

Massively parallel multi-target CRISPR system interrogates Cas9-based target recognition, DNA cleavage, and DNA repair

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Abstract

CRISPR-Cas9 nucleases, and particularly *Streptococcus pyogenes* Cas9, are widespread tools for genome editing. However, many aspects of intracellular Cas9 activity and the ensuing DNA damage response remain incompletely characterized. In order to address these issues, we developed a multiplexed CRISPR approach, where a single, degenerate multi-target gRNA (mgRNA) directs the Cas9 enzyme to target hundred endogenous sites at once. When combined with next-generation sequencing readouts, this system enables interrogation of Cas9 activity and DNA double-strand break (DSB) repair response in high-throughput. Here, we present a step-by-step protocol to deliver a Cas9:mgRNA ribonucleoprotein complex into cultured cells and measure key processes related to Cas9 activity and DSB repair.

Introduction

Reagents

Cell culture

1. Modified Eagle's Medium (DMEM, Corning 10-013-CV), Essential 8 Medium (E8; Thermo Fisher Scientific #A1517001).
2. FBS (Corning, 35-010-CV), Antibiotic-antimycotic (Thermo Fisher Scientific 15240062), PBS (Corning 21-040-CM), Trypsin (Corning 25-053-CI), Collagen I Rat Protein (Thermo Fisher Scientific A1048301).
3. Human embryonic cell (hES cell)-qualified matrigel (Corning #354277), ROCK inhibitor (Y-27632; STEMCELL #72308), accutase (Sigma #A6964).

Cas9 purification

1. Cas9 plasmid (Addgene #67881).
2. BL21-CodonPlus (DE3)-RIL competent cells (Agilent Technologies 230245).
3. cOmplete™ EDTA-free protease inhibitor tablet (Sigma-Aldrich 11836170001).
4. Syringe filter (Thermo Fisher Scientific F25006).
5. Ni-NTA agarose bead slurry (Qiagen 30210).
6. Coomassie blue (Bio-Rad 1610400).
7. 1 mL Q Sepharose® column (GE Healthcare 17051005).

8. 10 kDa SnakeSkin™ dialysis tubing (Thermo Fisher Scientific 68100).
9. Amicon® Ultra 10 kDa centrifugal filter unit (Millipore UFC801024).

Electroporation

1. crRNA (IDT 1072532) and tracrRNA (IDT)
2. SF Cell Line 4D-Nucleofector™ X Kit L (Lonza #: V4XC-2012). Solution and supplement
3. P3 Primary Cell 4D-Nucleofector™ X Kit L (Lonza #: V4XP-3024). Solution and supplement
4. Electroporation Enhancer (IDT 1075915)

Chromatin immunoprecipitation with sequencing (ChIP-Seq)

1. 16% formaldehyde methanol-free (Thermo Fisher Scientific 28908)
2. Halt™ Protease Inhibitor Cocktail (100X) (Thermo Fisher Scientific 78438)
3. Dynabeads™ Protein A for Immunoprecipitation (Thermo Fisher Scientific 10001D)
4. NEBNext® Ultra™ II End Repair/dA-Tailing Module (NEB E7546S), T4 DNA Ligase (NEB M0202)
5. Antibodies: Cas9 – Diagenode C15310258; MRE11 – Novus NB100-142; γH2AX – Abcam ab81299; 53BP1 – Novus NB100-305.

Genome-wide DSB detection with BLISS

1. 5' DNA Adenylation Kit (NEB E2610S), Quick Blunting Kit (NEB E1201L), dA-Tailing mix (NEB E6053L), Ligation mixture (NEB M0202L), IVT reaction (NEB E2050S).
2. T4 RNA Ligase 2, truncated KQ (NEB M0373S).
3. Q5 2x master mix (NEB M0492).
4. CutSmart Buffer (NEB B7204).
5. Monarch RNA cleanup kit (NEB T2040L).
6. RNaseOUT (Thermo Fisher Scientific 10777019).

7. SuperScript III (Thermo Fisher 18080044).

Assay for Transposase-Accessible Chromatin using sequencing (ATAC-Seq)

1. Tagment DNA Enzyme and Buffer Small Kit (Illumina 20034197).
2. NEBNext® High-Fidelity 2X PCR Master Mix (NEB M0541).
3. Invitrogen™ SYBR™ Green I Nucleic Acid Gel Stain (Invitrogen S7563).

