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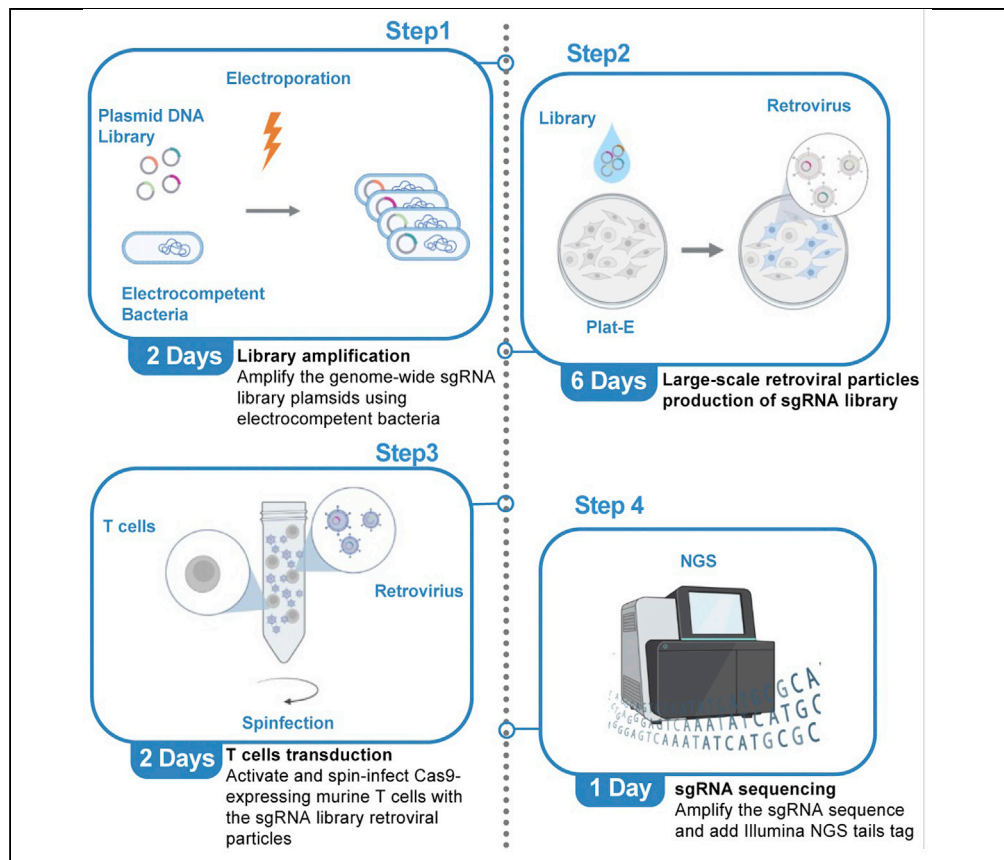
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Protocol

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Highlights

Detailed description of mouse genome-wide CRISPR knockout library amplification

Large-scale production of retroviral library

Optimized transduction of murine Cas9-expressing T cells

PCR amplification of sgRNA region for NGS analysis

In vivo genome-wide CRISPR screens in primary T cells allow the systematic and unbiased identification of non-redundant regulatory mechanisms shaping immune responses. Here, we present an optimized protocol for efficient generation of a pool of genome-wide inactivated Cas9-expressing T cells using a retroviral library of sgRNA. We detail the process of large-scale viral production and library integration in activated murine T cells as well as the two-step PCR approach for sgRNA recovery and abundance evaluation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Optimized protocol to generate genome-wide inactivated Cas9-expressing murine T cells

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SUMMARY

***In vivo* genome-wide CRISPR screens in primary T cells allow the systematic and unbiased identification of non-redundant regulatory mechanisms shaping immune responses. Here, we present an optimized protocol for efficient generation of a pool of genome-wide inactivated Cas9-expressing T cells using a retroviral library of sgRNA. We detail the process of large-scale viral production and library integration in activated murine T cells as well as the two-step PCR approach for sgRNA recovery and abundance evaluation.**

For complete details on the use and execution of this protocol, please refer to Sutra Del Galy et al. (2020).

