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TF and epigenetic modifier CRISPRi/a screens in human T cells

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We use this protocol and it's working

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Abstract

This protocol describes methods for CRISPR interference or activation screens identifying transcriptional and epigenetic regulators of human CD8+ T cell state.

Materials

pLV hU6-gRNA hUbC-dSaCas9-KRAB-T2A-Thy1.1 (Addgene 194278)
pLV hU6-gRNA hUbC-VP64-dSaCas9-VP64-T2A-Thy1.1 (Addgene 194279)

Troubleshooting

TF and epi-modifier CRISPRi/a gRNA library construction

1 The TSSs for each TF and epi-modifier were extracted using CRISPick and 1,000-bp windows were constructed around each TSS (−500 to +500 bp).

2 After establishing an SaCas9 gRNA database with the strict PAM variant (NNGRRT) using guideScan[66], the genomic windows were input into the guidescan_guidequery function to generate the gRNA library.

3 Any gRNA that aligned to another genomic site with fewer than four mismatches was removed from the library. The final gRNA library contained at least seven gRNAs targeting 120/121 target gene (there were no *PBX2*-targeting gRNAs) with an average of 16 gRNAs per gene.

4 A total of 120 NT gRNAs were included in the library for a total of 2,099 gRNAs (available in Supplementary Table 2, McCutcheon et al. Nature Genetics, 2023. https://doi.org/10.1038/s41588-023-01554-0)

gRNA library cloning

5 Oligonucleotide pools containing variable gRNA sequences and constant regions for polymerase chain reaction (PCR) amplification were synthesized by Twist Bioscience.

6 gRNA amplicons were gel extracted, PCR purified and input into 20 µl Gibson reactions (5:1 molar ratio of insert to backbone) with 200 ng of Esp3I digested and 1 × solid-phase reversible immobilization (SPRI)-selected (Beckman Coulter) plasmid backbone.

6.1 Addgene: pLV hU6-gRNA hUbC-dSaCas9-KRAB-T2A-Thy1.1 (Addgene 194278) and pLV hU6-gRNA hUbC-VP64-dSaCas9-VP64-T2A-Thy1.1 (Addgene 194279).

7 Gibson reactions were purified using ethanol precipitation and transformed into Lucigen's Endura ElectroCompetent Cells.

8 Transformed cells were cultured overnight and plasmids were isolated using Qiagen Midi Kits.

Transfections for high-titer lentiviral production

9 Plate 1.2 × 106 or 7 × 106 HEK293T cells in a 6 well plate or 10 cm dish in the afternoon with 2 mL or 12 mL of complete opti-MEM (Opti-MEM‰ I Reduced Serum Medium supplemented with 1x Glutamax, 5% FBS, 1 mM Sodium Pyruvate, and 1x MEM Non-Essential Amino Acids).

10 The next morning, transfect HEK293T cells with 0.5 µg pMD2.G, 1.5 µg psPAX2, and 0.5 µg transgene for 6 well plates or 3.25 µg pMD2.G, 9.75 µg psPAX2, and 4.3 µg transgene for 10 cm dishes using Lipofectamine 3000.

11 Exchanged media 6 hours after transfection and collect and pool lentiviral supernatant at 24 hours and 48 hours after transfection.

Transduction of primary human T cells

12 Centrifuged lentiviral supernatant at 600g for 10 min to remove cellular debris.

13 Concentrate lentivirus to 50–100× the initial concentration using Lenti-X Concentrator (Takara Bio).

14 Transduce T cells at 5–10% v/v of concentrated lentivirus at 24 h post-activation. For dual transduction experiments, T cells were serially transduced at 24 h and 48 h.

TF and epi-modifier CRISPRi/a gRNA screens

15 $CD8^{+}CCR7^{+}$ T cells were sorted and transduced with either CRISPRi or CRIPRa TF + epi-modifier gRNA libraries at a low MOI.

