Protocol for Paired-Damage-seq

Regents

Cell Culture and Processing:

- DMEM (Gibco, 10569010)
- Fetal Bovine Serum (FBS) (Gibco, 16000044)
- Penicillin-Streptomycin (Gibco, 10378016)
- Hydrogen Peroxide (H_2O_2) (Thermo Scientific Chemicals, 202465000)
- Phosphate-Buffered Saline (PBS) (Gibco, 10010-23)
- HEPES pH 7.3 (Thermo Scientific Chemicals, J16924.K2)
- NaCl (Sigma, S7653)
- $MgCl_2$ (Sigma, 63069)
- Protease Inhibitor (Roche, 05056489001)
- RNase OUT (Invitrogen, 10777-019)
- SUPERAse Inhibitor (Invitrogen, AM2694)
- IGEPAL CA630 (Sigma, I8896)
- Tween-20 (Sigma, P1379-25ML)

Biospecimen Processing:

- Sucrose (Sigma, S7903)
- KCl (Sigma, P9333)
- Tris-HCl pH 7.5 (Invitrogen, 15567027)
- DTT (Sigma, D9779)
- Triton-X100 (Sigma, T9284)

Cell Fixation:

- Formaldehyde (37%) (Sigma, F8775)

Enzymatic Labeling:

- NEBuffer r2.1 (NEB, B6002S)
- SDS (Invitrogen, 15553-035)
- BSA (10%) (Sigma, A1595-50ML)
- Fpg (NEB, M0240L)
- Endo IV (NEB, M0304L)
- NEBuffer 2 (NEB, B7002S)
- NEBuffer 3 (NEB, B7003S)
- Bst Full-Length Polymerase (NEB, M0328S)

- Taq DNA Ligase (NEB, M0208L)
- NAD+ (NEB, B9007S)
- dATP (NEB, N0440S)
- dCTP (NEB, N0441S)
- dGTP (NEB, N0442S)
- Biotin-dUTP (Thermo Scientific, R0081)

Antibody Staining and Tagmentation:

- Anti-Biotin Antibody (Abcam, ab234284)
- Protein A-Tn5 (0.5 mg/mL)
- HEPES pH 7.5 (Invitrogen, 15630106)
- Spermidine (Sigma, S2626-1G)
- Digitonin (Millipore, 300410-250MG)
- EDTA (Thermo Scientific Chemicals, 15575020)

Reverse Transcription:

- Betaine (Sigma, B0300-1VL)
- Maxima Reverse H Minus Reverse Transcriptase (Invitrogen, EP0751)

Ligation-Based Barcoding:

- NEBuffer r3.1 (NEB, B6003S)
- T4 DNA Ligase Buffer (NEB, B0202S)
- T4 DNA Ligase (NEB, M0202L)

Preamplification of Barcoded DNA/cDNA:

- 10× TdT Buffer (NEB, M0315S)
- 1 mM dCTP (NEB, N0447S)
- TdT (Terminal Deoxynucleotidyl Transferase) (NEB, M0315S)
- 5× KAPA HiFi Buffer (KAPA, KK2502)
- 10 mM dNTPs (Thermo Scientific)
- KAPA HiFi HS (KAPA, KK2502)
- 10× rCutSmart Buffer (NEB, B6004S)
- SbfI-HF (NEB, R3642)
- FokI (NEB, R0109S)
- SPRI Beads (Agencourt, A63881)
- 10× T4 DNA Ligase Buffer (NEB, B0202S)
- NotI-HF (NEB, R3189)

Indexing PCR:

- 5× Q5 Buffer (NEB, M0491)
- 10 mM dNTPs (Thermo Scientific)
- NEB Q5 DNA Polymerase (NEB, M0491)

Library Purification:

- SPRI Beads (Agencourt, A63881)

The reagents in this section can be stored at 4 °C for up to 6 months

Barcode Plates (R02, R03 plates):