Other general reagents

1. RNaseA (NEB T3018L), Proteinase K (NEB P8107S)
2. AMPure XP beads (Beckman Coulter)
3. Detergents: Igepal-CA630 (Millipore Sigma I8896-50ML), Tween-20 (Thermo Fisher Scientific BP337-100), Digitonin (Millipore Sigma CHR103), Triton X-100 (Millipore-Sigma T8787-50ML)
4. Minelute kit (Qiagen 28004)

General molecular biology reagents including KCl, Tris, NaCl, etc.

Equipment

1. 4D-Nucleofector™ Core Unit (Lonza)
2. Fisher 150E Sonic Dismembrator.
3. Qsonica Q125 sonicator.
4. QuBit 4 fluorometer (Thermo Fisher Scientific Q33238).
5. Bioanalyzer (Agilent)
6. qPCR instrument (BioRad)
7. Eppendorf ThermoMixer® C (Eppendorf 5382000023).
8. Access to Next-Seq and Nova-Seq sequencers (Illumina).

Procedure

SpCas9 purification

1. Transform BL21-CodonPlus (DE3)-RIL competent cells with Cas9 plasmid, then inoculate into 5 mL of LB media with Ampicillin at 1 μ L/mL concentration.
2. Grow the bacteria culture overnight (37 °C, 220 rpm), then transfer to 1 L of LB-Ampicillin media supplemented with 0.1% glucose until OD₆₀₀ of ~0.5.
3. Induce the cells with 0.2 mM IPTG and maintain overnight at 18 °C.
4. Pellet the bacteria at 4500 $\times g$, 4 °C for 15 min and resuspend in 20 mL of lysis buffer containing 20 mM Tris pH 8.0, 250 mM KCl, 20 mM imidazole, 10% glycerol, 1 mM TCEP, 1 mM PMSF, and cOmplete™ EDTA-free protease inhibitor tablet (Sigma-Aldrich).
5. Lyse this cell suspension using a microfluidizer and clarify the supernatant containing Cas9 protein by spinning down cell debris at 16,000 $\times g$, 4 °C for 40 min, then filtering with a 0.2 μ m syringe filter (Thermo Scientific). Pre-equilibrate Ni-NTA agarose bead slurry (Qiagen) with 5 column volumes of lysis buffer.
6. Load the clarified supernatant at 4 °C, and wash protein-bound Ni-NTA beads with 15 column volumes of wash buffer containing 20 mM Tris pH 8.0, 800 mM KCl, 20 mM imidazole, 10% glycerol, and 1 mM TCEP.
7. Perform gradient elution with buffer containing 20 mM HEPES pH 8.0, 500 mM KCl, 10% glycerol, and varying concentrations of imidazole (100, 150, 200, and 250 mM) at 7 mL collection volume per fraction.
8. Evaluate the eluted fractions on an SDS-PAGE gel and image with Coomassie blue staining.
9. To remove potential DNA contamination, charge 1 mL Q Sepharose® column with 1 M KCl and then equilibrate with elution buffer containing 250 mM imidazole. Pass the purified protein solution over the Q column at 4 °C. Collect the flow-through and dialyze in a 10 kDa SnakeSkin™ dialysis tubing against 2 L of 20 mM HEPES pH 7.5, and 500 mM KCl, 20% glycerol at 4 °C, overnight.
10. The next day, dialyze the protein for an additional 3 hours in fresh dialysis buffer.
11. Concentrate the final Cas9 protein to 10 μ g/ μ L using Amicon® Ultra 10 kDa centrifugal filter unit, aliquot, flash-freeze, then store at -80 °C.

Assembly of Cas9:mgRNA RNP.

1. Mix 2 μ L of 100 μ M crRNA with 2 μ L of 100 μ M tracrRNA and heat to 95 °C for 5 min in a thermocycler, then allow to cool on benchtop for 5 min. crRNA and tracrRNA sequences are in **Supplementary Table 2** of associated manuscript.