16 Cells were expanded for 10 days and then stained for Thy1.1 (a marker to identify transduced cells) and CCR7 (a marker associated with T cell state).

16.1

	Antibody Target	Fluorophore/ Sequence	Clone	Isotype	Dilution	Application	Manufacturer	Catalog #	Notes
	CCR7	FITC	150503	Mouse IgG2a	1:100	Flow cytometry	BD Biosciences	561271	Stain at 37C
	Thy1.1	PE	OX-7	Mouse IgG1, κ	1:300	Flow cytometry	StemCell Technologies	60024 PE	-

17 An SH800 FACS Cell Sorter (Sony Biotechnology) was used for cell sorting and analysis.

18 For antibody staining of Thy1.1 cells were collected, spun down at 300*g* for 5 min, resuspended in flow buffer (1× phosphate-buffered saline (PBS), 2 mM ethylenediaminetetraacetic acid and 0.5% bovine serum albumin) with the appropriate antibody dilutions and incubated for 30 min at 4 °C on a rocker. Antibody staining of CCR7 was carried out for 30 min at 37 °C.

19 Cells were then washed with flow buffer, spun down at 300*g* for 5 min and resuspended in flow buffer for cell sorting.

20 Transduced cells in the lower and upper 10% tails of CCR7 expression were sorted for subsequent gRNA library construction and sequencing. All replicates were maintained and sorted at a minimum of 300× coverage.

gRNA sequencing

21 Genomic DNA was isolated using Qiagen's DNeasy Blood and Tissue Kit. Genomic DNA was split across 100 µl PCR reactions (25 cycles at 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 20 s) with Q5 2× Master Mix and up to 1 µg of genomic DNA per reaction.

22 PCRs were pooled together for each sample and purified using double-sided (SPRI)bead selection at 0.6× and 1.8×.

23 Libraries were run on a High Sensitivity D1000 tape (Agilent) to confirm amplicon size and quantified using Qubit's dsDNA High Sensitivity assay.

24 Libraries were diluted to 2 nM, pooled together at equal volumes, and sequenced using Illumina's MiSeq Reagent Kit v2 (50 cycles).

25 Primers are available in Supplementary Table 5 of McCutcheon et al. Nature Genetics, 2023. https://doi.org/10.1038/s41588-023-01554-0

Processing gRNA sequencing and gRNA analysis

26 FASTQ files were aligned to custom indexes for each gRNA library (generated from the bowtie2-build function) using Bowtie 2 (ref. **67**).

27 Counts for each gRNA were extracted and used for further analysis in R.

28 Individual gRNA enrichment was determined using the DESeq2 (ref. **68**) package to compare gRNA abundance between groups for each screen.

Gene-level analysis for CRISPRi/a screens

29 DESeq2 *P* values were empirically transformed to cumulative probabilities using a midpoint linear interpolation of the 120 NT gRNA *P* values between 0 and 1. This transformation aligns the data with the null hypothesis that NT gRNA *P* values have a uniform distribution between 0 and 1.

30 Within each gene, transformed *P* values were aggregated using a modified robust rank aggregation method to detect genes with nonuniform (non-null) gRNA *P* values.

31 A gene-level *P* value was produced by comparison with 10 million gene-level null simulations of *P*values randomly sampled from a uniform distribution.

32 NT gRNAs were randomly grouped into NT control 'genes' (NTCs) and analyzed in the same way.

33 The number of gRNAs per NTC was sampled with replacement from the distribution of gRNAs per gene in the screen until all the NT gRNAs were used.

34 Genes were selected as hits if their Benjamini–Hochberg false discovery rate (FDR) was less than 0.05. Gene-level aggregation was done in Python.

35 Two effect sizes were computed for each gene by averaging gRNAs' unshrunk DESeq2 $\log_2$FoldChange within the gene, weighted by each gRNA's transformed one-sided *P* value.

36 The larger (absolute value) effect size was chosen for each gene. Effect sizes were estimated in R.

Protocol references

67. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357–359 (2012).

68. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).