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Supplemental Information

Genome-wide Nucleotide-Resolution Mapping of DNA Replication Patterns, Single-Strand Breaks, and Lesions by GLOE-Seq

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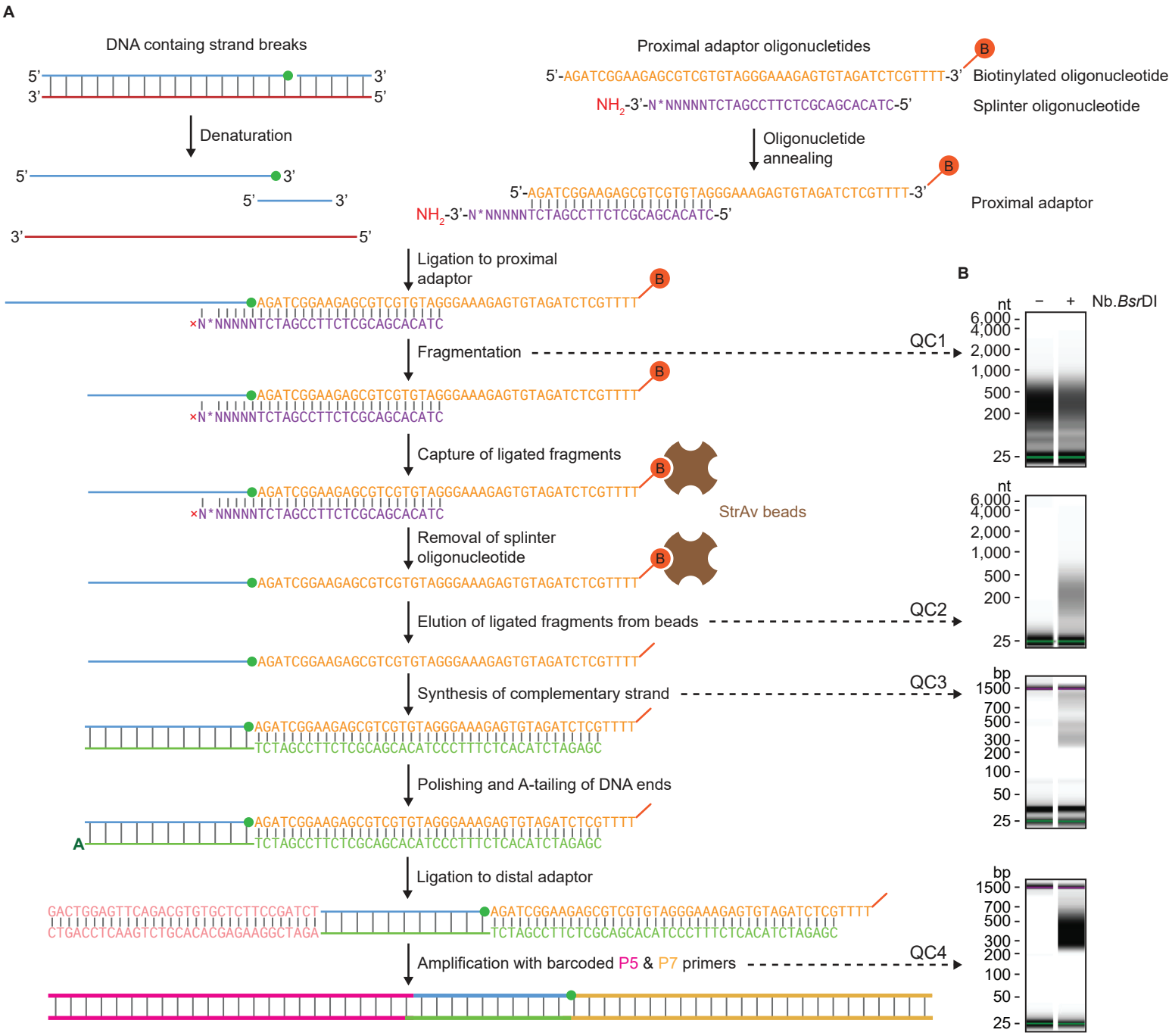

Figure S1. Related to Figure 1. GLOE-Seq workflow

A. Schematic step-by-step illustration of the GLOE-Seq procedure, indicating critical oligonucleotide sequences and modifications. Green circle: ligatable 3'-OH terminus; red circle (B): biotin-dT; -NH_2 (or x): 3'-amino modification; *: phosphorothioate bond; StrAv: Streptavidin.

B. Quality control samples were analysed on an Agilent TapeStation: QC1 – RNA ScreenTape of denatured genomic DNA (1 µL) after ligation of the proximal adaptor and fragmentation. QC2 – RNA ScreenTape of ligated single-stranded genomic DNA fragments (1 µL) captured on Streptavidin beads. QC3 – High Sensitivity DNA ScreenTape of captured DNA (2 µL) after second-strand synthesis. QC4 – High Sensitivity DNA ScreenTape of the libraries (1 µL) after ligation of the distal adaptor and amplification with Illumina primers P5 and P7.