1. Distribute the R02 and R03 barcoded oligos to two 96-well plates, respectively as: 6 μL barcoded oligos (R02 or R03, 100 μM), 5.5 μL Linker (R02 or R03, 100 μM), 38.5 μL H_2O.
2. Seal the plates, heat for 5 mins at 95 °C and slowly cool down to 20°C at the speed of 0.1 °C/s.
3. Aliquot the each of the annealed barcode plate into 10 working plates (each plate with 5 μL of annealed oligos in each well), sealed and stored at -20°C

P5 adaptor mix:

1. Mix the oligo DNA as (a) P5 complex: 25 μL P5-FokI (100 μM), 25 μL P5c-NNDC-FokI (100 μM); and (b) P5H complex: 25 μL P5H-FokI (100 μM), 25 μL P5Hc-NNDC-FokI (100 μM).
2. Heat the mixtures for 5 mins at 95 °C and slowly cool down to 20°C at the speed of 0.1 °C/s.
3. Mixed P5 complex with P5H complex (on the ice) at the ratio of 1:3, stored at -20 °C.

Barcoded RT primers:

Mix the oligo (RNA barcode R01) in 12 1.5 mL tubes: 12.5 μL RNA_RE (#01 to #12 in the 12 tubes, 100 μM), 12.5 μL RNA_NRE (#01 to #12 matched with RNA_RE, 100 μM), and 75 μL H_2O, stored at -20 °C.

Barcoded Protein A-Tn5:

1. Mix the oligo (DNA barcode R01) in 12 PCR tubes: 25 μL DNA_RE (#01 to #12 in the 12 tubes, 100 μM), 25 μL pMENTs (100 μM, same in the 12 tubes).
2. Heat the mixtures for 5 mins at 95 °C and slowly cool down to 20 °C at the speed of 0.1 °C/s.
3. Mix 1 μL of annealed transposome DNA with 6 μL of unloaded proteinA-Tn5 (0.5 mg/mL), briefly vortex and quickly spun down.

4. The mixtures were incubated at room temperature for 30 min then at 4 °C for an additional 10 min, stored at -20 °C.

Tn5-AdaptorA:

1. Mix the oligo as: 25 μL Adaptor A (100 μM), 25 μL pMENTs (100 μM).
2. Heat the mixture for 5 mins at 95 °C and slowly cool down to 20 °C at the speed of 0.1 °C/s.
3. Mix 1 μL of annealed transposome DNA with 6 μL of unloaded Tn5 (0.5 mg/mL), briefly vortex and quickly spun down.
4. The mixtures were incubated at room temperature for 30 min then at 4 °C for an additional 10 min. Dilute 10× with dilution buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 50% Glycol, 1 mM DTT), stored at -20 °C.

2× TB (Tagmentation buffer):

1. Prepare the 2× TB as: 660 μL Tris-Ac (pH 7.8, 1 M), 440 μL K-Ac (3 M), 3.2 mL DMF, H_2O (5.59 mL).
2. Aliquot the 2× TB to 1.5 mL tubes and stored at 4 °C.
3. To avoid contamination, each experiment should use a new tube of 2× TB.

Sample processing

Processing of culture cells

1. Wash collected cells with PBS and count using a BioRad TC20 cell counter.
2. Resuspend cells in 1 mL cold NIB-HEPES buffer (10 mM HEPES pH 7.3 (Thermo Scientific Chemicals, J16924.K2), 10 mM NaCl (Sigma, S7653), 3 mM $MgCl_2$ (Sigma, 63069), 1× Protease Inhibitor (Roche, 05056489001), 0.5 U/μL RNase OUT (Invitrogen, 10777-019) and 0.5 U μL^{-1} SUPERase Inhibitor (Invitrogen, AM2694)) with 0.1% IGEPAL CA630 (Sigma, I8896), 0.1% Tween-20 (Sigma, P1379-25ML)), incubate on ice for 10 minutes, and centrifuge at 600 g for 10 minutes at 4°C.
3. Wash cells with 1 mL cold NIB-HEPES buffer and centrifuge again at 600 g for 10 minutes at 4°C.
4. Proceed to Paired-Damage-seq experiments.

