: 2–3 weeks

The protocol below describes the specific steps using ER-A*si*SI U2OS cells.

5. Maintain the *AsiSI*-ER U2OS cell culture by working under a biosafety cabinet with sterile equipment and using an aseptic technique.

Note: U2OS cells are classified as Biosafety Level 1. These adherent cells can be conveniently maintained in 100 mm culture dishes with routine subculture at 1:5 to 1:10 dilutions when 90% confluence is reached.

Note: Cultures should be restarted from liquid nitrogen stocks once 30 passages have been exceeded.

6. Culture cells at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% charcoal-stripped fetal bovine serum (FBS).

⚠ CRITICAL: Charcoal-stripped FBS is used instead of regular FBS to avoid trace levels of estrogen or tetracycline, which may induce the activity of the *Asi*SI endonucleases in samples where the inducing agents (doxycycline, 4-OHT) are not added.

Note: Do not add chilled media to cells; always use media pre-warmed to 37°C.

Note: The media should be replenished for the cells every 3–4 days.

7. Appropriate selection markers should be added to maintain the stable expression of ER- A*si*SI by the cells. ER-*AsiSI* U2OS cells were grown in a DMEM medium supplemented with 1 μg/mL puromycin.

8. Ensure enough 150 mm dishes (60% confluence) have been seeded for the number of conditions required for the experiment, factoring in dishes for "not induced" controls (where 4-OHT are not added to the media, preventing the induced DSBs) and dishes for any experimental manipulations required (overexpression of other proteins, RNA interference, pharmacological inhibition, etc.).

Before starting the protocol

Timing: 2–3 h

9. Prepare (and chill to 4°C overnight, if necessary) all buffers as required (see materials and equipment) the day before performing the desired steps. While it is preferable to have all buffers freshly prepared, for convenience, some buffers may be stored long-term, as indicated in **Materials and Resources.**
10. For each day, ensure any centrifuges required are pre-chilled to 4°C at least 20 min before they are needed.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
RNase A	Thermo Fisher Scientific	# EN0531
HEPES	Sigma-Aldrich	Cat# H4034
Sodium chloride	Sigma-Aldrich	Cat# S9888
Calcium chloride	Sigma-Aldrich	Cat# C8106
Sucrose	Sigma-Aldrich	Cat# S5016
EDTA	Sigma-Aldrich	Cat# E9884
1 M Magnesium Chloride Solution	Sigma-Aldrich	Cat# 63069
Triton™ X-100	Sigma-Aldrich	Cat# X100
Potassium chloride	Sigma-Aldrich	Cat# P9541
Sodium phosphate dibasic solution	Sigma-Aldrich	Cat# 94046
Dulbecco's Modified Eagle's Medium	Sigma-Aldrich	Cat# D5030
Potassium phosphate monobasic	Sigma-Aldrich	Cat# P5655
4-Hydroxytamoxifen	Sigma	Cat#H7904; CAS: 68047-06-3
Proteinase K solution (20 mg/mL)	Thermo Fisher Scientific	#25530049
N-Lauroylsarcosine sodium salt	Sigma-Aldrich	#L9150
BsrGI-HF	New England Biolabs	# R3575S
BanI	New England Biolabs	# R0118S
Low Melting Point Agarose	Invitrogen™	16520050
Charcoal striped Serum	Sigma	F6765-100ML
Trypsin	Sigma-Aldrich	T4049-500ML
Critical commercial assays		
QIAquick PCR Purification Kit	Qiagen	Cat#28104
DNeasy Blood & Tissue Kit	Qiagen	Cat#69504
Bright Green 2× qPCR MasterMix-ROX	abm	MasterMix-R
Experimental models: Cell lines		
ER-AsiSI- U2OS	Gaelle Legube lab	N/A
Software and algorithms		
Prism	GraphPad	Ver-6
QuantStudio Real-Time PCR Software	Applied Biosystems	Version 1.3
Other		
QuantStudio 6 Flex Real-Time PCR System	Applied Biosystems	RRID: SCR_020239

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
NanoDrop ND-1000 Spectrophotometer	Thermo Scientific	Discontinued, currently replaced by Cat# 840-329700
Water bath	N/A	N/A
Model 200 Rocking Platform	VWR	Cat# 40000-304
Hematology / Chemistry Mixer Model 346	Fisher Scientific	Cat# 14-059-346