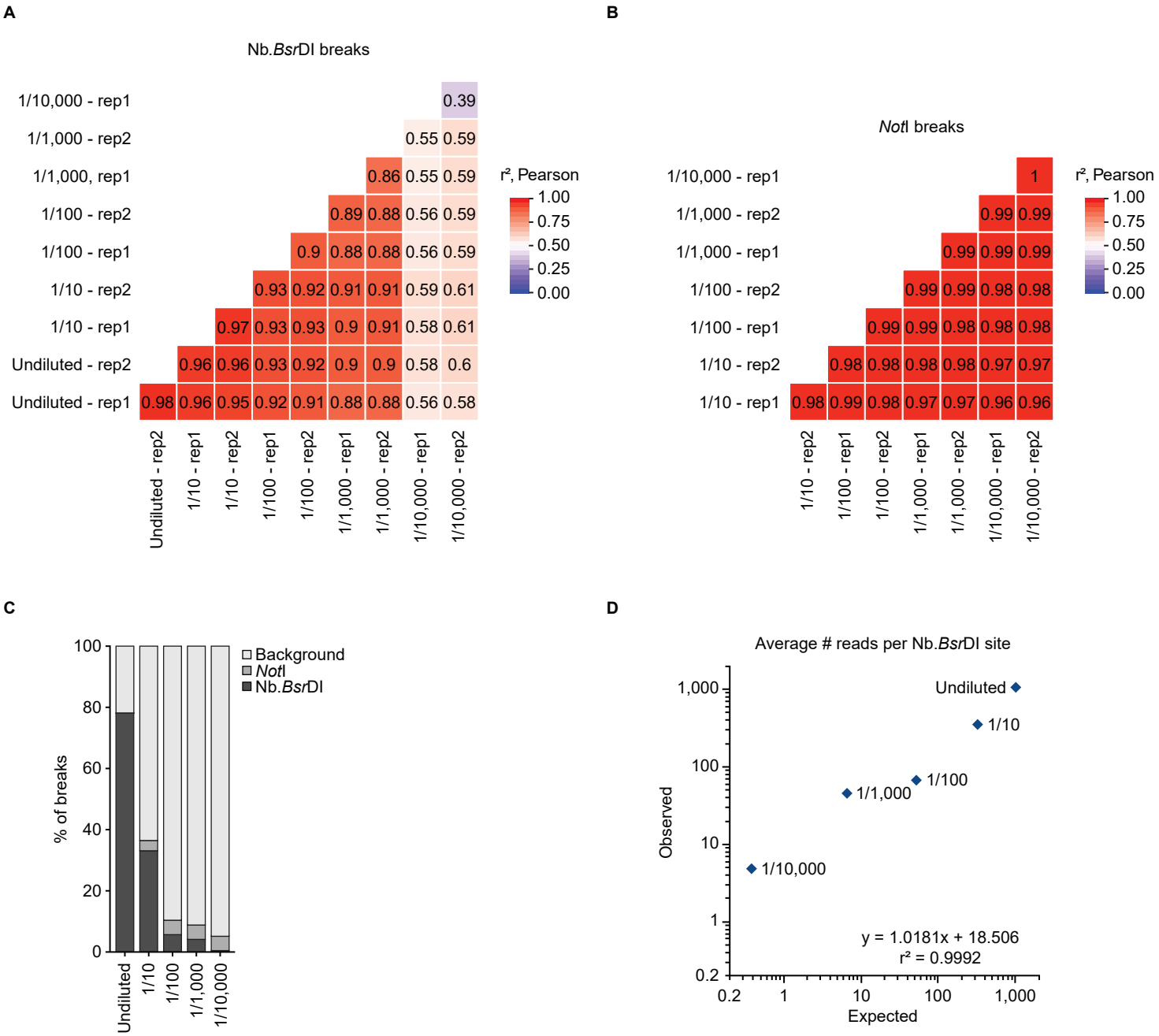

Figure S2. Related to Figure 1. Assessment of the sensitivity of GLOE-Seq

A. GLOE-Seq profiles of Nb.*Bsr*DI sites in independent replicates are highly correlated up to a dilution of 1:1000 (r^2: Pearson correlation coefficients).

B. GLOE-Seq profiles of *Not*I sites in independent replicates are highly correlated across all samples.

C. SSBs can reliably be detected at high dilution. The distribution of GLOE-Seq signals is shown for a series of Nb.*Bsr*DI/*Not*I-digested DNA diluted at the indicated ratios with *Not*I-digested DNA. The undiluted DNA was only treated with Nb.*Bsr*DI.

D. The observed numbers of reads per Nb.*Bsr*DI site correlate well with the expected numbers across all dilutions. The expected numbers were calculated as follows: $\frac{\#\text{ reads mapped to Nb.}BsrDI\text{ \& }NotI\text{ sites}}{\#\text{ Nb.}BsrDI\text{ 3'-ends}} \times \frac{\#\text{ Nb.}BsrDI\text{ 3'-ends} \times \text{Dil. factor}}{\#\text{ }NotI\text{ 3'-ends} + \#\text{ Nb.}BsrDI\text{ 3'-ends} \times \text{Dil. factor}}$, where # Nb.BsrDI 3'-ends = 6271, # NotI 3'-ends = 80 and Dil. factor = [1, 0.1, 0.01, 0.001, 0.0001].

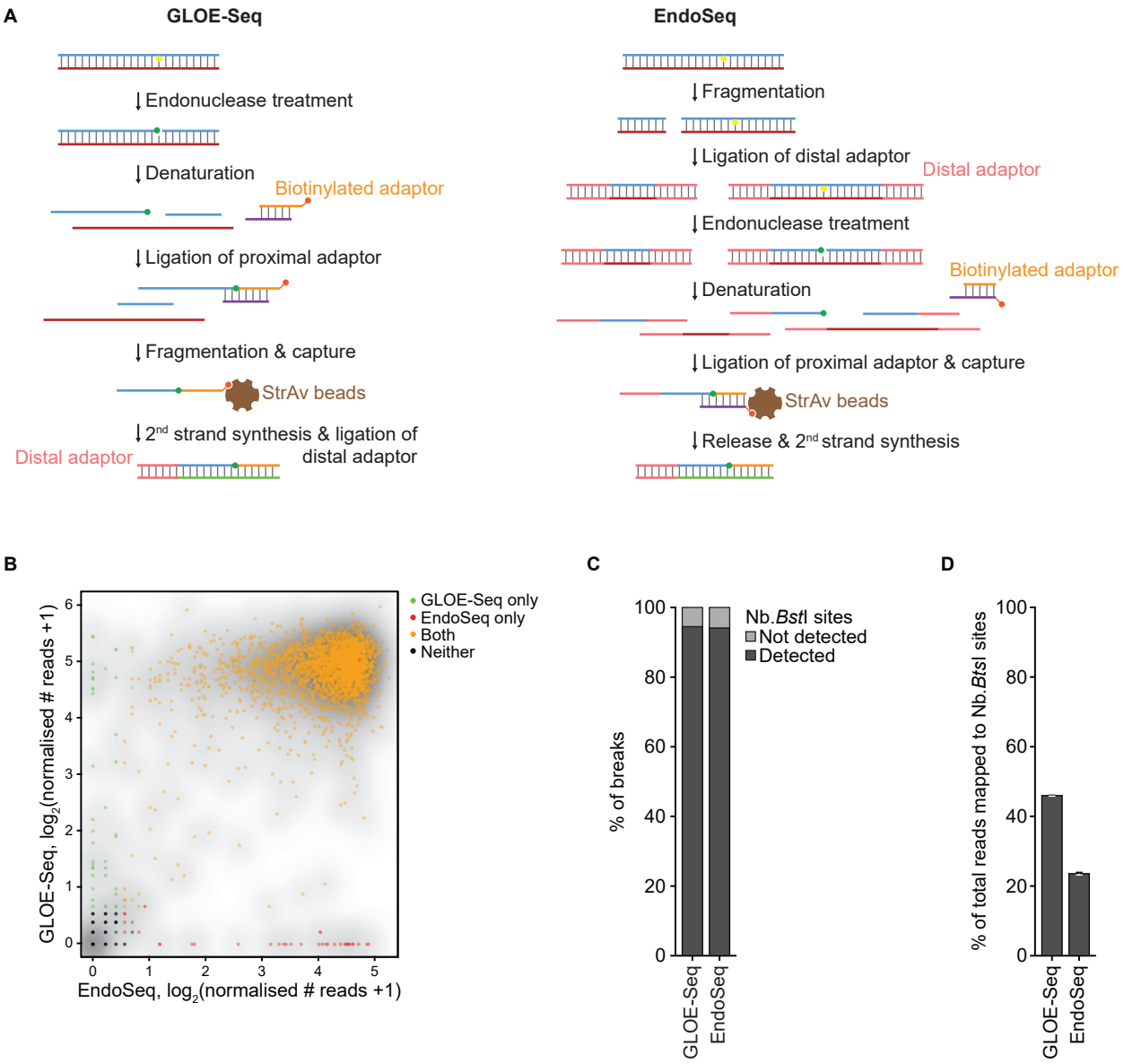

Figure S3. Related to Figure 2. Comparison of GLOE-Seq with a published EndoSeq data set

A. GLOE-Seq versus EndoSeq workflow. Yellow circle: base lesion; green circle: ligatable 3'-OH terminus; red circle: biotin.