BEFORE YOU BEGIN

This protocol describes an efficient method for incorporating the Teichmann Retroviral Mouse Genome-wide CRISPR Knockout (GW-KO) library of sgRNA (Addgene# 104861), covering 18,424 mouse genes into cas9-expressing murine T cells. Originally designed by Kosuke Yusa Laboratory, the library of sgRNA was cloned into the pMSCV-U6sgRNA(BbsI)-PGKpuro2ABFP retroviral vector (mock, Addgene #102796). This library presents numerous advantages for efficient genome-wide CRISPR screens in T cells. With 90 230 sgRNA targeting 18 424 murine genes (5sgRNA/gene), in a small retroviral vector (efficient transduction) with a Blue Fluorescent Protein reporter (integration tracking), a puromycin resistance cassette (mutated cells enrichment), it also possesses an improved scaffold for higher gene knockout efficiency.^{1,2} This method includes several crucial steps such as, mock and library plasmids amplification, large-scale viral production and transduction of activated recipients T cells. To confirm the integration of one sgRNA per cell, we also provide details on single infection percentage (SIP) calculation. Finally, to characterize the sgRNA library representation at each step of the screening process, we describe the two-step PCR conditions enabling its analysis by next generation sequencing (NGS).

Institutional permissions

All experiments involving animals are conducted with 6–12 weeks old mice, in an accredited animal facility by the French Veterinarian Department following ethical guidelines, approved by the relevant ethical committee (APAFIS #35057-2022013112272631 v4). To conduct this type of experiments you will need to acquire permissions from the relevant institutions.

Safety with retroviruses (RV)

Of note, it is mandatory to perform the steps involving RV supernatants and transduced samples following biosafety procedures.



All experiments should be carried out in BSL2 or more laboratory with a class II biological safety cabinet (BSC).

All operators must wear the following Personal Protective Equipment (PPE): double gloves, disposable lab coat. Surgical masks and goggles are also recommended.

Everything that comes in contact with retroviral material, as well as liquid or solid wastes must be decontaminated before exiting the BSC for at least 30 min in concentrated bleach solution.

The BSC surfaces must be disinfected with detergents or 70% ethanol.

Transportation or storage of any infected or transduced material shall be done in leak proof secondary container with explicit label of biohazard material.

Petri dishes for bacterial growth

⌚ Timing: 2 h

Prepare ten Petri dishes with solid LB+ Ampicillin medium for bacterial culture, to measure electroporation efficacy.

1. Prepare 1 L of sterile LB agar medium in a 2 L bottle following the [manufacturer's instructions](#).
2. Melt your bottle of gel mix at 60°C in a water bath.
3. Under sterile conditions, add 500 µL of Ampicillin at [50 mg/mL] for 1 L of medium.
4. Pour 10 mL of gel mix with Ampicillin in each 10 cm Petri dishes and let it cool until it solidifies.

Note: 8 plates will be needed for this protocol but you can store the remaining plates at 4°C up to two months.

Note: If you use larger plates make sure to adjust the volumes accordingly.

Culturing Plat-E cells

⌚ Timing: 1 week

Platinum-E (Plat-E) Retroviral Packaging Cell Line is a 293T cell derived line containing *gag*, *pol* and *env* genes maintained with antibiotics. It allows high retroviral yields (average titer 10^6 – 10^7 infectious units/mL) with a single plasmid transfection.

5. Thaw Plat-E cells stock (10×10^6 cells), stored at -80°C and liquid nitrogen (passage < 10).
6. Resuspend in 9 mL of Plat-E culture medium.
7. Spin down at 500 g for 5 min and remove supernatant.
8. Expand Plat-E cells in two 75 cm² TPP flask in Plat-E culture medium ([1 µg/mL puromycin], [10 µg/mL blasticidin]) in an incubator at 37°C with 5% CO₂ for a week.
9. Passage the Plat-E cells at confluence in Plat-E culture medium.

Mice

To overcome the delivery challenge of Cas9 in murine primary T cells, we recommend to use C57BL/6 Rosa26-Cas9-EGFP knock-in mice (#026179, Jackson laboratory). Alternatively, these mice can be bred to TCR-transgenic strains with congenic marker (CD45.1) to obtain a trackable monoclonal and specific T cell population expressing the Cas9. To study Tregs regulation, C57BL/6 Rosa-Cas9/Foxp3 mice have been generated.^{3,4} Of note, to maintain the colony, mice must be bred as