MATERIALS AND EQUIPMENT

Buffer recipes for quantifying DNA end resection intermediates by qPCR

ESP Buffer		
Reagent	Final concentration	Amount
Proteinase-K	1 mg/mL	10 mg
EDTA, 0.5 M	0.5 M	10 mL
N-lauroylsarcosine	2%	0.2 mg
CaCl2	1 mM	0.11 gm
Total		**10 mL**
Prepare fresh EPS buffer, Use ESP buffer only when genomic DNA isolated from cells embedded in agarose plug.		

HS Buffer		
Reagent	Final concentration	Amount
NaCl, 5 M	1.85 M	3.7 mL
KCl, 3 M	0.15 M	0.5 mL
MgCl2, 1 M	5 mM	0.05 mL
EDTA, 0.5 M	2 mM	0.04 mL
Tris, pH 7.5, 1 M	4 mM	0.04 mL
Triton X-100, 25%	0.5%	0.2 mL
ddH_2O		5.47 mL
Total		10 mL
Prepare fresh.		

1× PBS		
Reagent	Final concentration	Amount
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	10 mM	1.44 g
KH_2PO_4	1.8 mM	0.24 g
concentrated $HCl_{(aq)}$	use to adjust final pH to 7.4	
ddH_2O	N/A	dissolve solids in, then complete to 1,000 mL final volume
PBS can be stored up to a year upon autoclaving or filter sterilization with a 0.2 μm filter.		

Sequence of qPCR primers: AsiSI sites, DSB1 on Chromosome 1	
Reagent	Sequence 5′-3′
DSB1 335 bp: FW	GAATCGGATGTATGCGACTGATC
DSB1 335 bp: REV	TTCCAAAGTTATTCCAACCCGAT
DSB1 1618 bp: FW	TGAGGAGGTGACATTAGAACTCAGA
DSB1 1618 bp: REV	AGGACTCACTTACACGGCCTTT
DSB1 3500 bp: FW	TCCTAGCCAGATAATAATAGCTATACAAACA
DSB1 3500 bp: REV	TGAATAGACAGACAACAGATAAATGAGACA

Sequence of qPCR primers: AsiSI sites, DSB 2	
Reagent	Sequence 5′-3′
DSB 2 231 bp: FW	ACCATGAACGTGTTCCGAAT
DSB 2 231 bp: REV	GAGCTCCGCAAAGTTTCAAG
DSB 2 918 bp: FW	ACAGATCCAGAGCCACGAAA
DSB 2 918 bp: REV	CCCACTCTCAGCCTTCTCAG
DSB1 1656 bp: FW	CCCTGGTGAGGGGAGAATC
DSB1 1656 bp: REV	GCTGTCCGGGCTGTATTCTA

STEP-BY-STEP METHOD DETAILS

Genomic DNA extraction from "ER-A*si*SI" U2OS

Timing: 2.5 days

On this day, A*si*SI-mediated DSBs are induced in cells, after which genomic DNA is extracted.

Note: This protocol is described for cultured U2OS cells grown in 150 mm dishes, containing ~12–18 × 10^7 cells per dish. Genomic DNA is purified in agarose plugs to prevent DNA breakage or mechanical shearing.

⚠ CRITICAL: Always count cells and use an accurate and consistent cell number for all the samples to obtain the same amount of genomic DNA.

1. In the biosafety cabinet, remove media from each 150 mm dish of cells by tipping the dish towards the vacuum aspirator.
   a. Gently wash the cells once by adding 20 mL 24°C sterile phosphate-buffered saline (PBS).

   Note: Aim the pipettor to the wall of the dish to avoid dislodging the cells.

   b. Remove the PBS by vacuum aspiration.

   Note: These approaches for applying and aspirating solutions from cells should be maintained for the rest of the protocol.

   Note: PBS can be stored for up to 1 year at 4°C upon autoclaving or filter sterilization with a 0.2 μm filter.

2. **Induce DSBs.** To induce DSBs in ER-A*si*SI U2OS cells, replace the dish with 20 mL 37°C (DMEM + 10% charcoal-stripped FBS) containing 300 nM 4-OHT for 4 h.