B. A scatter plot shows the normalised numbers of reads at all Nb.*Bts*I sites for samples processed by GLOE-Seq in comparison to a published EndoSeq data set (Reijns et al., 2015), grouped by peak calling. **C.** GLOE-Seq and EndoSeq detect a similar percentage of Nb.*Bts*I sites.

D. Comparison of the percentage of total reads mapped to predicted Nb.*Bts*I sites by GLOE-Seq versus EndoSeq.

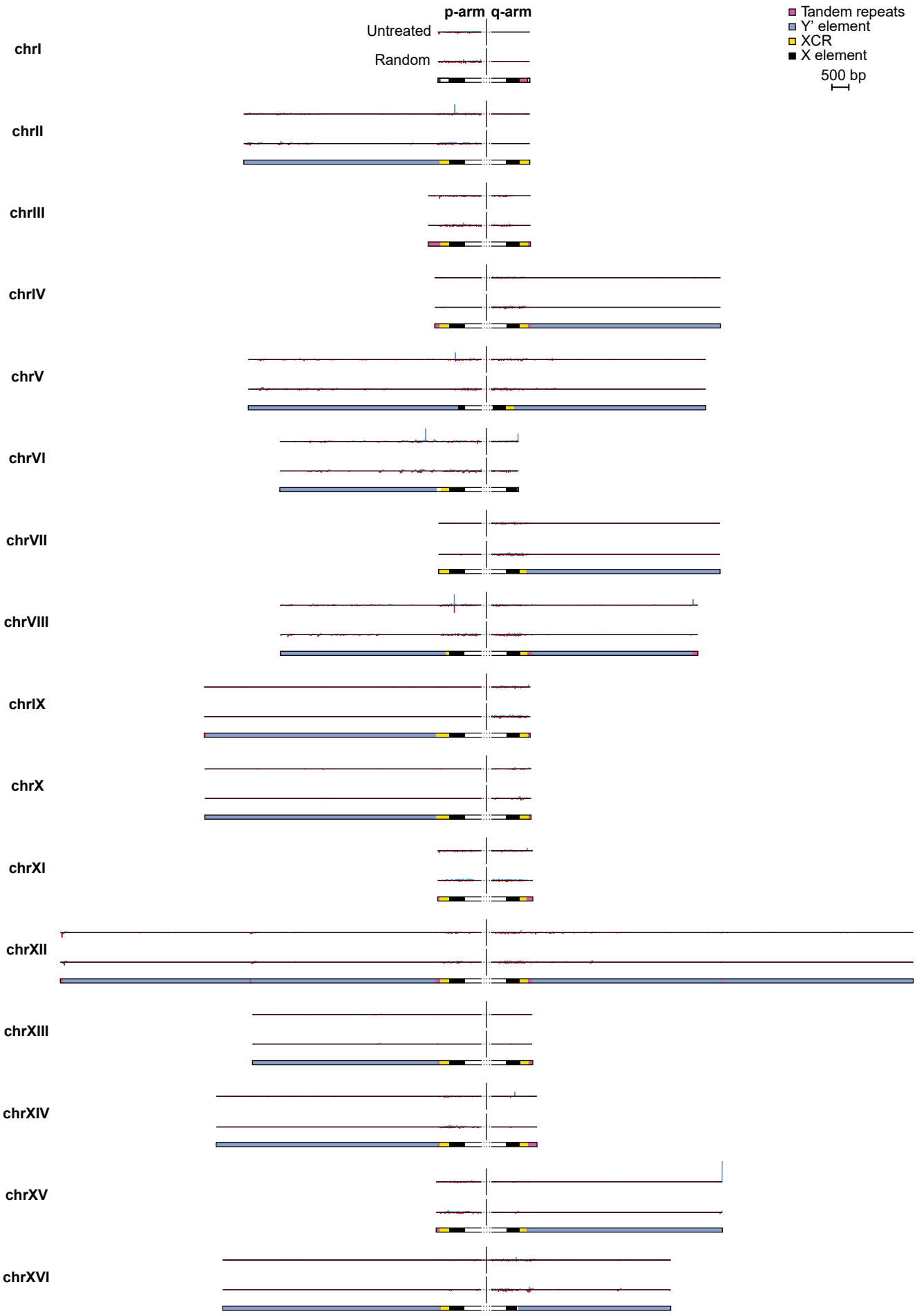
p-arm
q-arm
Untreated
Random
Tandem repeats
Y' element
XCR
X element
500 bp
chrI
chrII
chrIII
chrIV
chrV
chrVI
chrVII
chrVIII
chrIX
chrX
chrXI
chrXII
chrXIII
chrXIV
chrXV
chrXVI

Figure S4. Related to Figure 3. Distribution of breaks at yeast chromosome ends

Breaks are enriched at several yeast chromosome ends. Strand-specific GLOE-Seq signals of untreated and randomly fragmented DNA are shown in the subtelomeric regions of all 16 chromosomes. Defined sequence elements (X, XCR, Y') are indicated below each plot. The telomeric tandem repeats (magenta) are indicated as far as they are covered by uniquely mapping sequencing reads. Breaks are shown at the same scale as in Figure 3b.

