Superb-seq v1.0[1]

Experimental design

CRITICAL To maximize performance in the cell type of interest, optimization of CRISPR-Cas9 editing efficiency is strongly recommended. As needed, test different guide RNAs, Cas9 ribonucleoprotein and T7 promoter concentrations, electroporation settings (or instruments), and post-electroporation recovery conditions. See Parse user manuals for additional recommendations for maximizing cell fixation and single-cell library quality for particular cell types.

This full protocol has been validated for human HEK293T, K562, and U2OS cells. T7 promoter labeling with this protocol has been additionally validated for human GM12878, Jurkat, and primary T cells.

CRITICAL To analyze differential expression of detected on-target and off-target edits, include a control sample of cells with identical electroporation and T7 in situ transcription treatment, but without editing.

Identically treated cells, but without T7 RNA polymerase, can be included as an additional control sample. This can be useful for assessing sources of background T7 signal (e.g. endogenous T7 promoter-like sequences).

Materials

Biologicals

- Live cells amenable to electroporation of CRISPR-Cas9 ribonucleoprotein (RNP)

Reagents

Part 1 – Labeling Cas9 edits with T7 promoters

- All materials needed for culturing and expanding cells of interest (e.g. complete culture medium, antibiotics, supplements, culture flasks and plates)
- Sterile pipette tips for P2, P10, P20, P200, and P1000 (avoid wide-bore P1000 tips)
- Low-retension nuclease-free tips for P2, P10, P20, P200, and P1000 pipettes (avoid wide-bore P1000)
- Serological tips, sterile
- PCR tube strips, nuclease-free
- 15 mL, 50 mL conical tubes
- 6-well flat-bottom tissue culture plates
- Electroporation buffer (Maxcyte #EPB-1)

- S. p. Cas9 protein 10 µg/µL, (IDT #1081059)
- Electroporation enhancer 100 µM (IDT #1075916)
- OC-25x3 process assemblies (MaxCyte #SOC-25x3)
- Single-stranded guide RNA (sgRNA), synthesized and reconstituted to **125 µM** (4 µg/µL) in electroporation buffer, with the following sequence (5' to 3'):

mN*mN*mN*rNrNrNrNrNrNrNrNrNrNrNrNrNrNrNrNrGrUrUrUrCrArGrArGrCrUrArC
rArGrCrArGrArArArArUrGrCrUrGrUrArGrCrArArGrUrUrGrArArArUrArArGrGrCrUrAr
GrUrCrCrGrUrUrArUrCrArArCrUrUrGrArArArArArGrUrGrGrCrArCrCrGrArGrUrCrG
mG*mU*mG*rC

CRITICAL Replace "N" sequence with a 20-base guide sequence targeting an "NGG" PAM, selected for efficiency and specificity by GuideScan2[2] (https://www.guidescan.com) or other equivalent tool. The above sequence incorporates common scaffold and terminal base modifications[3]. Indicated are RNA bases (rN), 2-O-methyl bases (mN), and phosphorothioate links (asterisk).

- Superb-seq T7 promoter, pre-annealed, reconstituted to **100 µM** in electroporation buffer, with the following duplexed sequences (5' to 3'):

1. **/5Phos/GTGAATTTAATACGACTCACTATAGGGAGAGT*A*T**
2. **/5Phos/ATACTCTCCCTATAGTGAGTCGTATTAAATTC*A*C**

CRITICAL Include 5' phosphate (/5Phos/) and phosphorothioate links (asterisk).

- Evercode Cell Fixation v3 kit, 12 samples (Parse Biosciences #ECFC3300)
- All materials required by fixation kit user manual (link below)

Part 2 – In situ transcription of Cas9 edits

- HiScribe T7 Quick RNA synthesis kit (New England BioLabs #E2050S)
- RiboLock RNase inhibitor 40 U/µL (Thermo Scientific #EO0382)
- Sterile cell strainers 40 µm (Fisherbrand #22363547), or alternative size suitable for cell type of interest
- Trypan blue

Part 3 – Single-cell RNA-seq

- Evercode WT Mini v3 library kit, 12 samples (Parse Biosciences #ECWT3100)
- All materials required by library kit user manual (link below)
- UDI WT plate (Parse Biosciences #UDI1001)
- Magnetic rack (Parse Biosciences #SB1004)

Equipment

- Automated cell counter with imaging
- Centrifuge with temperature control and swinging bucket adapters for microwell plates and 1.5 mL, 15 mL, and 50 mL tubes
- Ice bucket
- Incubator
- Laminar flow hood
- Maxcyte ATx (or alternative nucleofection instrument)
- P2, P10, P20, P200, and P1000 pipettes
- PCR thermal cycler
- Racks for PCR tube strips and 1.5 mL, 15 mL, and 50 mL tubes
- Serological pipette controller