Table 1. NGS primer design

Sequence name	Product	Purification	Sequence
U6-Illumina-seq2	1 umole DNA Oligo	PAGE Purification	TCT TCC GAT CTC TTG TGG AAA GGA CGA AAC ACC G
iPCR-tagseq	1 umole DNA Oligo	PAGE Purification	AAG AGC GGT TCA GCA GGA ATG CCG AGA CCG ATC TC
GWKO_PCR1_Fw_HiSeq_u1	1 umole DNA Oligo	PAGE Purification	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CTT GTG GAA AGG ACG AAA CA
GWKO_PCR1_Rv_HiSeq_l1	1 umole DNA Oligo	PAGE Purification	TCG GCA TTC CTG CTG AAC CGC TCT TCC GAT CTC TAA AGC GCA TGC TCC AGA C
GWKO_PCR2_Fw_PE1.0	1 umole DNA Oligo	PAGE Purification	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC* T
GWKO_PCR2_Rv_iPCRtagT1	1 umole DNA Oligo	PAGE Purification	CAA GCA GAA GAC GGC ATA CGA GAT AAC GTG ATG AGA TCG GTC TCG GCA TTC CTG CTG AAC CGC TCT TCC GAT C*T
GWKO_PCR2_Rv_iPCRtagT2	1 umole DNA Oligo	PAGE Purification	CAA GCA GAA GAC GGC ATA CGA GAT AAA CAT CGG AGA TCG GTC TCG GCA TTC CTG CTG AAC CGC TCT TCC GAT C*T
GWKO_PCR2_Rv_iPCRtagT3	1 umole DNA Oligo	PAGE Purification	CAA GCA GAA GAC GGC ATA CGA GAT ATG CCT AAG AGA TCG GTC TCG GCA TTC CTG CTG AAC CGC TCT TCC GAT C*T
GWKO_PCR2_Rv_iPCRtagT4	1 umole DNA Oligo	PAGE Purification	CAA GCA GAA GAC GGC ATA CGA GAT AGT GGT CAG AGA TCG GTC TCG GCA TTC CTG CTG AAC CGC TCT TCC GAT C*T
GWKO_PCR2_Rv_iPCRtagT5	1 umole DNA Oligo	PAGE Purification	CAA GCA GAA GAC GGC ATA CGA GAT ACC ACT GTG AGA TCG GTC TCG GCA TTC CTG CTG AAC CGC TCT TCC GAT C*T
GWKO_PCR2_Rv_iPCRtagT6	1 umole DNA Oligo	PAGE Purification	CAA GCA GAA GAC GGC ATA CGA GAT ACA TTG GCG AGA TCG GTC TCG GCA TTC CTG CTG AAC CGC TCT TCC GAT C*T

heterozygotes. We have noticed breeding and birth rates issues with Cas9 homozygotes mice. For this experiment we used female mice from 8 to 12 weeks old.

Primers

sgRNA sequencing primers were purchased from Integrated DNA technologies (IDT) at 1 μ mole DNA oligo with PAGE purification (Table 1).

Timetable

Steps are shown in Figure 1.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Purified anti-mouse CD3 antibody (clone 17A2) [0.5 mg/mL]	BioLegend	100202
Purified anti-mouse CD28 antibody (clone 37.51) [0.5 mg/mL]	BioLegend	102102
Anti-mouse CD4 antibody (clone RM4-5) [0.5 mg/mL]	BioLegend	100505
Anti-mouse CD8 antibody (clone53-6.7) [0.5 mg/mL]	BioLegend	100701
Anti-mouse CD44 antibody (clone IM7) [0.2 mg/mL]	BioLegend	103011
Anti-mouse CD62L antibody (clone MEL-14) [0.2 mg/mL]	BioLegend	104407
Bacterial and virus strains		
Endura electrocompetent cells 24 Rxns (DUOs)	Sigma-Aldrich	LU-60242-2
Chemicals, peptides, and recombinant proteins		
Ampicillin sodium salt	Sigma-Aldrich	A19518-100G
TransIT®-293 transfection reagent	Mirus Bio	MIR-2700
Red blood cell lysis buffer	Sigma-Aldrich	11814389001
Recombinant murine IL-7	PeproTech	217-17
Recombinant murine IL-15	PeproTech	210-15
Gibco penicillin-streptomycin (10 000 U/mL)	Thermo Fisher	15140-122
Critical commercial assays		
EndoFree Plasmid Mega Kit	Qiagen	12381