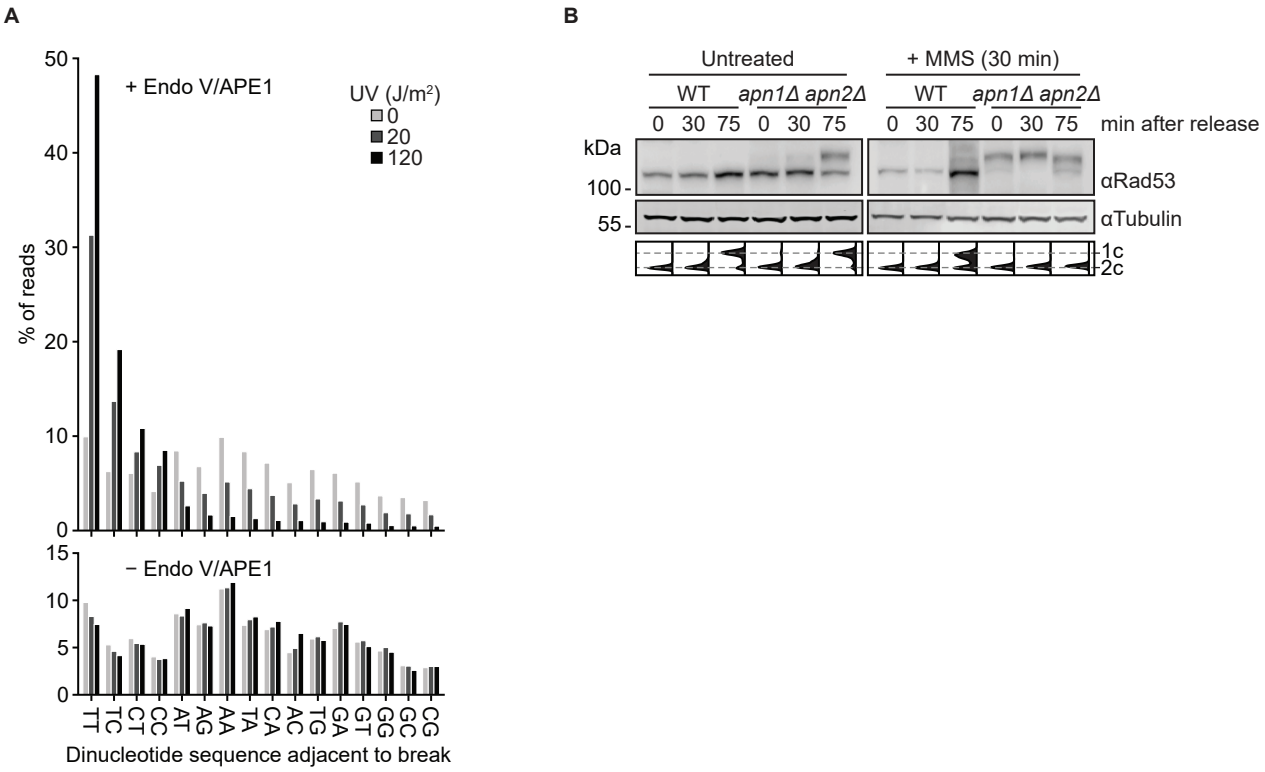

Figure S5. Related to Figure 3. GLOE-Seq analysis of DNA lesions and repair intermediates in yeast

A. GLOE-Seq detects UV-induced pyrimidine dimers in yeast. Plots show relative frequencies of dinucleotide sequences adjacent to the detected strand breaks. Data are identical to those shown in Figure 3D, but visualised in a different manner to show the percentages of individual sequences.

B. Checkpoint activation and cell cycle profiles in response to MMS treatment. Western blot images show Rad53 and its phosphorylated forms as well as tubulin (loading control) in total extracts of WT and *apn1Δ apn2Δ* cells from the experiment shown in Figures 3D and E. Corresponding flow cytometry profiles are shown beneath each lane.

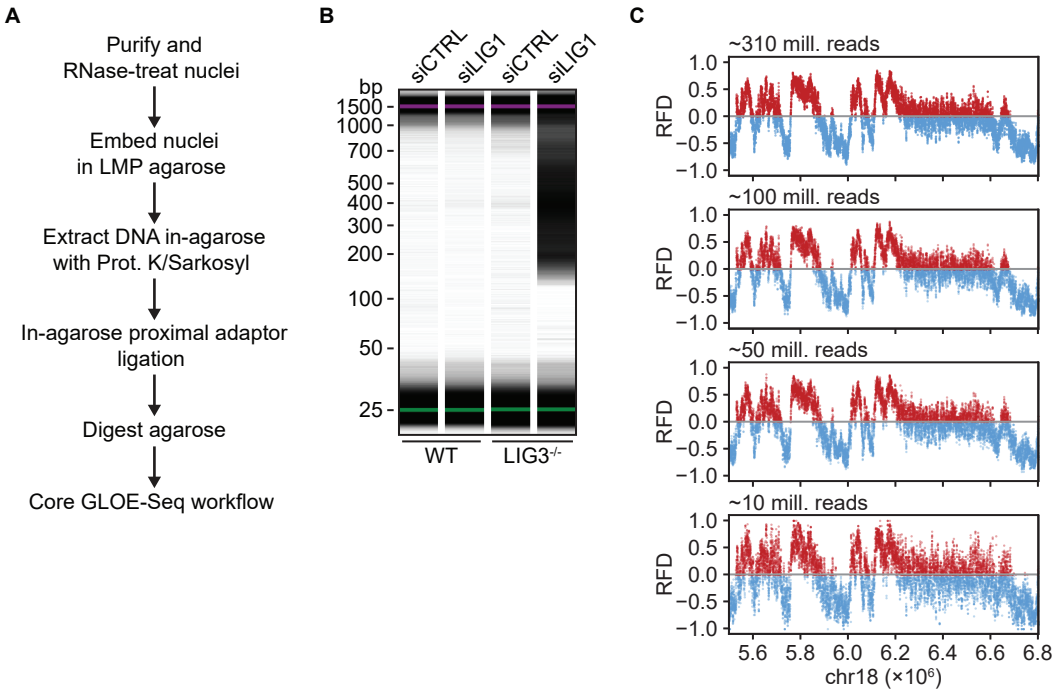

Figure S6. Related to Figure 6. GLOE-Seq analysis in human cells

A. Workflow for the preparation of mammalian DNA from agarose-embedded nuclei (see step-by-step protocol for details). LMP: low-melting point.

B. Size distribution and amount of DNA recovered after second strand synthesis in the core GLOE-Seq workflow (see Figure S1A) from HCT116 WT and LIG3$^{-/-}$:mL3 cells treated with either an unspecific (CTR) or a Ligase 1-specific siRNA (LIG1).

C. Effects of downsampling on RFD plots of GLOE-Seq data from HCT116 cells under conditions of DNA Ligase 1 inactivation (WT + siLIG1).

Methods S1. Related to Star Methods.

Step-by-step protocol for the preparation of GLOE-Seq libraries

Introduction

GLOE-Seq is a next generation sequencing method for the genome-wide mapping of 3'-OH termini, either resulting from single- or double-strand breaks or introduced by enzymatic conversion of lesions or modified nucleotides. This protocol provides step-by-step instructions starting with the isolation of genomic DNA up to the amplification of libraries in preparation for sequencing and includes explanatory notes, quality controls and a troubleshooting guide. Separate instructions are provided for isolation of DNA from budding yeast, accomplished by gentle lysis of spheroplasts, and from mammalian cultured cells, where lysis is induced in agarose-embedded nuclei (steps 1-28). The subsequent procedure (steps 29-50) follows a common protocol that is independent of the source of DNA.