   ⚠ CRITICAL: Keep the cells incubating at 37°C in a humidified atmosphere with 5% CO_2 for the duration of the induction.

   a. If multiple dishes are being treated, prepare a master mix of pre-warmed DMEM + 10% charcoal-stripped FBS with 300 nM 4-OHT to be split over all the dishes. This will prevent variations in 4-OHT concentration between samples.
   b. Avoid introducing air bubbles to the media. Please avoid vigorous vortexing, rough handling, and harsh pipetting during genomic DNA isolation.

   ⚠ CRITICAL: A 4 h incubation is required to quantify optimum DNA end resection. In our hands, we found that 4 h is better than the 2 h to monitor and measure DNA end resection

during HR repair. To measure initial end resection, 2 h is enough, but 4 h is typically better to measure extensive resection.

c. DSB induction can be verified in cells using immunofluorescence staining against DNA damage response proteins known to form ionizing radiation-induced foci (IRIF) such as γH2AX.

3. Remove media by vacuum aspiration and wash each dish twice with 20 mL of ice-cold PBS.

Note: Keep the bottle of refrigerated PBS on ice for the washes.

4. Harvest cells by using 2 mL of trypsin enzyme and incubate the cells for 5 min at 37°C, monitoring the cells periodically under the microscope.

⚠ CRITICAL: To avoid DNA degradation, keep the cells at 4°C after the trypsin digestion until the cells embedded in LMP agarose plugs are in a lysis buffer.

5. Once the cells round up, stop the trypsin digestion immediately with 5 mL of ice-cold DMEM media containing 10% FBS to prevent cells from lysing.
6. Transfer all cells to 15 mL centrifuge tubes and spin down at 1,000 rpm for 5 min at 4°C.
7. Wash cells once with 10 mL cold PBS, then suspend cells in ice-cold PBS at a concentration of 1.5×10^6 cells/mL.
8. Centrifuge tubes at 1,000 rpm for 5 min at 4°C.
9. Remove the PBS and resuspend the cells in 37°C 0.6% LMP agarose (Invitrogen™) in PBS at a concentration of 1.5×10^6 cells/mL (Figure 1).

Note: 0.6% LMP agarose was prepared by dissolving 0.06 grams of LMP agarose powder in 10 mL PBS, melted by boiling.

⚠ CRITICAL: The agarose solution should be maintained in the melted form at a 37°C water bath. It is advisable to use LMP agarose, as regular agarose solution may begin to harden at 37°C.

10. **Moulding cells.** Drop 50 μL of the cell suspension onto a piece of Parafilm (Pechiney) and place it at 4°C for 5–10 min to solidify. Repeat for the rest of the solution.

⚠ CRITICAL: Cells are embedded in LMP agarose ball before cell lysis to avoid mechanical shearing of genomic DNA during extraction.

a. Mixing cells with agarose solution must be done quickly before the solution begins to solidify.
b. Avoid having air bubbles in the agarose ball.

11. Immediately after the hardening of the agarose balls, transfer all of them, one at a time, into a 1.5 mL Eppendorf tube using a spatula.
12. Add 1 mL of ESP Buffer to the agarose balls in the tube. Ensure that the agarose balls are entirely immersed in the buffer to avoid the repair of DSBs.

Note: Prepare fresh EPS buffer.

13. Leave the tube for 20 h at 16°C on a mini rotary shaker at a rotation rate of 100 RPM.

14. Remove the ESP Buffer carefully from the agarose ball and add 1 mL of HS buffer to the tube for 20 h at 16°C with rotation (100 RPM).

⚠ CRITICAL: Just before use, add Proteinase K to the ESP buffer.

Note: Prepare fresh HS buffer.

15. Remove the HS buffer and add 1 mL ice-cold PBS buffer to the tube, then incubate for 1 h at 4°C with rotation (40 RPM).

⚠ CRITICAL: The temperature of his step is critical and must be done at 4°C to prevent the DNA from degradation.

16. Remove PBS buffer after 1 h incubation and repeat this wash step 6 times.

Note: HS buffer was used to achieve high purity of DNA without degradation of DNA.

17. Place the tube containing agarose balls in a 70°C heat block for 10 min to melt the agarose balls.

⚠ CRITICAL: Gently flick the tube after the gel is melted until you get the solution to the bottom of the tube.