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pan T Cell Isolation Kit II, mouse	Miltenyi Biotec	130-095-130
Herculase II Fusion Enzyme with dNTPs Combo	Agilent	600679
AMPure XP 5 mL	Beckman Coulter	A63880
DNA 1K / 12K / Hi Sensitivity Assay LabChip	Perkin Elmer	CLS138948
DNA High Sensitivity Reagent Kit	Perkin Elmer	CLS760672
DNeasy® Blood and Tissue Kit	Qiagen	69504
<i>Experimental models: Cell lines</i>		
Platinum-E Retroviral Packaging Cell Line, Ecotropic	Cell Biolabs	CB-RV-101
<i>Experimental models: Organisms/strains</i>		
B6J.129(Cg)-Gt(ROSA)26Sortm1.1(CAG-cas9*,-EGFP)Fehz/J	The Jackson Laboratory	026179
<i>Oligonucleotides</i>		
U6-Illumina-seq2	IDT	Table 1
iPCR-tagseq	IDT	Table 1
GWKO_PCR1_Fw_HiSeq_u1	IDT	Table 1
GWKO_PCR1_Rv_HiSeq_l1	IDT	Table 1
GWKO_PCR2_Fw_PE1.0	IDT	Table 1
GWKO_PCR2_Rv_iPCRtagT1	IDT	Table 1
GWKO_PCR2_Rv_iPCRtagT2	IDT	Table 1
GWKO_PCR2_Rv_iPCRtagT3	IDT	Table 1
GWKO_PCR2_Rv_iPCRtagT4	IDT	Table 1
GWKO_PCR2_Rv_iPCRtagT5	IDT	Table 1
GWKO_PCR2_Rv_iPCRtagT6	IDT	Table 1
<i>Recombinant DNA</i>		
pMSCV-U6sgRNA(BbsI)-PGKpuro2ABFP retroviral vector (mock)	Addgene	102796
Teichmann Retroviral Mouse Genome-wide CRISPR Knockout library of gRNA	Addgene	104861
<i>Other</i>		
SOC Outgrowth Medium	Sigma-Aldrich	S1797-10X5ML
LB Broth with Agar (Lennox)	Sigma-Aldrich	L2897-250G
LB Broth (Lennox)	Sigma-Aldrich	L3022-1KG
Gibco DPBS, w/o calcium, w/o magnesium	Thermo Fisher	14190-250
Gibco TrypLE Express (1x), phenol red	Thermo Fisher	12605-036
Dulbecco's Modified Eagle's Medium (DMEM) - high glucose	Sigma-Aldrich	D5796-24X500ML
Gibco FBS, Qualified (Origine Brazil)	Thermo Fisher	10270106
Sodium Pyruvate MEM 100 mM	Thermo Fisher	11360039
MEM Non-Essential Amino Acids (100x)	Thermo Fisher	11140-035
L-Glutamine 200 mM (100x)	Thermo Fisher	25030024
HEPES Buffer Solution 1 M, liquid	Invitrogen	15630-056
Opti-MEM I Reduced Serum Media	Thermo Fisher	31985062
EDTA UltraPure™ 0.5 M, pH 8,0	Thermo Fisher	15575020
Gibco 2-Mercaptoethanol (50 mM)	Thermo Fisher	31350-010
LS column Miltenyi	Miltenyi Biotec	130-042-401
MACS MultiStand	Miltenyi Biotec	130-042-303
DynaMag™-2	Invitrogen	12321D
CellStar dish 20 cm	Greiner Bio-One	639160
Millex-HV 0,45 µm PVDF filter	Millipore	SLHV033RS
Syringe PP/PE without needle	Sigma-Aldrich	Z116882-100EA
Pre-separation filters (30 µm)	Miltenyi Biotec	130-041-407
Phase lock gel light tube	QuantaBio	2302820
GeneElute-LPA	Sigma-Aldrich	56575
Tris	VWR	1.08382.0100
SDS	VWR	1.06022.1000
Phenol:chloroform:isoamyl	Thermo Fisher	15593031
Isopropanol	VWR	20922.320
NaCl	Sigma-Aldrich	S9888