Table of Contents **Page**

A. Reagents and Consumables

Commercial reagents

PEG 8000 (Sigma-Aldrich, 89510)
Zymolyase 20T (AMS Biotechnology, 120491-1)
β-mercaptoethanol (Sigma-Aldrich, M3148)
AMPure XP beads (Beckman Coulter, A63881)
RNase A (Sigma-Aldrich, 10109169001)
RNase H (New England Biolabs, M0288S)
Sorbitol (Sigma-Aldrich, S1876)
Sodium chloride (Sigma-Aldrich, S3014)
Sodium hydroxide (Sigma-Aldrich, S8045)
Sodium citrate (Sigma-Aldrich, W302600)
Ethanol (Fisher Scientific, 15643690)
Tris base (Sigma-Aldrich, T4661)
EDTA (Sigma-Aldrich, E6758)
20% (*w/v*) SDS (Sigma-Aldrich, 05030)
10× T4 DNA ligase buffer (New England Biolabs, B0202S)
T4 DNA ligase, 20,000,000 U/mL (New England Biolabs, M0202T)
Q5® High-Fidelity DNA polymerase with reaction buffer (New England Biolabs, M0491)
NEBNext® Ultra™ II DNA Library Prep kit for Illumina® (New England Biolabs, E7645)
Phusion Flash high-fidelity PCR master mix (Thermo Fisher Scientific, F-548)
Deoxynucleotide (dNTP) Solution Mix, 10 mM (New England Biolabs, N0447S)
Dynabeads™ MyOne™ Streptavidin C1 (Life Technologies, 65001)
Potassium acetate (VWR International, 236497-500G)
High Sensitivity D1000 ScreenTape (Agilent Technologies, 5067-5584)
High Sensitivity D1000 ScreenTape reagents (Agilent Technologies, 5067-5585)
RNA ScreenTape (Agilent Technologies, 5067-5576)
RNA ScreenTape sample buffer (Agilent Technologies, 5067-5577)
RNA ScreenTape ladder (Agilent Technologies, 5067-5578)
Qubit™ dsDNA HS Assay Kit (Invitrogen, Q32854)
Agilent Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies, 5067-4646)
NextSeq 550 System High-Output Kit (Illumina, 20024906)
NuSieve™ GTG™ Agarose (Lonza, 859081)
Proteinase K (Roche, 3115801001)
Sarkosyl (Sigma-Aldrich, 61743)
β-Agarase I, 1000 U/mL (New England Biolabs, M0392S)
Trypan blue solution, 0.4% (*w/v*) (Thermo Fisher Scientific, 15250061)
Phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, P7626)

Consumables

Microtubes for Bioruptor® Pico, 0.65 mL (Diagenode, C30010011)

Filter Tips 10/20 μL, 20 μL, 200 μL, 1000 μL (TipOne, S1120-3810, S1120-1810, S1120-8810, S1126-7810)

Centrifuge Tubes (Corning® 50 mL PP, 430829)

Microcentrifuge Tubes, 2 mL (Eppendorf, 0030120094)

DNA LoBind Tubes, 1.5 mL (Eppendorf, 0030108051)

Axygen 0.2 mL 8-strip PCR Tubes (Thermo Fisher Scientific, 14-222-252)

Qubit Assay Tubes (Life Technologies, Q32856)

Tubes, 12 mL (Sarstedt, 60.9922.937)

Buffers and solutions

Water	sterile, deionized (e.g. Milli Q-purified)
0.5 M EDTA	pH 8.0
Y1 buffer	1 M sorbitol 100 mM EDTA 14 mM β-mercaptoethanol (added immediately before use)
Lysis buffer	50 mM Tris-HCl, pH 8.0 50 mM EDTA
TE buffer	10 mM Tris-HCl, pH 8.0 1 mM EDTA
5 M potassium acetate	
1% (*w/v*) SDS	
70% (*v/v*) ethanol	
10 mM Tris-HCl	pH 8.5
Bind & Wash buffer	10 mM Tris-HCl, pH 8.5 2 M NaCl
50% (*w/v*) PEG 8000	
5 M NaCl	
20 mM NaOH	
1× SSC	150 mM sodium citrate, pH 7.0 15 mM NaCl
Nuclear isolation buffer	10 mM Tris, pH 8.0 50 mM NaCl 50 mM EDTA 0.34 M sucrose 10% (*v/v)* glycerol 0.1% (*v/v)* Triton X-100
PBS, pH 7.4	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4
Proteinase K solution	1 mg/mL Proteinase K 1% (*w/v)* sarkosyl 125 mM EDTA, pH 9.0
Plug wash buffer	10 mM Tris-HCl, pH 8.0 100 mM NaCl 1 mM EDTA

Oligonucleotides:

Proximal adaptor:	40 μM oHU3898 40 μM oHU3899 60 mM NaCl	(annealing programme: see below)
Distal adaptor:	40 μM oHU3791 40 μM oHU3792 60 mM NaCl	(annealing programme: see below)

Oligonucleotides were HPLC-purified (IDT).

Name	**Sequence (5' – 3')**
3898	CTACACGACGCTCTTCCGATCTNNNNN*N-NH_2 (*: phosphorothioate bond, IDT code *; NH_2: 3'-amino modification, IDT code /3AmMO/)
3899	PO_4-AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGTTTT-Bio (PO_4: 5'-phosphorylation, IDT code /5Phos/; T-Bio: 3'-biotin-dT, IDT code /3BiodT/)
3790	CGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
3791	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
3792	GATCGGAAGAGCACACGTCTGAACTCCAGTC
P5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
P7	CAAGCAGAAGACGGCATACGAGAT$(X)_6$GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Annealing Programme:

T (°C)	**min:s**	**ΔT (°C)**	**Ramp rate (°C/s)**
95	05:00	-20	0.1
75	00:01	-20	0.1
55	00:01	-20	0.1
35	00:01	-10	0.1
25	∞	-	6.0

B. Equipment

Stereomicroscope (Leica DM1000 LED, Leica Biosystems)
Bioruptor® Pico (Diagenode, B01060010)
Tube holders for Bioruptor®, 0.5/0.65 mL (Diagenode, B01200043)
Agilent 2200 TapeStation System (Agilent Technologies, G2964AA)
Agilent 2100 Bioanalyzer Instrument (Agilent Technologies, G2939AA)
Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, cat. #Q32866)
Thermocycler (Biometra TRIO, 070-723)
Magnetic rack (DynaMag™-2 Magnet, Thermo Fisher Scientific, 12321D)
Rotating wheel (Stuart, SB2)
Benchtop Centrifuge (Heraeus™ Multifuge™ X3R, VWR International, 97040-234)
Refrigerated Centrifuge (Heraeus Fresco 21, Thermo Fisher Scientific, 75002555)
Centrifuge (Sorvall™ RC 6 Plus, Thermo Fisher Scientific, 36-101-0816)
Illumina NextSeq 550 System