User Manuals

- Parse Cell Fixation v3 kit user manual, current version (https://support.parsebiosciences.com/hc/en-us/articles/23914547728660-Evercode-Cell-Fixation-v3-User-Guides)
- Parse WT v3 library user manual, current version (https://support.parsebiosciences.com/hc/en-us/articles/23911840786196-Evercode-WT-v3-User-Guides)

Software

- Split-pipe (https://support.parsebiosciences.com/hc/en-us/articles/17200056667924-Pipeline-Download-Current-Version)
- Sheriff (https://github.com/BradBalderson/Sheriff)
- Superb-seq differential expression analysis scripts (https://github.com/BradBalderson/superb_analysis)

Note – Login is required for Split-pipe download. Contact Parse Biosciences for access.

Note – This protocol has been validated for v1.1.1 of Split-pipe. Downgrade Split-pipe if the current release generates errors.

Procedure

Part 1 – Labeling of Cas9 edits with T7 promoters (3 days)

Cell preparation

1. Per sample, expand a minimum of **2.5 million** cells of interest, plus up to 50% extra to accommodate loss during spin-down and washing.

Note – A typical requirement is 2.5 million cells per sample, to perform a 25 µL ATx electroporation at a cell concentration of 100 million cells/mL. For other nucleofection devices, consult technical support or publications. Optimize as necessary.

2. Incubate a 6-well tissue culture plate(s) with complete cell culture medium, to receive post-electroporation samples of 2.5 million cells (or alternative determined cell count) at the desired recovery concentration (e.g. 0.5–1 million cells/mL).

RNP preparation

3. Fill an insulated bucket with ice.

4. In a laminar flow hood, prepare RNP in a PCR tube, **2 µL** per sample (2 to 1 molar ratio of sgRNA to Cas9). Mix gently and fully, avoiding bubbles. Scale by number of samples per sgRNA:

    a. **1 µL** sgRNA

    b. **1 µL** Cas9 protein

Note – Add sgRNA first, then mix in the more viscous Cas9 to minimize volume loss. If treating multiple samples with the same RNP, prepare a scaled-up RNP master mix.

Note – This RNP concentration is optimal for ATx electroporation of many cell types. Consult instrument support or publications for your cell type. Optimize as necessary.

5. Incubate RNP at room temperature for 15 minutes. Immediately store on ice.

Cell washing and counting

6. Harvest cells into 15 mL or 50 mL conical tubes. Spin down cells at 300 x *g* for 5 minutes, or another minimally sufficient force for the cell type. Aspirate supernatant.

7. Resuspend cells in **1 mL** of electroporation buffer. Add **4–9 mL** of electroporation buffer. If cells are in one or multiple 50 mL tubes, consolidate cells into one 15 mL tube. Record final volume.

8. Count resuspended cells with an automated counter (with trypan blue or other viability dye). Record cell concentration and percent viability. Calculate and record the total resuspension volume for **125 million cells/mL**.

9. Spin down cells at 300 x *g* for 5 minutes (or alternative chosen force). During spin-down, start **step 10**.

10. Aspirate spun-down cells, completely and carefully. Fully resuspend cells in **~50%** of the calculated resuspension volume of electroporation buffer. Check the total volume by pipette. Add more electroporation buffer to the exact total volume for 125 million cells/mL.

Note – This cell concentration is optimal for ATx electroporation of many cell types. Consult instrument support or publications for your cell type. Optimize as necessary.

Note – At this high cell concentration, cells can compose ~50% or more of the total final volume. To avoid over-diluting cells during resuspension, initially resuspend in a fraction of the total final volume, then add more volume as necessary. If the cell slurry is too viscous to resuspend to the planned cell concentration, add a minimum sufficient additional volume of electroporation buffer, and record the new cell concentration.

Example – To resuspend 10 million K562 cells to 125 million cells per mL, the final total volume will be 80 µL. First resuspend cells in 40 µL of electroporation buffer, check the total volume by pipette, and add more electroporation buffer to exactly 80 µL total.

ATx electroporation

11. Within each tube of RNP, prepare a loading agent master mix, **5 uL** per sample. Mix gently and fully. Scale by RNP volume per sample:
    a. **2 µL** RNP
    b. **1 µL** T7 promoter
    c. **1 µL** Electroporation enhancer
    d. **1 µL** Electroporation buffer

Note – This master mix is optimal for edit labeling of many cell types. Consult instrument support or publications for your cell type. Optimize as necessary.

12. Gently resuspend cell slurry immediately before pipetting.
13. With the same pipette tip, add **20 µL** of cell slurry (2.5 million cells) to each **5 µL** loading mix (RNP and T7 promoter), and immediately transfer each sample to one well of an OC-25x3 process assembly (or alternative nucleofection cassette). Avoid bubbles. Finish loading all samples. Final concentrations will be:
    a. 100 million cells/mL
    b. 2.5 µM Cas9
    c. 5 µM sgRNA
    d. 4 µM T7 promoter
    e. 4 µM Electroporation enhancer

Note – If preparing multiple cell types, load one cell type per process assembly, to apply cell-type specific ATx electroporation settings.