Processing of biospecimens

1. Prepare single-nuclei suspensions by douncing frozen tissue in Douncing Buffer (DBI: 0.25 M sucrose (Sigma, S7903), 25 mM KCl (Sigma, P9333), 5 mM $MgCl_2$ (Sigma, 63069), 10 mM Tris-HCl pH 7.5 (Invitrogen, 15567027), 1 mM DTT (Sigma, D9779), 1× Protease Inhibitor (Roche, 05056489001), 0.5 U/μL RNase OUT (Invitrogen, 10777-019) and 0.5 U/μL SUPERase Inhibitor (Invitrogen, AM2694)), supplemented with 0.1% Triton-X100 (Sigma T9284).
2. Filter cell suspension using a 30-μm Cell-Tric filter and centrifuge at 1,000 g for 10 minutes at 4°C.
3. Wash cell pellets with Douncing Buffer and centrifuge again.
4. Resuspend nuclei pellets in cold NIB-HEPES buffer, incubate on ice for 10 minutes, and centrifuge at 600 g for 10 minutes at 4°C.
5. Wash with 1 mL cold NIB-HEPES buffer and centrifuge again at 600 g for 10 minutes at 4°C.
6. Count cells using BioRad TC20 cell counter and proceed to Paired-Damage-seq experiments.

Paired-Damage-seq

Nuclei preparation:

1. Suspend nuclei in 2.5 mL NIB-HEPES buffer.
2. Add 123 µL of 37% formaldehyde (Sigma, F8775) to the nuclei suspension and incubate at room temperature for 10 minutes with gentle rotation.
3. Spin down fixed cells at 4°C, 600 g for 8 minutes.
4. Wash cells with cold NIB-Tris Buffer (10 mM Tris-HCl pH 7.5 (Invitrogen, 15567027), 10 mM NaCl (Sigma, S7653), 3 mM $MgCl_2$ (Sigma, 63069), 1× Protease Inhibitor (Roche, 05056489001), 0.5 U/µL RNase OUT (Invitrogen, 10777-019) and 0.5 U/µL SUPERase Inhibitor (Invitrogen, AM2694), 0.1% IGEPAL CA630 (Sigma, I8896), 0.1% Tween-20 (Sigma, P1379-25ML)), and spin down at 4°C, 600 g for 10 minutes.
5. Resuspend nuclei in 800 µL 1× NEBuffer r2.1 (NEB, B6002S) with 0.3% SDS (Invitrogen, 15553-035) and incubate at 42°C, 300 rpm for 30 minutes in a Thermomixer (Eppendorf).
6. Spin down nuclei at 100 g for 10 minutes and wash twice with 200 µL cold NIB-Tris buffer for SDS quenching.
7. Wash once with 200 µL 1× NEBuffer r2.1 containing 0.1% Triton X-100 (Sigma T9284).

Enzymatic labelling:

1. Suspend nuclei in 84 µL enzymatic labeling buffer (71 µL H_2O, 10 µL 10× NEBuffer 2 (NEB, B7002S), 1 µL SUPERase Inhibitor, 0.5 µL RNase OUT, 1 µL 10% BSA (Sigma, A1595-50ML), and 0.1% Triton X-100).
2. Add 8 µL Fpg (NEB, M0240L) and 8 µL Endo IV (NEB, M0304L) to nuclei suspension and incubate at 37°C for 4 hours.
3. Spin down nuclei at 600 g for 10 minutes.
4. Resuspend nuclei in 79 µL enzymatic labeling buffer (66 µL H_2O, 10 µL 10× NEBuffer 3 (NEB, B7003S), 1 µL SUPERase Inhibitor, 0.5 µL RNase OUT, 1 µL 10% BSA (Sigma, A1595-50ML), and 0.1% Triton X-100).
5. Add 5 µL Bst full-length polymerase (NEB, M0328S), 10 µL Taq DNA Ligase (NEB, M0208L), 2 µL NAD^+ (NEB, B9007S), 4 µL mixed dNTPs (5 µM each: dATP, dCTP, dGTP, biotin-dUTP), and incubate at 37°C for 6 hours.
6. Spin down nuclei at 600 g for 10 minutes.