2. To form the RNP complex, mix 3 μL of 10 $\mu\text{g}/\mu\text{L}$ ($\sim 66 \mu\text{M}$) of purified Cas9 with the annealed 4 μL 50 μM cr:tracrRNA, and add 8 μL of dialysis buffer (20 mM HEPES pH 7.5, and 500 mM KCl, 20% glycerol) for a total of 15 μL . Mix well and incubate this solution for 20 min at room temperature to allow for RNP formation.

Electroporation of HEK293T cells.

1. Culture HEK293T cells (ATCC® CRL-3216™) cells at 37 °C under 5% CO₂ in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic.
2. Maintain HEK293T cells in to a confluency of $\sim 90\%$ prior to electroporation. Trypsinize 12 million cells with 5 min incubation in the incubator, then add 1:1 of complete DMEM to inactivate trypsin.
3. Centrifuge this mixture (3 min, 200 $\times g$), remove the supernatant, resuspend the cell pellet in 1 mL PBS, centrifuge (3 min, 200 $\times g$), and remove completely the supernatant.
4. Resuspend cell pellet in 90 μL of SF solution from SF Cell Line 4D-Nucleofector™ X Kit L nucleofection solution (16.2 μL of Supplement solution mixed with 73.8 μL of SF solution) and mix thoroughly.
5. Add the 15 μL RNP solution and 2 μL of Cas9 Electroporation Enhancer (IDT). Mix gently.
6. Transfer the entire final solution (approximately 125 μL) to one well of a provided cuvette rated for 100 μL . Perform electroporation according to the manufacturer's instructions on the 4D-Nucleofector™ Core Unit (Lonza) using code CA-189. Some white residue may appear in the cell mixture after electroporation, but that is completely normal.
7. Use a total of 400 μL of complete DMEM to transfer the cells out of the cuvette, before plating to culture wells pre-coated with 1:100 collagen in PBS.

Electroporation of WTC-11 iPSCs

1. Alternatively, Cas9:mgRNA RNP can be electroporated into an induced pluripotent stem cell (hiPSC), WTC11 cell line¹.
2. For culture of WTC11, we followed the guidelines of Johns Hopkins Medical Institute. Briefly, thaw frozen WTC11 cells in 37 °C water bath and wash in Essential 8 Medium by centrifugation. After resuspension, plate WTC cells onto a 6 cm cell culture dish pre-coated with human embryonic cell (hES cell)-qualified matrigel (1:100 dilution). Plate coating should be performed for at least 2 h. Subsequently, supplement 10 μM ROCK inhibitor into the E8 medium to promote cell growth and survival.

3. For subculture, dissociate WTC11 cells from the plate using accutase and passage every 2 days. Maintain WTC11 cells in an incubator at 37 °C with 5% CO₂.
4. Perform electroporation using the Lonza P3 Primary Cell 4D-Nucleofector™ X Kit L using code CA-137, on 10 million cells, and using 65 µL of the P3 solution mixture with electroporation enhancer per electroporation cuvette (compared to 90 µL of comparable SF solution mixture for HEK293T cells).
5. After electroporation, resuspend cells in E8 medium supplemented with 10 µM ROCK inhibitor, and plate onto a 10 cm cell culture dish pre-coated with human embryonic cell (hES cell)-qualified matrigel (1:100 dilution) for at least 2 hours.

Chromatin immunoprecipitation with sequencing (ChIP-Seq)

1. In order to measure Cas9 binding and/or recruitment of double-strand break (DSB) repair proteins, ChIP-Seq was performed after Cas9:mgRNA delivery. We recommend using at least 4 million cells per ChIP reaction. For time-resolved experiments, this means one electroporation equates to 3 ChIP samples.
2. The ChIP protocol was adapted from previous literature². This protocol describes the reagents for one ChIP. When more than one ChIP can be performed, reagent master mixes can be prepared whenever appropriate.
3. Beads pre-loaded with antibodies were prepared before cell harvesting. 50 µL Protein A beads were used per IP and transferred to a 2 mL Eppendorf tube on a magnetic stand. Beads were washed twice with blocking buffer BB (0.5% BSA in PBS), then resuspended in 100 µL BB per IP. 3 µL of antibody per IP was added and placed on rotator for 1-2 h.
4. Briefly, cells were washed once with room temperature PBS, then washed off the plate with 10 mL DMEM and transferred to 15 mL falcon tubes. 721 µL of 16% formaldehyde (methanol-free) was added and incubated for 12 min at room temperature. 750 µL of 2 M glycine was added to quench the formaldehyde and incubated for 3 min at 4°C.
5. Cells were spun down with 1,200 × *g* at 4 °C for 3 min and washed with ice-cold PBS twice, spinning down with the same centrifugation conditions. Pellet can be decanted, flash-frozen and stored in -80 °C for later use.
6. Cells were then resuspended in 4 mL lysis buffer LB1 (50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Igepal CA-630, 0.25% Triton X-100, pH to 7.5 using KOH, add 1x protease inhibitor right before use) for 10 min at 4 °C, then spun down 2,000 × *g* at 4 °C for 3 min. The supernatant was decanted.