C. Protocol

Steps y1 – 22: Extraction of genomic DNA from budding yeast **(Note 1)**

1. Collect ca. 6×10^9 cells (300 OD_{600}) by centrifugation in a 50 mL tube (2,000×*g*, 5 min), resuspend the pellet in 25 mL of 10 mM Tris-HCl pH 8.5, and centrifuge again. Discard the supernatants.
2. Resuspend the pellet in 4 mL of Y1 buffer and add 400 μL of Zymolyase 20T (10 mg/mL). Incubate at 30°C and monitor formation of spheroplasts under a microscope. **(Note 2)**
3. Pellet the spheroplasts at 2,000×*g* for 5 min, wash once with 4 mL of Y1 buffer without β-mercaptoethanol and resuspend in 4.75 mL of lysis buffer. Initiate lysis by adding 250 μL of 20% SDS and incubating at 37°C until the solution turns transparent. **(Note 3)**
4. Centrifuge the sample at 5,000×*g* for 10 min and transfer the supernatant (by pouring) to a fresh 50 mL tube. **(Note 4)**
5. Add 1.666 mL of 5 M potassium acetate, invert the tube to mix and incubate on ice for 45-60 min.
6. Centrifuge the sample at 17,000×*g* for 15 min at room temperature, transfer the supernatant (by pouring) to a fresh 50 mL tube and centrifuge again at 17,000×*g* for 15 min. **(Note 5)**
7. Transfer the supernatant to a fresh 50 mL tube, add 19.5 mL of 100% ethanol and swirl the tube to precipitate the DNA.
8. Centrifuge the sample at 8,000×*g* for 15 min and discard the supernatant.
9. Rinse the pellet by adding 25 mL of 70% ethanol and centrifuging again at 8,000×*g* for 10 min. Discard the supernatant.
10. Add 4 mL of 70% ethanol to the pellet and distribute the suspension with a wide-bore pipette (cut the tip off a 1 mL pipette tip) into two 2 mL microcentrifuge tubes. **(Note 6)**
11. Centrifuge at 21,100×*g* for 10 min and discard the supernatants.
12. Air-dry the pellets and resuspend in 245 μL of TE each by incubation overnight at 4°C.
13. Add 5 μL of 5 M NaCl and 2.5 μL of RNase A to each DNA sample and incubate at 37°C for 1 h.
14. Add 1.5 mL of 100% ethanol to each tube, invert to mix, centrifuge at 21,100×*g* for 10 min and discard the supernatant.
15. Rinse the pellets once with 1 mL of 70% ethanol.
16. Air-dry the pellets and resuspend overnight at 4°C by gentle rocking in 250 μL of 10 mM Tris-HCl pH 8.5.
17. Add 250 μL AMPure beads to a 1.5 mL DNA LoBind microcentrifuge tube and use a magnetic rack to remove 125 μL of the storage buffer.
18. Add the DNA sample (250 μL) to the bead suspension and incubate at room temperature for 10 min.
19. Using a magnetic rack, remove and discard the supernatant.
20. While keeping the tube on the magnetic rack, rinse the beads twice with 500 μL of 70% ethanol each.
21. Remove the supernatant and add 125 μL of 10 mM Tris-HCl pH 8.5.
22. Elute the DNA by incubating at room temperature for 10 min and collecting the supernatant using a magnetic rack.

#Pause point – At this point, the extracted genomic DNA can be stored at 4°C for up to 6 months. When mapping lesions not involving strand breaks, additional treatments (followed by renewed purification using AMPure beads) are applied at this stage to generate the corresponding 3'-OH termini, e.g.:

- pyrimidine dimers: T4 Endonuclease V + AP endonuclease (APE1)
- abasic (AP) sites: AP endonuclease (APE1)
- ribonucleotides: RNAase H

Steps y23 – 28: Denaturation and ligation of 3'-OH termini (yeast)

23. Incubate 2.5 μg of genomic DNA (treated as described above if relevant) at 95°C for 10 min, followed by incubation on slushy ice for 5 min to denature the DNA.
24. Set up the ligation reaction in a PCR tube in the specified order at room temperature:

Denatured DNA	2.5 μg
10× T4 DNA ligase buffer	6.5 μL
Proximal adaptor	3.55 μL **(Note 7)**
50% PEG 8000	19.5 μL
T4 DNA ligase	3 μL

Adjust the total volume to 65 μL with water and incubate in a thermocycler as follows:

T (°C)	min:s
25	60:00
22	60:00
22	60:00
16	∞

25. Add 100 μL of AMPure beads to a 1.5 mL DNA LoBind microcentrifuge tube and remove the storage buffer, using a magnetic rack.
26. Add 35 μL of 5 M NaCl and 35 μL of water to the ligation mix. Transfer the entire mix to the tube containing the AMPure beads.
27. Incubate at room temperature for 5 min. Using a magnetic rack, remove the supernatant from the beads and rinse the beads twice with 500 μL of 70% ethanol while keeping the tube on the rack.
28. Elute the DNA from the beads by adding 103 μL of water and incubation at room temperature for 5 min. Transfer 100 μL to a fresh tube, using a magnetic rack.

#Pause point – At this point, the ligated DNA fragments can be stored at -20°C for up to 3 days.

Steps m1 – 21: Isolation of genomic DNA from mammalian cell culture

1. Harvest up to 10 million cells by trypsinization.
2. Wash the cells twice in ice-cold PBS + 5 mM EDTA pH 8.0.
3. Resuspend cells in 1 mL of nuclear isolation buffer and incubate them on ice for 10 min to dissolve the plasma membrane.
4. Harvest nuclei by centrifugation in a swing-out rotor at 1,200×*g* for 3 min at 4°C and wash them once with 1 mL of nuclear isolation buffer. Loosen the pellet by inversion or tapping, rather than pipetting.

5. Mix the nuclei with nuclear isolation buffer supplemented with RNase A at a final concentration of 100 μg/μL at a ratio of 100 μL of buffer per 20 μL of nuclear pellet and incubate at 37°C for 15 min.
6. Count the nuclei with a haemocytometer in the presence of Trypan blue (0.2% (*w/v*) final).
7. To prepare agarose plugs, harvest the desired number of nuclei (~700,000 per plug) by centrifugation in a swing-out rotor at 1,200×*g* for 3 min.
8. Remove all but ~10 μL of supernatant, taking care not to disturb the pellet of nuclei.
9. Resuspend the nuclei (by tapping) at a density of about 700,000 nuclei per 22.5 μL of suspension in PBS + 25 mM EDTA pH 8.0, prewarmed to 50°C.
10. Mix the suspension 1:1 (*v/v)* with molten 1.2% (*w/v*) low-melting point agarose prepared in PBS + 25 mM EDTA pH 8.0, equilibrated to 50°C.
11. Immediately pipette the cell-agarose mixture into the wells of a 45 μL plug mould. **(Note 8)**
12. Allow the agarose to solidify for 1 h at 4°C.
13. Combine the plugs into 12 mL tubes filled with 7-8 mL of Proteinase K solution and incubate overnight at 42°C (no more than 3 plugs per tube).
14. Exchange the Proteinase K solution for a fresh aliquot of 7-8 mL and perform a second overnight incubation at 42°C.
15. Wash the plugs three times with 7-8 mL of plug wash buffer + 1 mM PMSF for 10 min each.
16. Wash the plugs once with 7-8 mL of plug wash buffer + 1 mM PMSF for 1 h.
17. Wash the plugs once with 7-8 mL of plug wash buffer for 1 h.
18. Transfer each plug to a separate 1.5 mL DNA LoBind tube and wash twice with 1 mL of 10 mM Tris-HCl pH 8.0 + 0.1 mM EDTA pH 8.0.
19. Wash each plug once with 1 mL of 10 mM Tris-HCl pH 8.0 + 0.1 mM EDTA pH 8.0 for 30 min.
20. Wash each plug twice with 1 mL of 0.1 mM EDTA pH 8.0 for 15 min each.
21. Quickly rinse the plugs with water and remove as much residual water as possible.