Immuno-staining:

1. Assemble anti-biotin antibody (Abcam, ab234284) and barcoded protein A-Tn5 (1 μL of 0.5 mg/mL) in 20 μL 1× Med Buffer #1 (20 mM HEPES pH 7.5 (Invitrogen, 15630106), 300 mM NaCl, 0.5 mM Spermidine (Sigma, S2626-1G), 1× Protease Inhibitor cocktail, 0.5 U/μL SUPERase IN, 0.5 U/μL RNase OUT, 0.01% IGEPAL CA630, 0.01% Digitonin (Millipore, 300410-250MG) and 2 mM EDTA (Thermo Scientific Chemicals,15575020)).
2. Rotate the antibody-protein A-Tn5 mixture for 60 minutes at room temperature.
3. Spin down nuclei at 600 g for 10 minutes.
4. Wash nuclei with 200 μL Med Buffer #1 twice.
5. Aliquot 3.6 million nuclei into 12 Maximum Recovery tubes (Axygen, MCT-150-L-C) and resuspend in 50 μL Med Buffer #1.
6. Mix antibody-protein A-Tn5 complex with nuclei suspension and incubate at 4°C overnight with rotation.
7. Spin down nuclei at 600 g, 4°C for 10 minutes.

Nuclei washing and tagmentation:

1. Resuspend nuclei in 50 μL Med Buffer #2 (same composition as Med Buffer #1, but without EDTA).
2. Repeat step 8 twice.
3. Initiate tagmentation by adding 2 μL of 250 mM $MgCl_2$ and incubate at 550 rpm at 37°C for 60 minutes in a ThermoMixer.
4. Quench the reaction by adding 16.5 μL of 40.5 mM EDTA.
5. Spin down nuclei at 1,000 g, 4°C for 10 minutes.

Reverse transcription:

1. Resuspend nuclei pellets in 20 μL reverse transcription mix containing #1–#12 barcoded primers (1× RT Buffer, 1 M Betaine (Sigma, B0300-1VL), 2 μM TSO, 5 mM DTT (Sigma, D9779), 6.25 mM $MgCl_2$, 0.5 mM dNTPs (NEB, N0447S), 0.5 U/μL SUPERase IN, 0.5 U/μL RNase OUT, 2.5 μM barcoded T15 primer and 2.5 μM barcoded N6 primer, and 1 U/μL Maxima Reverse H Minus Reverse Transcriptase (Invitrogen, EP0751)).
2. Perform reverse transcription in a thermocycler with the specified program: Step 1: 42 °C × 90 mins; Step 2: 50 °C × 10 mins; Step 3: 8 °C × 12 s, 12°C × 45 s, 25 °C × 45 s, 37 °C × 45 s, 45 °C × 45 mins, go to Step 2 for additional two times; Step 3: 50 °C × 10 mins and hold at 4 °C.

3. After reverse transcription, transfer and pool nuclei into pre-washed 1.5 mL tubes with 5% BSA in PBS.
4. Add 4.8 µL of 5% Triton X-100 and spin down nuclei at 1,000 g, 4°C for 10 minutes.

Ligation-based combinatorial barcoding

1. Resuspend nuclei in 1 mL of 1× NEBuffer r3.1 and transfer to ligation mix (2,262 µL of H_2O, 500 µL of 10× T4 DNA Ligase Buffer (NEB, B0202S), 50 µL of 10 mg/mL BSA, 100 µL of 10× NEBuffer r3.1 and 100 µL of T4 DNA Ligase (NEB, M0202L)).
2. Distribute 20 µL of the ligation reaction mix to Barcode-plate-R02 and incubate at 300 rpm at 37°C for 30 minutes.
3. Add 5 µL of R02-Blocking-Solution (264 µL of 100 µM Blocker-R02 oligo, 250 µL of 10× T4 DNA Ligase Buffer, 486 µL of H_2O) to each well and incubate for an additional 30 minutes.
4. Spin down nuclei at 1,000 g, 4°C for 10 minutes.
5. Perform the second round of ligation as described, followed by termination with Quencher-R03 solution (264 µL of 100 µM Quencher-R03, 250 µL of 0.5 M EDTA and 236 µL of H_2O).
6. Pool nuclei and resuspend in PBS, count, and aliquot to sub-libraries (5,000–10,000 nuclei each).