14. Serially load process assemblies onto the ATx instrument and run electroporation with recommended settings.

15. If required, perform post-electoporation recovery procedure (e.g. incubation of cells in process assembly at 37°C for 15 minutes).

Note – Consult instrument support or publications for electroporation settings and post-electroporation cell recovery protocols for your cell type. Optimize as necessary.

16. Transfer cells to pre-warmed 6-well plate(s). Transfer residual cells by washing each process assembly well with 20 µL of cell culture medium, and transferring.

17. Incubate cells for 3–4 days under normal conditions (e.g. 37°C, 5% CO_2).

18. Harvest ~0.5 million cells per sample for genomic DNA extraction.

19. **Optional stopping point**: harvest cells and cryopreserve normally.

20. Proceed immediately to **step 21**.

Quality control – Extract genomic DNA. Assess T7 promoter labeling efficiency by TIDE analysis of genomic DNA samples (see manuscript[1]).

Cell fixation

21. Prepare an RNase-free working space.

22. Perform cell fixation according to Parse Cell Fixation v3 kit user manual. Input 1 million cells per sample. Keep fixed cells on ice.

Note – 40 µM cell strainers are suitable for isolating singlet fixed cells from many cell types. If necessary, optimize cell strainer pore size for singlet purity and yield.

23. Count fixed cells. Per sample, prepare 2–3 aliquots of **35 µL** of ~200,000 cells per sample (150,000 to 250,000 cells) in “cell storage mix” prepared according to the user manual.

CRITICAL Save leftover “cell storage mix” components for Section 2.

Note – Typical yield is ~500,000 fixed cells from 1 million input cells (~50%).

24. **Optional stopping point:** Transfer aliquots to Mr. Frosty containers. Store at -80°C.

25. Proceed immediately to **step 26**.

CRITICAL If proceeding directly to Section 2, ensure that all materials have been obtained for Section 3, which is performed immediately afterward. Carefully review Parse WT Mini v3 user manual, used for this section.

Part 2 – In situ transcription of Cas9 edits (1.5 days)

Overnight T7 in situ transcription (IST)

26. Prepare an RNase-free working space.

27. Fill a bucket with ice.

28. Prepare an IST master mix on ice, **27.5 µL** per sample. Scale by total samples:

    a. **22 µL** 2X HiScribe NTP-cofactor buffer mix

    b. **4.4 µL** 10X HiScribe T7 polymerase

    c. **1.1 µL** RiboLock

Note – If fixed cell counts are sufficient, double master mix volume and prepare duplicate IST reactions per sample. This will help ensure sufficient post-IST cell counts for library generation.

Note – For a typical Superb-seq library of 10,000 cells across 12 samples (1600 cells per sample), 1–2 IST reactions per sample is sufficient to yield the required 300 cells/µL concentration (0.3 million cells/mL) for library input. If sequencing more cells per sample, scale the number of IST reactions per sample to 2 or more as necessary to meet cell concentration requirements. See library kit user manual for more information.

29. Thaw fixed cells from **step 24**, or obtain fresh cells from **step 25**. Keep on ice.

30. Gently resuspend cells by pipetting 3 times. Add **15 µL** of fixed cells (>50,000 cells) to a PCR tube, per IST reaction.

31. Add **25 uL** of IST master mix. Mix gently by pipetting 5 times.

Note – Avoid splashing and centrifugation before incubation.

32. Incubate in a thermal cycler at **40°C** overnight, up to 24 hours.

33. ~1 hour before incubation end, prepare **50 µL** of fresh “cell storage mix” per sample, according to the fixation kit user manual. Pre-chill centrifuge with PCR plate adapter to 4°C.

Note – Alternatively, thaw leftover “cell storage buffer” from fixation kit, if the volumes of other cell storage mix components are insufficient.

34. Transfer IST reactions to ice. Proceed immediately to **step 35**.

Post-IST cell washing

35. Pool all IST reactions intended for each sequencing sample in PCR tubes (e.g. duplicate IST reactions per sample, if prepared).

CRITICAL Do NOT pool across intended sequencing samples.

36. Spin down pooled IST samples at 300 x *g* for 5 minutes at 4°C.

37. Carefully remove supernatant by P200 pipette, without disturbing cell pellet. Transfer supernatant to a low-bind 1.5 mL tube.

QC: Extract total RNA from IST supernatants. Measure T7 RNA levels by RT-qPCR (**Methods**).