7. Cells were then resuspended in 4 mL LB2 (10 mM Tris-HCl pH 8, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, pH to 8.0 using HCl, add 1x protease inhibitor right before use) for 5 min at 4 °C, spun down with the same protocol, and the supernatant decanted.
8. Cells were then resuspended in 1.5 mL LB3 (10 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, 0.5% N-lauroylsarcosine, pH to 8.0 using HCl, add 1x protease inhibitor right before use) and transferred to 2 mL Eppendorf tubes for sonication with 50% amplitude, 30 s ON, 30 s OFF for 12 min total time (Fisher 150E Sonic Dismembrator).
9. Sample was spun down with 20,000 × *g* at 4 °C for 10 min, and supernatant was transferred to 1.5 mL LB3 in a 15 mL tube. 300 μL of 10% Triton X-100 was added, and the solution was well mixed by gentle inversion.
10. For WTC11 iPSCs, the only difference is a 7 min fixation time versus 12 min for HEK293T cells. Empirically, 7 min reduced overfixing for iPSCs resulting in better MRE11 ChIP signal.
11. Right before IP, the 2 mL tube containing the antibody-beads mixture was placed on a magnetic rack and washed 3x with BB, before resuspending in 50 μL BB per EP. 50 μL of beads in BB were transferred to each IP and placed in 4 °C rotator for 6+ hours.
12. Samples were transferred to 2 mL Eppendorf tubes on a magnetic stand, washed 6x with 1 mL RIPA buffer (50 mM HEPES, 500 mM LiCl, 1 mM EDTA, 1% Igepal CA-630, 0.7% Na-Deoxycholate, pH to 7.5 using KOH), then washed 1x with 1 mL TBE buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl), before decanting.
13. Beads containing ChIP-ed DNA were mixed with 70 μL elution buffer EB (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) and incubated 65 °C for 6+ hours. 40 μL of TE buffer was mixed to dilute the SDS, followed by 2 μL of 20 mg/mL RNaseA for 30 min at 37 °C.
14. 4 μL of 20 mg/mL Proteinase K was added and incubated for 1 h at 55 °C. The genomic DNA was purified using Minelute kit (Qiagen) and eluted in 35 μL nuclease free water.
15. Oligonucleotide sequences for library preparation are in **Supplementary Table 3** of associated manuscript. End-repair/A-tailing was performed on 17 μL of ChIPed DNA using NEBNext® Ultra™ II End Repair/dA-Tailing Module, followed by ligation (MNase_F/MNase_R) with T4 DNA Ligase. 10, 13, 13 and 13 cycles of PCR using PE_i5 and PE_i7XX primer pairs were performed for γH2AX, 53BP1, Cas9, and MRE11 ChIP samples, respectively to amplify libraries.
16. Samples were pooled, quantified with QuBit (Thermo), Bioanalyzer (Agilent) and qPCR (BioRad), then sequenced on a NextSeq 500 (Illumina) using high-output paired 2x36bp reads. Reads were demultiplexed after sequencing using bcl2fastq. Paired-end reads were aligned to hg19 or hg38 using bowtie2. Samtools was used to filter for mapping quality >= 25, remove singleton reads, convert to BAM format, remove potential PCR duplicates, and index reads.