Lysis and DNA cleanup:

1. Dilute sub-libraries to 35 µL with 1× NIB-Tris buffer.
1. Add 5 µL of 4 M NaCl, 5 µL of 10% SDS, and 5 µL of 10 mg/mL Protease K.
2. Lyse nuclei at 850 rpm at 55°C for 2 hours in a ThermoMixer.
3. Cool the lysed solution to room temperature and purify with 1× SPRI beads.
4. Elute the purified DNA in 12.5 µL of H_2O and store at −20°C or −80°C for up to 4 weeks.

<u>Pause Point: Purified DNA can be stored at -20 °C or -80 °C for up to 6 months.</u>

Preamplification of barcoded DNA/cDNA:

1. Add 1 µL of 10× TdT Buffer and 0.5 µL of 1 mM dCTP to 12.5 µL of purified DNA. Incubate at 95 °C for 5 minutes, then quickly cool on ice for 5 minutes.
2. Add 1 µL of TdT to the mixture. Incubate at 37 °C for 30 minutes, followed by inactivation at 75 °C for 20 minutes.

3. Add the Anchor Mix (6 µL of 5× KAPA Buffer, 0.6 µL of 10 mM dNTPs, 0.6 µL of 10 µM Anchor-FokI-GH, 7.3 µL of H_2O, and 0.6 µL of KAPA HiFi HS DNA polymerase) directly to the reaction mix. Mix by pipetting up and down. Perform the following program in a thermocycler: Step 1: 95 °C × 3 mins; Step 2: 95 °C × 15 s, 47 °C × 60 s, 68 °C × 120 s, 47 °C × 60 s, 68 °C × 120 s; repeat Step 2 for an additional 15 cycles; Step 3: 72 °C × 10 mins; Step 4: hold at 12 °C.
4. Add the Preamp Mix (4 µL of 5× KAPA Buffer, 0.5 µL of 10 mM dNTPs, 2 µL of 10 µM PA-F, 2 µL of 10 µM PA-R, 11 µL of H_2O, and 0.5 µL of KAPA HiFi HS DNA polymerase) directly to the reaction mix. Mix by pipetting up and down. Perform the following program in a thermocycler: Step 1: 98 °C × 3 mins; Step 2: 98 °C × 20 s, 62 °C × 20 s, 72 °C × 150 s; repeat Step 2 for an additional 9 cycles; Step 3: 72 °C × 120 s; Step 4: hold at 12 °C.
5. Purify the reaction using 0.2× + 0.65× SPRI beads (10 µL + 32.5 µL) for double-size selection. Elute in 36 µL of ultra-pure H_2O, and use 1 µL for Qubit quantification. Record the concentration in ng/µL. Depending on the cell type and the number of cells per sub-library, the concentration typically ranges from 1 ng/µL to 30 ng/µL.

2nd adaptor tagging and digestion

1. Transfer 17 µL of the purified amplified product into two 200 µL PCR tubes for DNA and RNA, respectively.
2. For DNA part: add 2.5 µL 10× Cutsmart buffer, 1 µL SbfI-HF, 1 µL FokI, and 3.5 µL H_2O.
3. For RNA part: add 2 µL 10× Cutsmart buffer, 1 µL NotI-HF.
4. Incubate at 37 °C for 60 mins.
5. Use 1.25× SPRI beads (31.3 µL for DNA, 25 µL for RNA). Elute both DNA and RNA in 10 µL H_2O.

Pause Point: Purified DNA can be stored at -20 °C or -80 °C for up to 6 months.