18. Dilute it 15-fold with 70°C ddH2O, mix the solution well, and wait until it cools down to 24°C.

⚠ CRITICAL: Always mix the solution with gentle pipetting and do not vortex the solution to avoid shearing the genomic DNA.

19. Add an equal volume of appropriate 2× rCutSmart™ NEB restriction enzyme buffer.

Note: Store the buffer at 4°C for future use.

20. **Measure DNA concentration.** Determine the DNA concentration for all samples by measuring the absorbance of the samples at 260 nm using a spectrophotometer (we use the NanoDrop ND-1000 spectrophotometer). TE buffer should be used to blank the spectrophotometer.

Pause point: Genomic DNA can be stored at −20°C for at least 12 months. To avoid multiple freeze-thaw cycles upon proceeding with qPCR, the sample can be aliquoted into multiple tubes before freezing.

Measurement of DNA resection by qPCR amplification

Timing: 12–18 h

As mentioned earlier, we measured DNA end resection adjacent to two A*si*SI cut sites on Chromosome 1. To analyze the proximal DSBs, we used a procedure described by Zhou et al. (2014)[2] with the following modifications:

21. We purified gDNA from cells using the Qiagen DNeasy kit according to the manufacturer's instructions and recommendations to add the RNase A incubation step.
22. To digest or mock digest our gDNA, we added 20 units of restriction enzymes *Bsr*GI-HF enzyme for DSB1 or BanI enzyme for DSB2 (New England Biolabs) to a 20 μL gDNA sample

(140 ng in 1× NEB restriction enzyme buffer) as described below at 37°C for 12–16 h. Samples were heat-inactivated at 65°C and analyzed by qPCR.

Reaction Mix of the Enzyme Digestion BsrGI-HF		
Components	Amount	Final concentration
2× rCutSmart™ buffer	5 μL	1×
BsrGI-HF (20,000 units/mL)	1 μL	20 units
Template DNA	140 ng template DNA per reaction	N/A
Nuclease-free ddH_2O	to complete to 10 μL	N/A

Reaction Mix of the Enzyme Digestion BanI		
Components	Amount	Final concentration
2× rCutSmart™ buffer	5 μL	1×
BanI (20,000 units/mL)	1 μL	20 units
Template DNA	140 ng template DNA per reaction	N/A
Nuclease-free ddH_2O	to complete to 10 μL	N/A

23. Use 3 μL of mock or BsrGI / BanI digested samples as templates in 10μL of a qPCR reaction to measure resection at loci 335 nt to Chr 1 DSB and 231 nt to Chr 22 DSB. The following **qPCR** reaction and cycling program was used for all reactions:

SYBR Green qPCR Reaction for resection		
Components	Amount	Final concentration
BrightGreen 2× qPCR MasterMix	5 μL	1×
Forward Primer ("For", 10 μM)	0.3 μL	300 nM
Reverse Primer ("Rev", 10 μM)	0.3 μL	300 nM
Template DNA	30 ng template DNA per reaction	N/A
Nuclease-free ddH_2O	to complete to 10 μL	N/A

qPCR Cycling Conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	95°C	15 s	40 cycles
Annealing	58°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	2 min	1
Hold	4°C	forever	

EXPECTED OUTCOMES

As shown in Figure 2, we selected a restriction site in the DNA region of interest to measure the amount of ssDNA. In Figure 2B, we show DSB1 and BsrGI and DSB2 and BanI. Using three pairs of primers across restriction sites, we performed a qPCR analysis on the mock digestion and digestion of gDNA samples across the restriction sites. There are two possible outcomes: the DNA remains dsDNA at the region of interest or is resected into ssDNA (Figure 2A). If the DNA remains double-stranded, we will have no PCR products because the restriction enzymes are geared toward chewing up dsDNA. On the other hand, if the region of interest is resected into ssDNA, the restriction enzymes will not be able to cut, and therefore, PCR products will be generated (Figure 2A). Other restriction enzymes like BamHI can be used for this application.

QUANTIFICATION AND STATISTICAL ANALYSIS

1. The amount of DNA is represented by the Ct (threshold cycle) value, which denotes the number of PCR cycles needed to reach a set threshold of detecting SYBR Green fluorescence above the background.