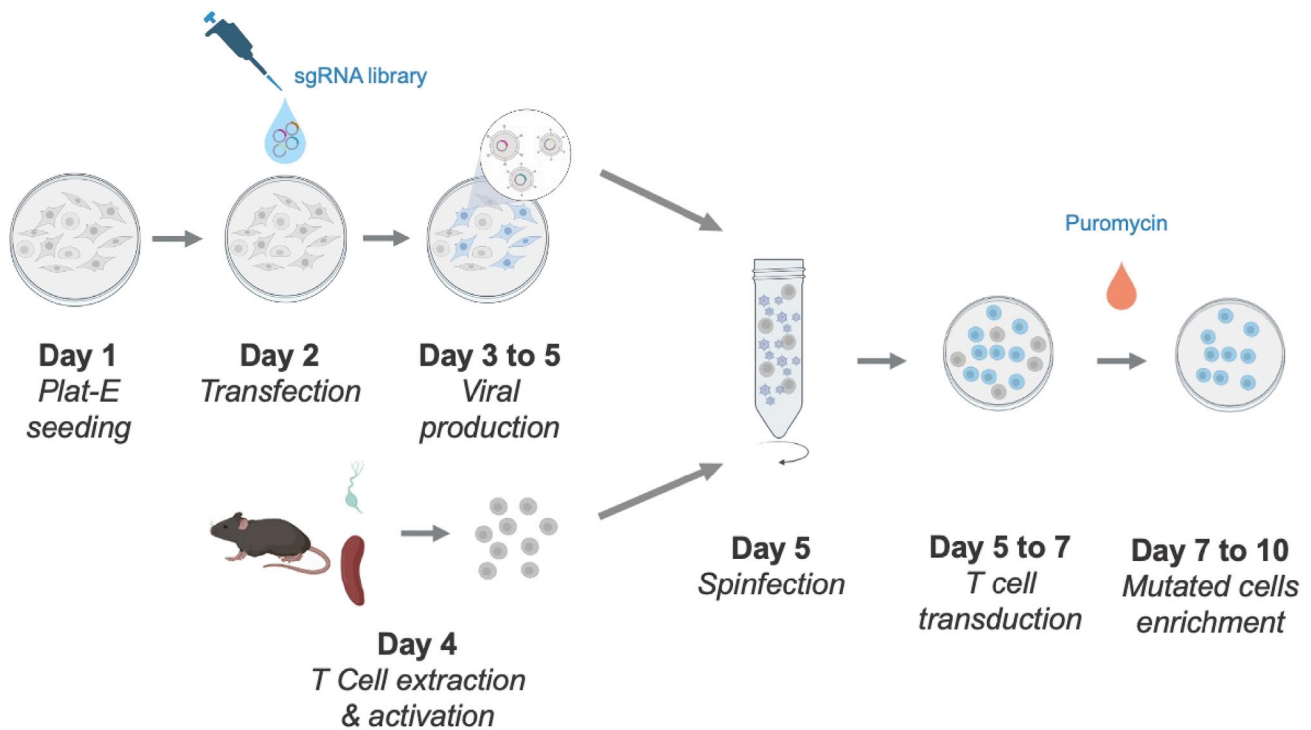


Figure 1. Synchronized timeline for fresh virus production and T cell transduction

MATERIALS AND EQUIPMENT

LB medium

Reagent	Final concentration	Amount
Lenox Broth (LB) powder (100 μ M)	20 g/L	20 g
ddH ₂ O	1 kg/L	1 L
Total		1 L

Autoclave and store at 22°C up to several months.

Plat-E culture medium

Reagent	Final concentration	Amount
Heat-Inactivated Fetal Bovine Serum (FBS)	10%	50 mL
Penicillin-streptomycin [10 000 U/mL]	1%	5 mL
L-Glutamine [200 mM]	1%	5 mL
Blasticidin S HCl [10 mg/mL]	1 μ L/mL	500 μ L
Puromycin Dihydrochloride [10 mg/mL]	0.1 μ L/mL	50 μ L
DMEM – High glucose	N/A	To 500 mL
Total		500 mL

Store at 4°C for up to one month.

PlatE viral production medium

Reagent	Final concentration	Amount
Heat-Inactivated Fetal Bovine Serum (FBS)	10%	50 mL
MEM Non-Essential Amino Acids [100 \times]	1%	5 mL

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Reagent	Final concentration	Amount
HEPES [1 M]	1%	5 mL
Sodium Pyruvate MEM [100 mM]	1%	5 mL
L-Glutamine [200 mM]	1%	5 mL
DMEM – high glucose	N/A	To 500 mL
Total		500 mL

Store at 4°C for up to one month.

MACS buffer

Reagent	Final concentration	Amount
Heat-Inactivated Fetal Bovine Serum [FBS]	0,5%	2,5 mL
EDTA [0,5 M]	5 mM	5 mL
DPBS, w/o calcium, w/o magnesium	N/A	To 500 mL
Total		500 mL

Store at 4°C for up to one month.

T cell culture medium

Reagent	Final concentration	Amount
Heat-Inactivated Fetal Bovine Serum (FBS)	10%	50 mL
HEPES [1 M]	1%	5 mL
Penicillin-streptomycin [10 000 U/mL]	1%	5 mL
Sodium Pyruvate MEM [100 mM]	1%	5 mL
2-Mercaptoethanol [50 mM]	0,1%	500 µL
RPMI medium 1640	N/A	To 500 mL
Total		500 mL

Store at 4°C for up to one month.

STEP-BY-STEP METHOD DETAILS

Mock and library plasmids amplification

⌚ **Timing: 2 days**

The sgRNA Library amplification is a crucial step to ensure sufficient DNA quantity (approximately 75 µg of DNA are required per screen) for performing multiple CRISPR screens (recommended to reduce the risk of false targets identification) and maintaining the genome-scale coverage.