#Pause point – At this point, the plugs can be stored at 4°C for up to 3 days. When mapping lesions not involving strand breaks, additional treatments could be applied at this stage to generate the corresponding 3'-OH termini, e.g.:

- pyrimidine dimers: T4 Endonuclease V + AP endonuclease (APE1)
- abasic (AP) sites: AP endonuclease (APE1)
- ribonucleotides: RNAase H

Inactivation of the enzymes after this step could be accomplished by heat denaturation in the presence of EDTA of by Proteinase K treatment.

Steps m22 – 28: Denaturation and ligation of 3'-OH termini (mammalian cells)

22. Denature the DNA by incubation at 95°C for 4 min and quenching on slushy ice for 5 min.
23. Melt the agarose at 65°C for 5 min and equilibrate to 37°C for 15 min.
24. Thoroughly mix the agarose, by tapping, with 17 μL of a prewarmed ligation mix consisting of:

10× T4 DNA ligase buffer	6.5 μL
Proximal adaptor	3.5 μL
Water	7 μL

and with 3 μL of T4 DNA ligase (added last).

25. After an overnight incubation at 16°C, supplement the ligation reaction with 2 μL of 0.5 M EDTA pH 8.0 and 30 μL of water.
26. Incubate the mixture at 65°C for 10 min to inactivate the ligase and melt the agarose.
27. Allow the agarose to equilibrate to 42°C for 20 min, supplement with 3 μL of β-agarase and incubate at 42°C for 3-4 h.
28. Adjust the sample volume to 100 μL with water and transfer to a 0.5 mL Bioruptor tube.

Steps 29 – 37: Fragmentation and capture of biotinylated ssDNA

29. Sonicate the eluate from step 28 (100 μL) to a fragment size of 200 nt, using a Bioruptor Pico (4 cycles for yeast DNA, 16 cycles for mammalian DNA, 30 s on/30 s off). **(QC sample 1) (Note 9)**
30. Clean up the DNA by two rounds of binding to an equivalent of 100 μL of AMPure beads, each time adding NaCl and PEG 8000 to final concentrations of 0.9 M and 7.5% (*w/v*), respectively, and elution in 100 μL of water as described in steps y27-28. **(Note 10)**
31. Transfer the sonicated DNA to a microfuge tube containing 20 μL of Streptavidin MyOne C1 Dynabeads (prewashed once with 1 mL Bind & Wash buffer).
32. Incubate for 15 min at room temperature on a rotating wheel.
33. Pulse-spin the tube and collect the beads on a magnetic rack. Remove the supernatant.
34. Wash the beads twice with 50 μL of 1× SSC buffer each for 5 min at room temperature on a rotating wheel. **(Note 11)**
35. Wash the beads once with 25 μL of 20 mM NaOH for 10 min at room temperature on a rotating wheel.
36. Wash the beads briefly with 100 μL water.
37. Elute the DNA by adding 16 μL of water, incubating at 95°C for 5 to 10 min and collecting the supernatant using the magnetic rack. **(QC sample 2) (Note 12)**

Steps 38 – 46: Second strand synthesis, end polishing and ligation of the distal adaptor

38. Combine the following reagents in a PCR tube to perform the second strand synthesis reaction:

Eluate from step 37	14.85 μL
Phusion FLASH mix	15 μL
100 μM oHU3790	0.15 μL

(Note 13)

Incubate the reaction mixture in a thermocycler with the following programme:

T (°C)	min:s
95	02:00
60	00:30
72	02:00
4	∞

39. Purify the resulting double-stranded DNA using 54 μL of AMPure beads in their supplied buffer as described in step y27-28. **(Note 14)**

40. Release the DNA by addition of 19 μL of water to the washed AMPure beads and incubation for 5 min at room temperature. Do not remove the eluate from the beads. **(QC sample 3)**

41. Add the following reagents (from the NEBNext® Ultra™ II DNA Library Prep kit) to perform the end polishing reaction:

    Eluate (bead suspension) 17 μL
    End Prep Reaction Buffer 2.3 μL
    End Prep Enzyme Mix 1 μL

    Incubate the reaction mixture in a thermocycler with the following programme:

T (°C)	**min:s**
20	30:00
65	30:00
4	∞

42. Add the following reagents from the NEBNext® Ultra™ II DNA Library Prep kit to perform the distal adaptor ligation reaction:

    Ligation Master Mix 13.5 μL
    Ligation Enhancer 0.45 μL
    Distal adaptor 2.25 μL
    Water 8.8 μL

    Incubate the reaction mixture in a thermocycler with the following programme:

T (°C)	**min:s**
20	20:00
4	∞

43. Add the following reagents:

    5 M NaCl 13.5 μL
    50% PEG 8000 11.25 μL
    Water 5.25 μL

44. Rinse the beads as described in step y27.

45. Elute the purified DNA from the beads in 50 μL of water as described in step y28 and repeat the purification as described in steps y27-28, using 80 μL of fresh AMPure beads in their supplied buffer.

46. Elute the DNA from the beads in 20 μL of water by incubation for 5 min at room temperature.

#Pause point – At this point, the ligated DNA fragments can be stored at -80°C.