6. For DNA part: add 2 µL 10× T4 DNA Ligase Buffer, 1.5 µL P5 Adaptor Mix, 5 µL H_2O and 1.5 µL T4 DNA Ligase. Incubate with the ligation program (4 °C × 10 mins, 10 °C 1 × 5 min, 16 °C × 15 min, 25 °C × 45 min).
7. For DNA part: cleanup the ligation mixture with 1.25 × (25 µL) SPRI beads and elute in 30 µL H_2O.
8. For RNA part: second round-preamplification mix (4 µL of 5× KAPA HiFi buffer, 0.5 µL of 10 mM dNTPs, 2 µL of 10 µM PA-F and TSO primers, 0.5 µL KAPA HiFi HS) was then added

and preamplification was performed with the following program: Step 1: 98 °C × 3 mins; Step 2: 98 °C × 20 s, 65 °C × 20 s, 72 °C × 2.5 mins, and repeat Step 2 an additional 6 times; Step 3: 72 °C × 2 mins and hold at 12 °C. Amplified products were purified with 1.8× SPRI beads and were eluted in 17 μL of H2O.

9. For RNA part: add 2 μL 10× Cutsmart buffer, 1 μL NotI-HF.
10. For RNA part: use 1.25× SPRI beads to purify and elute RNA in 10 μL H_2O.
11. For RNA part: add 10.5 μL 2× TB, y μL 1:10 diluted Tn5-Adaptor A (y = X × 0.2, titration may be needed for different batch of Tn5).
12. For RNA part: the tagmentation reaction was carried out 550 r.p.m., 37 °C for 30 mins in a ThermoMixer followed by cleaned up using QIAquick PCR purification kit and eluted in 30 μL 0.1× EB (QIAGEN).

Pause Point: Purified DNA can be stored at -20 °C or -80 °C for up to 6 months.

Indexing PCR and sequencing:

1. For DNA libraries, prepare the PCR mix as: 30 μL purified template, 2 μL 10 μM TruSeq i7 indices, 2 μL 10 μM Universal P5 primer, 1 μL 10 mM dNTP, 10 μL 5 × NEB Q5 Buffer, 4 μL H_2O and 1 μL Q5 HiFi DNA polymerase. Run the program in a thermocycler: Step 1, 98 °C × 3 mins; Step 2, 98 °C × 10 s, 63 °C × 30 s, 72 °C × 60 s; repeat Step 2 for 8 cycles; Step 3, 72 °C × 60 s; Step 4, hold at 12 °C.
2. For RNA libraries, prepare the PCR mix as: 30 μL purified template, 2 μL 10 μM TruSeq i7 indices, 2 μL 10 μM NextEra N5 indices, 1 μL 10 mM dNTP, 10 μL 5X NEB Q5 Buffer, 4 μL H_2O and 1 μL Q5 HiFi DNA polymerase. Run the program in a thermocycler: Step 1, 72 °C × 5 mins, 98 °C × 30s; Step 2, 98 °C × 10 s, 63 °C × 30 s, 72 °C × 60 s; repeat Step 2 for 8 cycles; Step 3, 72 °C × 60s; Step 4, hold at 12 °C.
3. Use 0.5 μL (dilute 1,000X) to run a qPCR to estimate the additional cycles needed (typically 0-4) to reach 10 nM. Run the additional cycles in a thermocycler: Step 1, 98 °C × 3 mins; Step 2, 98 °C × 10 s, 63 °C × 30 s, 72 °C × 60 s; repeat Step 2 for desired cycles; Step 3, 72 °C × 60s; Step 4, hold at 12 °C.
4. Cleanup the libraries using 0.9 × (45 μL) SPRI beads, elute in 15-30 μL H_2O. Use qPCR to quantify libraries. Store at -20 °C.

Pause Point: Purified libraries can be stored at -20 °C or -80 °C for up to 6 months.

5. Use Agilent Fragment Analyzer to analysis the fragment distribution of libraries.
6. Multiplex libraries and sequence with standard Illumina sequencing primers on commercial sequencing platforms. We have tested sequencing of Paired-Tag libraries on NextSeq 550 or NovaSeq 6000 platforms. Libraries should be loaded at recommended concentrations according to manufacturer's instructions. At least 50 and 100 sequencing cycles are needed for Read1 and Read2, respectively. For example: using PE 50 + 8 + 100 cycles (Read1 + Index 1 + Read2) on a NextSeq 550 platform with 150-cycle sequencing kits, or PE 100 +8 +100 cycles on a NovaSeq 6000 platform with 200-cycle sequencing kits.