1. Library electroporation and amplification.
 - a. Prepare two 2 L Erlenmeyer with LB+ Ampicillin medium by adding in each 250 µL of Ampicillin at [50 mg/mL] for 500 mL of LB medium and incubate at 37°C while performing the electroporation.
 - b. Pre-warm eight petri dishes at 37°C for electroporation efficiency measurement.
 - c. Prepare 5 pre-chilled microcentrifuge tubes, one with 1 µL of mock DNA plasmid, three with library DNA plasmid at [10 ng/µL] and one with 1 µL of control DNA pUC19 (furnished with the Endura Cell kit) at [10 pg/µL].
 - d. Thaw electrocompetent Endura bacteria cells on ice (20 min).
 - e. While the cells are thawing on ice, set the electroporator parameters as following: 10 µF ; 600 Ω ; 1,800 V.
 - f. Add 25 µL of electrocompetent Endura cells to each tube containing the DNA plasmids. Mix gently.
 - g. Transfer 25 µL of the DNA/bacteria cells mix to a 1.0 mm pre-chilled cuvette without introducing bubbles.

- h. Proceed to the electroporation and immediately add 1 mL of SOC medium at 22°C. Pipet up and down to resuspend the cells. Transfer the whole mix to a 15 mL propylene tube.
- i. Reproduce steps g to h with the other tubes of library DNA and pool them in the same 15 mL tube.
- j. Perform the last electroporation with the control DNA and pour the electroporated cells in a different 15 mL tube.
- k. Incubate in a shaking incubator for 1 h at 37°C, 250 rpm.

Note: The settings for the shaking speed is dependent on the orbital diameter of the shaker and the size of the flasks. The incubator used here has an orbital diameter of 19 mm. If using a shaker with different orbital diameter, the shaking speed needs to be adjusted. The new speed can be calculated using the formula $r2 = R2 \times (d1/d2)$. d1 = the orbital diameter for the old shaker, d2 = the diameter for the new shaker, R = the rpm for the old shaker and r = the rpm for the new shaker.

- l. Collect 20 µL of each condition to measure electroporation efficiency.
 - m. Transfer the bacteria electroporated with the mock and library plasmids into two different 500 mL pre-warmed LB medium and incubate 16 h at 37°C, 250 rpm.
2. Electroporation efficiency measurement.
- a. For Electroporation efficiency measurement, put 180 µL of SOC medium into 2 rows of 5 wells each, of a 96-well PCR plate (for example: A1-A5 and B1-B5).
 - b. Add 20 µL of the bacteria electroporated with the library into the first well of the first row (A1). Then add 20 µL of the bacteria electroporated with the control into the first well of the second row (B1).
 - c. Mix the wells and then transfer 20 µL from Column 1–2. Proceed to a successive dilution from Column 2–5.
 - d. Collect 50 µL from each well of columns 2–5 and spread them on the petri dishes with Agar LB+ Ampicillin and incubate at 37°C for 16 h.
 - e. Count the colonies formed the next day. From pUC19 transformation, calculate the transformation efficiency in colony forming unit (cfu)/µg using the following formula: $Efficacy (CFU/\mu g) = X \times 10^Y \times (Z/50)$ with X: number of colony, Z: µL of bacteria/SOC and Y: the column corresponding to the dilution.

Note: The required efficacy should be $> 5 \times 10^7$ cfu/µg for conserved library amplification.^{1,2}

3. Plasmid DNA extraction.
 - a. The next morning, centrifuge 30 min at 5,000 g to obtain the bacterial pellet containing the plasmid DNA and proceed to purification.

▣ Pause point: if needed, you can freeze the bacterial pellet obtained after amplification at –20°C for up to one month before plasmid purification.

- b. Perform plasmid purification using Qiagen EndoFree® Plasmid Mega Kit, [as instructed by the manufacturer](#). One column is necessary for 500 mL of LB. The expected yield of DNA retrieved should be > 3.5 mg.

△ CRITICAL: Plasmid DNA purification should include an endotoxin removal step. Endotoxins strongly influence transfection of DNA into primary cells and sensitive cultured cells leading to reduced transfection efficiencies.

- c. Resuspend your DNA pellet in 250 µL of endotoxin-free buffer TE from the kit and freeze at –20°C.
 - d. Thaw and measure DNA concentration with NanoDrop spectrophotometer.

e. Aliquot the mock and library DNA plasmids at [1 µg/µL] and keep at –20°C.

Note: At this step, the coverage of the amplified library has to be confirmed before proceeding to making virus to ensure the sgRNA are well represented, see PCR amplification of sgRNA region for NGS analysis section.

Mock and library retroviral virus production

⌚ Timing: 2 days

Viruses later use for transduction are produced here by Plat-E packaging cell lines, that encapsulated the sgRNA library into retroviruses. Thus, efficient transfection of Plat-E cells with DNA plasmid is mandatory for large-scale production of retroviral particles. [Troubleshooting 1](#).