Genome-wide DSB detection with BLISS

1. BLISS is performed in order to measure the generation of Cas9 DSBs. The BLISS protocol was adapted from previous literature³. All oligonucleotide sequences are provided in **Supplementary Table 4** of associated manuscript.
2. After 'Electroporation of Cas9 RNP', plate 400,000 cells each to 24-well.
3. BLISS adapters are annealed by mixing 5 μ L of Top, 5 μ L of Bottom, with 40 μ L of nuclease free water (NFW) in a PCR tube, heated to 95 $^{\circ}$ C for 5 min, then cooled to 4 $^{\circ}$ C at -0.5 $^{\circ}$ C per 30 seconds.
4. 5' Phosphorylated RA3 oligonucleotides were adenylated using 5' DNA Adenylation Kit (NEB), by mixing 1 μ L of 100 μ M of 5' Phosphorylated RA3, 13 μ L of NFW, 2 μ L of 10x reaction buffer, 2 μ L of 1 mM ATP, and 2 μ L of Mth RNA ligase to a total 20 μ L volume in a PCR tube. Samples were heated in a thermocycler to 65 $^{\circ}$ C for 1 hour, then 85 $^{\circ}$ C for 5 minutes, then ethanol precipitated to 10 μ L of NFW.
5. All PBS washes were performed by gently adding 500 μ L of 1xPBS to a 24-well at room temperature (RT), incubating at RT, then gently removing all liquid from the well. Cells were washed 1x with PBS, fixed in the 24-well with 4% paraformaldehyde at RT for 10 min, then washed 3x with PBS. Cells were incubated with lysis buffer BLISS-LB1 (10 mM Tris-HCl pH 8, 10 mM NaCl, 1 mM EDTA, 0.2% Triton X-100) for 1 hour at 4 $^{\circ}$ C, washed with PBS, then incubated with BLISS-LB2 (10 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 0.3% SDS) for 1 hour in a 37 $^{\circ}$ C incubator. Cells were then washed 2x with PBS.
6. Wash 2x with 200 μ L of 1x CutSmart Buffer (NEB) with 2 min incubation RT. Add 150 μ L of Quick Blunting Kit (NEB) (115.5 μ L NFW 15 μ L 10x blunting buffer, 1.5 μ L 10 mg/mL BSA, 15 μ L 1 mM dNTPs, 3 μ L Blunt Enzyme mix), incubate 1 hour at RT. Wash 2x with 200 μ L of 1x CutSmart Buffer with 2 min incubation at RT. Add 150 μ L of dA-Tailing mix (NEB) (131 μ L NFW, 15 μ L of 10x Tailing buffer, 4 μ L Klenow Fragment), incubate 30 min at 37 $^{\circ}$ C. Wash 2x with 200 μ L of 1x CutSmart Buffer with 2 min incubation RT. Wash with 200 μ L 1x T4 Ligase Buffer with 5 min incubation at RT. Add 150 μ L of Ligation mixture (NEB) (110 μ L NFW, 15 μ L of 10x T4 Ligase Buffer, 12 μ L 10 mM ATP, 7.5 μ L 20 mg/mL BSA, 4 μ L of 10 μ M ligated BLISS adapter, 1.5 μ L T4 Ligase). Incubate for 2 hours at RT. To remove unligated adapters, wash 4x with high salt wash buffer (10 mM Tris-HCl pH 8, 2 M NaCl, 2 mM EDTA, 0.5% Triton X-100) with 15 min incubation at 37 $^{\circ}$ C, 1x with PBS at RT (2 min incubation), 1x with NFW at RT (2 min incubation). Then, add 95 μ L of DNA extraction buffer (10 mM Tris-HCl pH 8, 100 mM NaCl, 50 mM EDTA, 1% SDS), add 5 μ L Proteinase K (NEB), scrape cells from 24-well, transfer 50 μ L each to two PCR tubes, incubate overnight at 55 $^{\circ}$ C. The next day, purify with spin columns (Qiagen MinElute), elute in 300 μ L TE buffer.
7. Sonicate samples using sonicator (Qsonica Q125), with 30% amplitude, 10s ON, 10s OFF, 1.5 min total time. Run samples on 2% gel to verify sonication size to 300-500 bp. Clean up reaction with 0.8x

volume AMPure XP beads, elute to 25 μ L NFW.