   Note: As SYBR Green fluoresces upon binding dsDNA, a plot of fluorescence over the number of PCR cycles represents the amount of DNA over the PCR amplification (Figure 3).

2. QuantStudio Real-Time PCR Software (Applied Biosystems) is used to calculate the Ct values for each qPCR reaction (Figure 3).

   Note: The qPCR reaction can be performed using commercial real-time PCR master mixes. We use an SYBR-based master mix that contains all the reagents for qPCR.[1] Other qPCR protocols require sequence-specific fluorescent probes, such as TaqMan probes can also be used.[2]

3. To calculate ΔCt for each sample, ΔCt is calculated by subtracting the Ct value of the mock-digested sample from the Ct value of the restriction enzyme (BsrGI-HF or Ban1) digested sample. The percentage of ssDNA generated by resection at selected sites (ssDNA %) is calculated using Equation 1.
4. The percentage of ssDNA (ssDNA %) generated by resection proximal to two A*si*SI-induced DSBs within the genome "DSB1", Chr 1: 89231183; "DSB2", Chr 1: 109838221) is calculated with the following equation:

$$ssDNA\ \% = \frac{1}{2^{(\Delta Ct-1)+0.5}} \times 100 \qquad \text{(Equation 1)}$$

ΔCt is calculated by subtracting the mock-digested sample's Ct value from the digested sample's Ct value.

5. Using Graphpad (Prism, Version 9), we generated our statistical analyses and graphs with error bars representing the standard deviation from the mean value. We determined statistical significance using a two-tailed, unpaired, parametric t-test or unpaired t-test.

LIMITATIONS

Ionizing radiation (IR) randomly damages the human genomes in a sequence-independent manner.[23] However, since most of the DSBs induced by IR are complex and contain non-ligatable DNA ends, successful repair of DSB by HR must require DNA end processing before ligation. This assay can measure the resection of DSBs induced by an *Asi*SI endonuclease. Restriction enzymes cause clean and ligatable DSBs, unlike the DSBs generated by IR and reactive oxygen species. Therefore, DNA end processing factors required to restore ligatable ends may not be necessary for DSB repair induced by restriction enzymes. Another limitation of the *Asi*SI system is that it cannot be used for dose-dependent studies like the increasing doses of genotoxins, and the efficacy of nuclease cleavage is highly variable among the different genomic loci.[24] Furthermore, kinetic information for DSB resection may be more challenging to obtain with this assay, as genomic loci are constantly cut by the enzymes and repaired by the cell throughout the induction period. This problem can be overcome by fusing auxin-inducible degron to the ER-A*si*SI, thus allowing the degradation of the A*si*SI enzyme upon auxin addition and controlling the number of DSBs.[25]

The A*si*SI expression system should generate 1,000 randomly distributed sequence-specific DSBs[26] in the human genome.[2,20] Still, only around ~150 DSBs arise upon its translocation into the nucleus upon exposure to 4-OHT.[20] This discrepancy in the number of DSBs could be attributed to two reasons: 1) rapidly repaired DSBs would not be detected in this method, and 2) the cell cycle phase and

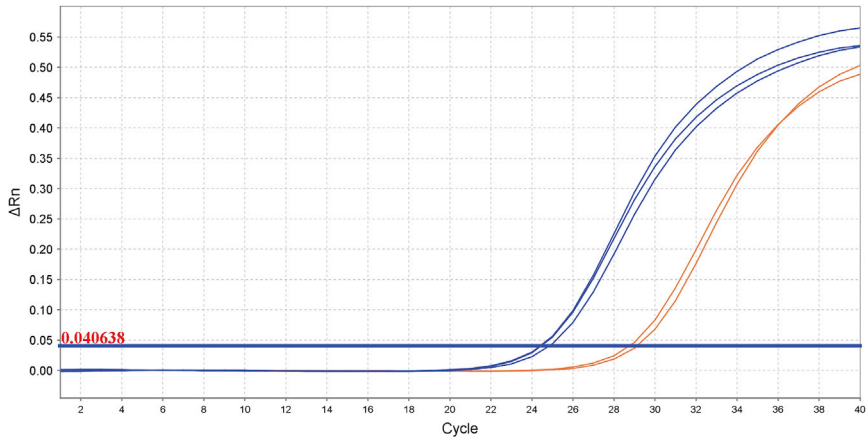

Figure 3. Representative qPCR Results

.qPCR amplification blot in ER-*Asi*SI U2OS-DSB reporter cells from QuantStudio Real-Time PCR Software. The horizontal blue line is the threshold the software sets to determine the Ct value for each reaction curve. qPCR reactions for samples in triplicate are represented in different colours: A*si*SI damage digested with *Bsr*GI enzyme (blue) and *Asi*SI damage mock (brown).