Day 1: Plat - E seeding

Before starting, ensure that you have enough plasmid and transfection reagent.

4. Remove Plat-E culture medium from two 75 cm² flasks and rinse the cells with 10 mL of PBS.
5. Detach the cells using 2.5 mL of TrypLE™ Express 1× and incubate 5 min at 37°C 5% CO₂.
6. Harvest the cells and centrifugate at 500 g 5 min.
7. Resuspend the cells in 20 mL of seeding medium and count.
8. Seed 16 × 10⁶ Plate-E cells in five CellStar dish 20 cm (Greiner for cell culture) and incubate 24 h at 37°C 5% CO₂.

Day 2: Transfection

9. Per 20 cm dish, add 3 mL of Opti-MEM in a 15 mL polypropylene sterile tube with 15 µg of plasmid DNA and mix with up and down.
10. Add 80 µL of TransIT®-293 reagent to the mix with gentle up and down and incubate at 22°C for 30 min.
11. Replace the Plat-E medium from each 20 cm dish with 16 mL of fresh Plat-E viral production medium.
12. Add dropwise the transfection mix of OptiMEM, plasmid and TransIT®-293. Gently swirl the dish and incubate at 37°C 5%CO₂.
13. 24 h after the transfection, replace the medium with 16 mL of fresh Plat-E viral production medium and incubate at 37°C 5%CO₂ for 24 h.

Note: Depending on the viral production efficacy, one to two 20 cm dishes are required for mock virus and between four to six dishes for the library viral production to perform a genome-wide CRISPR-Cas9 screen, with the transduction of at least 200 × 10⁶ activated T cells.

T cell extraction and activation

⌚ Timing: 3 h

T cells extracted from harvested spleen and lymph nodes must be activated 24 h for efficient transduction.

14. Coat a 6 well plate with anti-CD3 antibody [2.5 µg/mL] by diluting 5 µL of anti-CD3 in 1 mL of PBS per well and incubate 30 min at 37°C.

Note: Make sure to cover the plate with parafilm to avoid drying. The coating can also be prepared the day before and conserved at 4°C.

Note: Instruction are given for 1×10^7 cells. Adjust the volumes according to your cell count. The Miltenyi Pan T Cell isolation kit II provides T cells negative selection. Thus, T cells will be retrieved from the eluted fraction. You will obtain approximately 90×10^6 to 100×10^6 cells from one immunocompetent C57BL6-Cas9 mouse and isolate 20% of T cells.

15. Sacrifice the mice according to operative legal and ethical recommendations.
16. Collect spleen and lymph nodes (inguinal, mesenteric, axillary, mandibular) in microcentrifugation tubes filled with sterile MACS buffer and keep on ice.
17. In sterile condition, place 70 μ m cell strainers on a 6 well plate and add 2 mL of MACS buffer in each well.
18. Proceed to organ dissociation using a syringe plunger.
19. Rinse the strainer with up to 5 mL of MACS buffer and transfer the cell suspension in a 15 mL sterile tube.
20. Spin down 5 min at 500 g.
21. Proceed to spleen red blood lysis with 1 mL of Red Blood Cell Lysis Buffer incubation for 1 min at 22°C and add then 5 mL of MACS buffer.
22. Spin down 5 min at 500 g.
23. Pool the cells from lymph nodes and spleen and count.
24. Proceed to T cell isolation using the Miltenyi Pan T Cell isolation kit II [following the manufacturer's instruction](#), with MACS MultiStand magnet, LS column and 30 μ m pre-separation filters.
25. Rinse the anti-CD3 coated plate with 1 mL of PBS/well and add the T cell suspension at 1.5×10^6 T cells/mL with 5 mL per well in T Cell culture medium.
26. Perform T cell activation⁵ by adding [2 μ g/mL] of anti-CD28 antibody, [10 ng/mL] of murine IL-7 and [5 ng/mL] of murine IL-15, gently mix and incubate 24 h at 37°C, 5%CO₂.

Note: Split the medium and add cytokines every two days or when the medium is turning yellow.

T cells transduction with retroviruses

⌚ Timing: 7 h

This section details the transduction of 24 h activated murine Cas9-expressing T cells by single spinfection of viruses harvested 48 h–72 h after transfection with the Teichmann Retroviral Mouse Genome-wide CRISPR Knockout library and the pMSCV-U6sgRNA(BbsI)-PGKpuro2ABFP vectors (See timeline). [Troubleshooting 2](#).

Before starting, pre-heat the centrifuge at 32°C.