8. Assemble 20 μ L IVT reaction (NEB) (8 μ L of template in NFW, 10 μ L NTP reaction buffer, 2 μ L T7 RNAP mix), incubate at 37 °C for 4 hours in thermocycler. Add 2 μ L DNaseI to remove DNA template. Cleanup IVT reaction using Monarch RNA cleanup kit (NEB), eluting in 12 μ L NFW. Prepare RA3 adapter ligation with T4 RNA Ligase 2, truncated KQ (NEB) (5 μ L IVT RNA product, 1 μ L 10x reaction buffer, 2 μ L of 50% PEG8000, 1 μ L of 5' adenylated RA3 primer, 0.5 μ L of T4 RNA Ligase 2 truncated KQ (NEB), 0.5 μ L RNaseOUT (Thermo)). Incubate 2 hours in RT, perform RNA cleanup with Monarch RNA cleanup kit, eluting in 12 μ L NFW.

9. Prepare RT reaction with SuperScript III (Thermo Fisher; all 12 μ L of RA3 adapter-ligated sample, 1 μ L of 10 μ M RTP primer, 1 μ L of 10mM dNTP mix). Heat to 65 °C for 5 min, then transfer to ice for 1 min. Add 4 μ L 5x First-Strand Buffer, 1 μ L 100mM DTT, 1 μ L RNaseOUT, 1 μ L SuperScript III. Incubate 50 °C for 1 hour, then 70 °C for 15 min, store in -20 °C.

10. For library amplification PCR, add 1 μ L of RT sample, 9.5 μ L of NFW, 1 μ L of 10 mM RP1 primer, 1 μ L of 10 mM RP_X primer, 12.5 μ L of Q5 2x master mix (NEB). Cycle on thermocycler: 10s at 98 °C, 15 cycles of 30s at 60 °C and 30s at 72 °C, then 72 °C for 10 min.

11. Perform 0.8x volume AMPure XP cleanup to 20 μ L of elution buffer. Run on a 2% agarose gel to remove low molecular weight adapter dimers, isolating for fragments between 300 bp and 1 kb.

12. Samples were pooled, quantified with QuBit (Thermo), Bioanalyzer (Agilent) and qPCR (BioRad), then sequenced on a NextSeq 500 (Illumina) using high-output paired sequencing, with 64 bp for read1 and 36 bp for read2. The reason for this difference is that read1 starts with a 12 bp unique molecular index (UMI), followed by a 13 bp constant adapter region (CGCCATCACGCCT). This leaves 39 bp of genome-specific reads for read1. Read2 still has 36 bp genome-specific reads.

13. Reads were demultiplexed after sequencing using bcl2fastq. Only the subset of reads with the correctly matching 13 bp constant adapter region (CGCCATCACGCCT) in read1 was used for subsequent genome-side alignment. Paired-end reads were aligned to hg19 or hg38 using bowtie2. Samtools was used to filter for mapping quality \geq 25, remove singleton reads, convert to BAM format, remove potential PCR duplicates, and index reads.

Assay for Transposase-Accessible Chromatin using sequencing (ATAC-Seq)

1. In order to measure changes in accessible chromatin associated to DSB repair we performed ATAC-Seq following the Omni-ATAC protocol from⁴ with minimal modifications.

2. 900,000 HEK293T cells were electroporated as described above (Electroporation of Cas9 RNP section), but using reduced reagents' volumes: 1 μ L of crRNA, 1 μ L of tracrRNA, 1.5 μ L of Cas9, 1.5 μ L of

dialysis buffer and 20 μL of nucleofection solution, using SF Cell Line 4D-Nucleofector™ X Kit S (3.6 μL of Supplement solution and 16.4 μL of SF solution). After electroporation, cells were transferred to 3 mL of complete media and plated into two wells of a 12-well plate pre-coated with collagen. In parallel, 400,000 non-electroporated cells were harvested and were plated in another well of the same 12-well plate. Three hours after electroporation, cells were washed with PBS and harvested via scraping. Cells were counted and 50,000 cells were used for ATAC for each condition (electroporation and no-electroporation).