the chromatin structure at each site would affect the efficiency of DSB resection. This quantitative method, although helpful, is limited by the requirement for sequence specificity of the cut site. Consequently, it becomes challenging to determine resection efficiency at A*si*SI inaccessible sites on the human genome.

TROUBLESHOOTING

Problem 1

Problems with PCR amplification.

Potential solution

Problems with PCR amplification may be due to the non-specific or star effect of restriction enzyme digestion. Non-specific or star effects of enzymes can be prevented using high-fidelity restriction enzymes and optimum buffer.

Problem 2

A low signal of qPCR (products) or Ct value is high.

Potential solution

There are many reasons why sometimes we can get a low signal of qPCR, some of which are: reduced or loss of A*si*SI expression from stably expressing U2OS cells and DNA shearing during the isolation of genomic DNA. To avoid these issues, always ensure that you grow your ER-A*si*SI reporter U2OS cell line in the appropriate selection media to maintain the expression of A*si*SI. You should also carefully and gently embed your cells in agarose before lysis to avoid DNA shearing that results in a low signal. Vortexing should be avoided to preserve genomic DNA from shearing.

Problem 3

No or low DSBs induction.

Potential solution

There are many reasons why we sometimes can't get a DSB signal upon adding 4-OHT. These could include loss of expression of the A*si*SI enzyme in the stable cell line, the concentration of the 4-OHT not being sufficient to induce the translocation of the A*si*SI enzyme to the nucleus, or the 4-OHT incubation time not being long enough. To avoid these issues, always check the expression levels of

the A*si*SI enzyme. This can be done either by immunostaining of the A*si*SI enzyme in the stable cell lines or by monitoring the induction of DSBs using a phospho-specific H2AX antibody (via immunostaining or Western Blot analysis). Always grow the ER-A*si*SI U2OS cell line in the appropriate selection media to maintain the expression of the A*si*SI enzyme. Moreover, incubating the ER-A*si*SI U2OS cell line in media supplemented with 4-OHT for a longer time (approximately 8–12 h) will increase the number of DSBs induced.

Problem 4

High background in the non-damaged samples.

Potential solution

High background results from DSB induction in samples not treated with 4-OHT, which could be potentially due to steroid contaminants in the fetal bovine serum (FBS). Steroid contaminants could result in translocation of the AsiSI enzyme to the nucleus and induce DSBs. To overcome this problem, grow the ER-AsiSI U2OS cells in DMEM medium containing charcoal-stripped 10% FBS.

Problem 5

Amplification is detected for negative control samples during qPCR.

Potential solution

If amplification does occur, this suggests the PCR master mix, tubes, or primers may be contaminated with genomic DNA, or that the primers chosen are dimerizing and being amplified by PCR ("primer-dimers"). As SYBR Green binds to nucleic acids without sequence specificity, an amplification of SYBR Green fluorescence upon qPCR may not mean the amplicon of interest has been amplified. Further validation may be required, such as resolving the PCR product by agarose gel electrophoresis and verifying the size of the amplicon is as expected. The amplicon can also be sequenced by the Sanger method to ensure the correct genomic locus is being amplified. Amplicons also have a higher melting temperature than primer dimers. This is a result of increased thermostability, as an amplicon is typically longer than a primer dimer and both strands are complementary to each other. In contrast, primer dimers exhibit low complementarity to each other and are much shorter (30–50 bp in size).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and fulfilled by the lead contact, Ismail H. Ismail (iismail@ualberta.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

A.K.S. established the protocol and wrote the first draft of the manuscript. A.M.F. and I.H.I. extensively edited the first draft and generated the final version. J.Y.H.A. made figures.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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