38. Resuspend each IST pellet in **40 µL** cell storage by gently pipetting 10 times. Immediately filter through 40 µM cell strainer (or alternative optimized pore size) into a low-bind 1.5 mL tube. Store strained cells on ice.

CRITICAL Pipette fast and forcibly to minimize volume loss.

39. Prepare a 1:2 diluted aliquot of cells for counting by mixing **5 µL** of cells with **5 µL** of cell storage mix (or cell storage buffer). Add **10 µL** of trypan blue.
40. Count cells with an automated counter. Record undiluted cell concentration, percent viability (0% expected), and rate of singlets, cell clusters, and debris. Record image if able.
41. Proceed immediately to **step 42**.

Part 3 – Combinatorial single-cell RNA-seq (3 days)

Generate sequencing libraries

42. Prepare an RNase-free working space.
43. Generate single-cell sequencing libraries according to the Parse WT v3 library kit user manual.

Check library quality

44. Measure library concentration by Qubit fluorometer, according to the user manual.
45. Measure library size distribution by TapeStation or BioAnalyzer, according to the user manual.
46. Store sequencing libraries at -20°C.

Sequence libraries

47. Submit individual or pooled libraries to an Illumina sequencing service provider according to their instructions, with the following sequencing run configuration:
    a. 100/8/8/58 cycles for Parse v3 library (Read 1, i7 index, i5 index, Read 2)
    b. > 50,000 reads per cell (e.g. 500 million reads for a 10,000 cell library)
    c. 5% PhiX

Part 4 – Superb-seq analysis

Run Split-pipe

48. Install Split-pipe according to documentation instructions.
49. Build alignment index according to documentation instructions.

50. Run Split-pipe on raw paired-end reads (R1 and R2 FASTQ files) in a Linux or macOS command line terminal:

```
split-pipe \
    --mode all --nthreads 16 --chemistry v3 \
    --genome_dir 'PATH/TO/GENOME' --output_dir 'PATH/TO/OUTPUT' \
    --fq1 'PATH/TO/READS/R1.fastq' --fq2 'PATH/TO/READS/R2.fastq' \
    --sample SAMPLE_A A1-A2 \
    --sample SAMPLE_B A3-A7 \
    --sample SAMPLE_C A8-A12
```

CRITICAL Replace `PATH/TO/GENOME`, `PATH/TO/OUTPUT` , and `PATH/TO/READS`, with your directory and file paths.

CRITICAL Update `SAMPLE_A A1-A2`, etc., with your sample definitions. This example command defines `SAMPLE_A` as round 1 plate wells A1–A2, `SAMPLE_B` in wells A3–A7, and `SAMPLE_C` in wells A8–A12. See Split-pipe documentation for more information about sample definition.

Note – Split-pipe can take many hours to complete, depending on software version and CPU performance. Higher thread count may reduce run time.

Run Sheriff

51. Install Sheriff according to repository instructions.
52. Run Sheriff according to repository instructions.

Note – Sheriff requires two output files from Split-pipe:

- The BAM file of aligned reads, `barcode_headAligned_anno.bam`
- A text file list of valid cell barcodes from column `bc_wells` from `cluster_assignment.csv`, `cluster_umap.csv`, or `tscp_counts.csv` (see theexample in the Sheriff repository).

Perform differential expression analysis

53. Install software dependencies according to repository instructions.
54. Run differential expression analysis according to repository instructions.

Troubleshooting

Issue	*Action*
Low editing or donor labeling efficiency.	Screen alternative guide RNA designs. Optimize concentration of cells, Cas9, guide, and/or T7 promoter. Optimize electroporation device settings.
High rate of cell clusters after fixation or overnight IST.	Optimize cell strainer pore size. See the Parse fixation user manual for additional instructions.
Insufficient cell concentration for library preparation.	Increase the number of IST reactions per sample. Optimize cell strainer pore size and centrifugation force for the cell type of interest. See the Parse user manuals for additional instructions.
Split-pipe generates an error.	Contact Parse Biosciences.
Sheriff generates an error.	Submit an issue on the Sheriff repository. (https://github.com/BradBalderson/Sheriff/issues)
Differential expression analysis generates an error.	Submit an issue on the repository. (https://github.com/BradBalderson/superb_analysis/issues)

References

1. Lorenzini, M. H., Balderson, B., Sajeev, K., Ho, A. J. & McVicker, G. Joint single-cell profiling of Cas9 edits and transcriptomes reveals widespread off-target events and effects on gene expression. *bioRxivorg* 2025.02. 07.636966 (2025) doi:10.1101/2025.02.07.636966.
2. Schmidt, H. *et al.* Genome-wide CRISPR guide RNA design and specificity analysis with GuideScan2. *Genome Biol.* **26**, 41 (2025).
3. Hendel, A. *et al.* Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat. Biotechnol.* **33**, 985–989 (2015).