3. Harvested cells were centrifuged for 5 min at $500 \times g$, 4°C in a swinging-bucket rotor. The supernatant was carefully removed so as not to perturb the small, barely visible pellet. Cells were then resuspended in 50 μL of cold lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, and 3 mM MgCl_2 supplemented with 0.1% Igepal-CA630, 0.1% Tween-20, and 0.01% digitonin), gently mixed (pipetting up and down three times) and incubated on ice for 3 min.
4. 1 mL of wash buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, and 3 mM MgCl_2 supplemented with 0.1% Tween-20) was then added and was mixed by inverting the tube three times. Nuclei were then centrifuged at $500 \times g$ for 10 min, 4°C in a swing-bucket rotor.
5. The supernatant was carefully discarded and the nuclei were resuspended in 50 μL of transposition reaction (25 μL 2xTD buffer, 16.5 μL PBS, 5 μL NF H₂O, 1 μL 10% tween-20, 1 μL 1% digitonin, 2.5 μL transposase) and incubated at 37°C for 30 min in a Thermomixer with shaking at 1,000 rpm.
6. After transposition, DNA was purified using a Qiagen MinElute PCR purification kit. The product was eluted in 21 μL of elution buffer.
7. Transposed DNA was amplified using the conditions and primers described in⁵. Primer sequences can also be found in **Supplementary Table 9** of associated manuscript. We first pre-amplified the DNA product, by mixing 19 μL of transposed DNA, 6.25 μL of primer set F/R (10 μM) and 25 μL of NEBNext® High-Fidelity 2X PCR MM. The sample was well mixed and cycled as follows: 5 min 72°C , 30 sec 98°C , 5 cycles: [10 sec 98°C , 30 sec 63°C , 1 min 72°C].
8. A qPCR reaction was then run to determine the number of cycles needed for final amplification. Mix: 5 μL of pre-amplified DNA (from previous step), 0.63 μL PCR Primer Set F/R 10 μM , 4.4 μL SYBR Green 2x and 5 μL NEBNext® High-Fidelity 2X PCR MM. Using a qPCR (BioRad) thermocycler, cycle as follows: 30 sec 98°C , 20 cycles: [10 sec 98°C , 30 sec 63°C , 1 min 72°C].
9. Determine the cycle number that corresponds to one-fourth of the maximum fluorescent intensity. We found that this threshold is reached with very few cycles (typically two or three).
10. Run the remaining PCR reaction to the cycle number determined. Cycle as: 30 sec 98°C , N cycles: [10 sec 98°C , 30 sec 63°C , 1 min 72°C].
11. While PCR is running, take AMPure XP beads to room temperature, and vortex to resuspend. Purify the amplified product using double-sided AMPure bead purification (0.5x and 1.3x) to remove both

primers and large products (>1 kb).

12. Elute final DNA libraries were eluted in 32 μ L of IDTE. Load 5 μ L in a 2% agarose gel to check for the quality of the library.

13. Samples were pooled, quantified with QuBit (Thermo), Bioanalyzer (Agilent) and qPCR (BioRad), then sequenced on a NovaSeq 500 (Illumina) using paired 2x50 bp reads. Reads were demultiplexed after sequencing using bcl2fastq. Paired-end reads were aligned to hg19 or hg38 using bowtie2. Samtools was used to filter for mapping quality \geq 25, remove singleton reads, convert to BAM format, remove potential PCR duplicates, and index reads. Finally, because NovaSeq outputs two lanes for each sample, the two lanes for each sample were merged using samtools.

Troubleshooting

Time Taken

Anticipated Results

References

1. Kreitzer, F.R. *et al.* A robust method to derive functional neural crest cells from human pluripotent stem cells. *Am J Stem Cells* **2**, 119-131 (2013).
2. Wienert, B. *et al.* Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq. *Science* **364**, 286-289 (2019).
3. Yan, W.X. *et al.* BLISS is a versatile and quantitative method for genome-wide profiling of DNA double-strand breaks. *Nature Communications* **8**, 15058 (2017).
4. Corces, M.R. *et al.* An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. *Nature Methods* **14**, 959-962 (2017).
5. Buenrostro, J.D., Wu, B., Chang, H.Y. & Greenleaf, W.J. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. *Current Protocols in Molecular Biology* **109**, 21.29.21-21.29.29 (2015).