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CRISPRi tiling screens

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We use this protocol and it's working

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Abstract

This protocols describes methods for characterizing the activity of dSaCas9 as a repressor using promoter tiling guide RNA screens in primary human T cells.

Materials

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

Primary human CD8+ T cell cultures

1 Isolated CD8+ T cells from individual donors were obtianed directly from vials purchased from StemCell Technologies.

2 Culture T cells were in PRIME-XV T cell Expansion XSFM (FujiFilm) supplemented with 5% human platelet lysate (Compass Biomed), 100 U ml−1 penicillin and 100 µg ml−1 streptomycin. All media were supplemented with 100 U ml−1 human IL-2 (Peprotech).

Designing CD2 and BM2 CRISPRi gRNA libraries

3 Saturation *CD2* and *B2M* CRISPRi gRNA libraries were designed to tile a 1,050-bp window (−400 bp to 650 bp) around the transcription start site (TSS) of each target gene using CRISPick[61].

4 Each gRNA library was designed to target dSaCas9's relaxed protospacer adjacent motif (PAM) variant: 5′-NNGRRN-3′. NT gRNAs were generated for each library to match the nucleotide composition of the targeting gRNAs.

5 CD2 and B2M gRNA libraries are available in Supplementary Table 1 of McCutcheon et al. *Nature Genetics*, 2023. https://doi.org/10.1038/s41588-023-01554-0

gRNA library cloning

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

7 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, pLV hU6-gRNA hUbC-dSaCas9-KRAB-T2A-Thy1.1 (Addgene 194278).

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

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

Lentiviral transduction of T cells

10 CD8$^+$ T cells from pooled peripheral blood mononuclear cell donors (see step 1) were transduced with all-in-one lentivirus encoding for dSaCas9–KRAB–2A–GFP and either CD2 or B2M gRNA libraries.

11 Lentivirus was produced as previously described[60].

12 Lentiviral supernatant was centrifuged at 600*g* for 10 min to remove cellular debris and concentrated to 50–100× the initial concentration using Lenti-X Concentrator (Takara Bio).

13 T cells were transduced at 5–10% v/v of concentrated lentivirus.

CRISPRi tiling screens

14 Following transduction, cells were expanded for 9 days and then stained for the target gene.

15 For antibody staining of surface markers, 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.

15.1

	Antibody Target	Fluorophore/Sequence	Clone	Isotype	Dilution	Application	Manufacturer	Catalog #
	CD2	PE	RPA-2.10	Mouse / IgG1, kappa	1:50	Flow cytometry	Thermo	12-0029-42
	B2M	PE	A17082A	Mouse IgG1, κ	1:50	Flow cytometry	Biolegend	395704

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

17 An SH800 FACS Cell Sorter (Sony Biotechnology) was used for cell sorting and analysis. Fluorescent minus one (FMO) controls were used to set appropriate gates for all flow panels.

18 Transduced cells in the lower and upper 10% tails of target gene expression were sorted for subsequent gRNA library construction and sequencing. All replicates were maintained and sorted at a minimum of 350× coverage.

gRNA sequencing

19 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.

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

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

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

23 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

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

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

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

Protocol references

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