Steps 47 – 50: Library amplification

47. Add the following reagents to a PCR tube to amplify the libraries:

DNA (from step 46)	7.25 μL
5× Q5 reaction buffer	5 μL
1 μM P5	2.5 μL
1 μM P7	2.5 μL
10 mM dNTP solution	0.5 μL
Q5 DNA polymerase	0.25 μL
Water	7 μL

(Note 15)

Perform 8 cycles of PCR amplification, using the following programme:

T (°C)	min:s
95	02:00
95	00:15
60	00:30
72	00:20
4	∞

7×

48. Purify the amplified library using 25 μL of AMPure beads in their supplied buffer as described in step y27 and elute in 25 μL of water as described in step y28.
49. Repeat the purification (step 48) and elute with 20 μL of water. **(QC sample 4) (Note 16)**
50. Determine the concentration the library sample using a Qubit fluorometer and reagents for quantifying dsDNA and prepare a sample pool with a total concentration of 4 nM for loading onto the sequencer. **(Note 17)**

D. Notes

Note 1 – The protocol described here is recommended for preparing genomic DNA for detection of strand breaks, as it minimizes the introduction of additional nicks and breaks during the extraction procedure. For detection of DNA lesions involving an intact backbone, steps 1-22 can be replaced by the use of a standard genomic DNA extraction kit (e.g. Qiagen). The protocol can be scaled.

Note 2 – Spheroplast formation takes ~60-90 min. Its efficiency is monitored by adding 2 μL of 1% SDS to 2 μL of cell suspension on a microscope slide and observing the decrease in the number of intact cells under a stereomicroscope. Samples not treated with SDS can be used as a control. Efficient spheroplasting should result in close to 100% lysis within 2 min of incubation with SDS.

Note 3 – This should normally take about 45 min.

Note 4 – Repeat this step if the supernatant is not cleared of debris.

Note 5 – When handling more than 4 samples, pellets may detach from the wall of the tube over time. In this case, repeat the centrifugation to clear the supernatant of any visible debris.

Note 6 – If the DNA pellet does not resuspend well, it can be incubated overnight in the 50 mL tube and split into two 2 mL microcentrifuge tubes the next day before RNase A treatment. After splitting the original sample into two tubes, each of these is treated separately and in parallel. Hence, each sample of yeast cells eventually gives rise to two identical samples of genomic DNA. Alternatively, the initial steps can be scaled down to prepare a single sample of DNA.

Note 7 – The amount of adaptor can be calculated based on the approximate number of nicks expected in 2.5 μg of genomic DNA. A five-fold molar excess of adaptor should then be used. If the expected number of breaks is unknown, use 3.55 μL (resulting in a final concentration of 2.185 μM). If the excess of unligated adaptor presents a problem (e.g. in native, undigested DNA), this amount can be reduced.

Note 8 – A custom mould with pockets of ~45 μL is necessary to cast properly shaped plugs of 45 μL. Common commercially available moulds have pockets of ~90 μL (Bio-Rad, 1703713), which produce unevenly shaped plugs that easily break during handling. See Supplementary Information for a 3D printer template suitable for 45 μL moulds.

Note 9 – Make sure the Bioruptor Pico is cooled to 4°C before sonication. Incubation of the purified samples on ice is not required.

Note 10 – This additional clean-up step can be omitted in the yeast protocol, but at least one round of purification should be carried out if unligated adaptors present a problem (see Troubleshooting).

Note 11 – When processing more than 5 samples in parallel, reduce the incubation time to 2.5 min in order to account for the increased processing time due to buffer addition and resuspension of the samples.

Note 12 – Incubating the samples for 10 min does not affect the subsequent steps.

Note 13 – When performing more than one reaction, a master mix should be prepared using a 100 μM stock of oHU3790 in order to keep the total reaction volume as close to 30 μL as possible.

Note 14 – If a sample is expected to contain few nicks, sample volume can be increased to 50 μL by addition of water, and 90 μL of AMPure beads should then be used for purification. This minimises loss of library.

Note 15 – Oligonucleotide P7 contains a barcode, $(X)_6$, for multiplexing, which should be applied according to the number of samples to be included in the experiment.

Note 16 – While removing the eluate, collect only 18 μL and pipette very slowly to avoid transferring any beads along with the supernatant.

Note 17 – Sequencing conditions will depend on the available platform (e.g. Illumina NextSeq) as well as the nature and number of the samples to be analysed. We recommend following the manufacturer's specifications for single-end sequencing. For GLOE-Seq analysis in yeast, 3 million reads per sample are sufficient. For analysis of mammalian cells, a minimum of 50 million reads is recommended.

E. Quality Control

QC sample 1 – In order to verify appropriate fragmentation to an average fragment size of 200 nt, analyse 1 μL of the fragmented DNA either on an Agilent RNA ScreenTape (Agilent 2200 Tapestation) or an Agilent 2100 Bioanalyzer (using an RNA chip).

QC sample 2 – In order to verify efficient capture of biotinylated DNA and removal of excess unligated adaptor, analyse 1 μL of the eluted DNA either on an Agilent RNA ScreenTape (Agilent 2200 Tapestation) or an Agilent 2100 Bioanalyzer (using an RNA chip).

QC sample 3 – The efficiency of second strand synthesis and ligation of the distal adaptor should be monitored by analysing 2 μL of the eluate on an Agilent High Sensitivity D1000 ScreenTape (Agilent 2200 tape station).

QC sample 4 – In order to ensure that the sample is free of unligated adaptors or adaptor concatemers, analyse 1 μL of the purified DNA on an Agilent High Sensitivity DNA chip (Agilent 2100 Bionanalyzer).

F. Troubleshooting

QC Sample	Expected result	Possible deviation	Possible problem	Action
1	ssDNA in a size range of 160-450 nt (average: 200)	Improper size range	Improper fragmentation conditions	Adjust/standardise fragmentation conditions.
2	ssDNA in a size range of 100-300 nt (average: 200), free of unligated adaptor	No product	Sample contains very few nicks	No action required.
			Inefficient capture of biotinylated DNA	Verify the quality of Streptavidin beads using unligated proximal (biotinylated) adaptor as control. Make sure you use a "3'-Biotin-dT" label (IDT) on the adaptor. "Standard Biotin" has caused problems in our hands.
		Presence of unligated adaptor	Inefficient removal of unligated adaptor	Additional clean-up of the sonicated fragments with 1.6 volumes of AMPure beads, eluting in 50 μL of water. Alternatively: Beads used in step y25 can be reused to purify fragmented DNA by adding NaCl and PEG 8000 to final concentra-tions of 1.25 M and 7.5% (*w/v*), respectively, eluting in 50 μL of water as described in steps y27-28 and proceeding with step 31.
			Inefficient ligation of 3'-OH ends to the biotiny-lated adaptor	Verify ligation efficiency by separating a small sample on a TBE-urea gel, blotting onto a Nitrocellulose membrane and probing with anti-Streptavidin antibody. If needed, adjust ligation conditions.
3	dsDNA in a size range of 150-450 bp (average: 350)	No product	Inefficient 2nd strand synthe-sis due excess unligated adaptor	Additional purification using AMPure beads will remove excess adaptor.
4	Library in a size range of 250-700 bp, free of adaptor concatemers	Presence of adaptor concatemers	Presence of excess unligated proximal and distal adaptors	Additional purification using AMPure beads at a beads: sample ratio of 1:1 (*v/v*) will remove adaptor concatemers.