27. Harvest Plat-E cells supernatant 48 h–72 h after the transfection, pass it through a PVDF 0,45 μ m filter using a 20 mL syringe, collect in 50 mL tube and keep on ice.
28. Remove 4,5 mL of medium from each well of T cells culture.
29. Add polybrene to a final concentration of [8 μ g/mL] in each well of the 6 well plates.
30. Titrate the quantity of virus to add to obtain only 35% of BFP+ T cells, which corresponds to a low multiplicity of infection (MOI<0.3) associated with only one sgRNA integration per cell, two days after spinfection (typically: 25%, 50%, 75% or 100% of final volume).
31. In our hands, we add 2 mL of filtered supernatant and 2 mL of Plat-E viral production medium.
32. Centrifuge at 1,000 g during 2 h at 32°C.
33. Place the 6 well plates back in the incubator at 37°C with 5% CO₂.

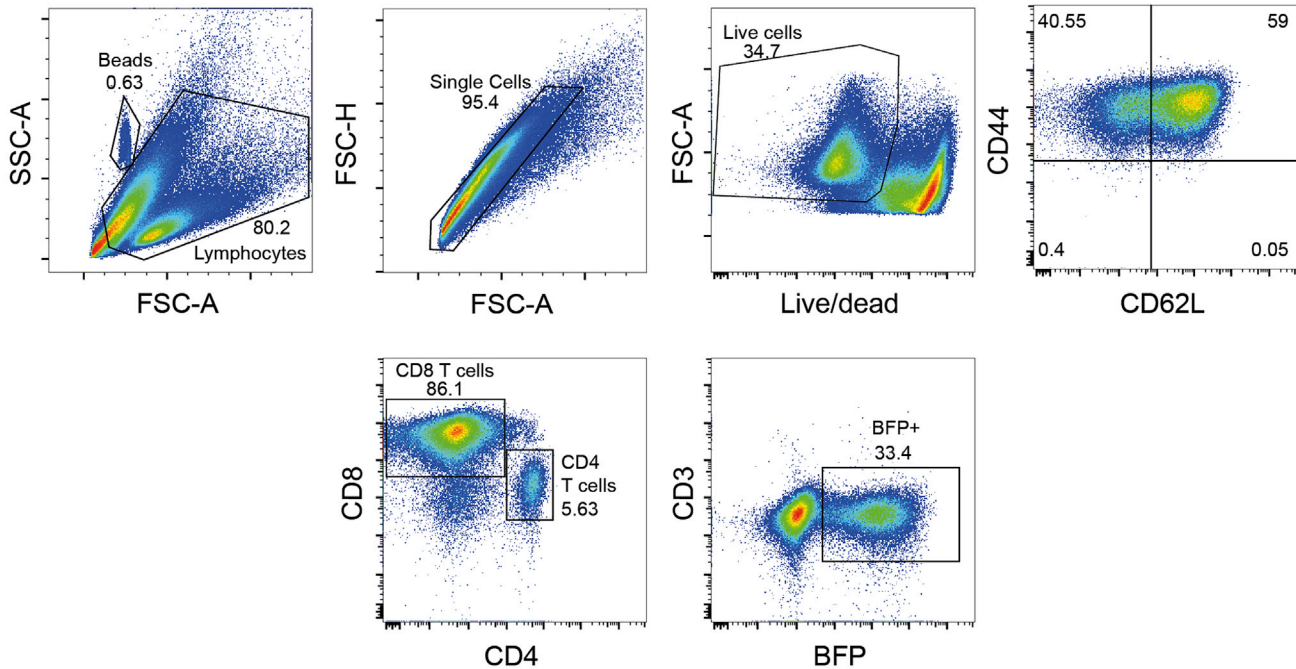


Figure 2. Flow cytometry gating strategy to assess the transduction, survival and activation of genome-wide mutated T cells at day 4 after extraction from C57BL/6 Rosa26-Cas9-EGFP mice

34. Remove 4.5 mL of medium from T cells culture after 3–4 h of incubation.
35. Add 4 mL of fresh T cell culture medium complemented with IL-7 [10 ng/mL] and IL-15 [5 ng/mL] and incubate for two days at 37°C with 5% CO₂.

Note: Renew half of the culture medium with appropriate dose of cytokine every two days, or sooner if your pH indicator is showing signs of acidification (yellow).

Puromycin selection and SIP calculation

⌚ Timing: 6 days

To ensure the single integration of one sgRNA per T cell, it is important to infect the cells at low MOI. Following Chen et al., protocol,⁶ we calculate a Single Infection Percentage (SIP) using the survival ratio of transduced T cell after Puromycin exposure. If the infection events are considered as independently-occurring, the SIP is the probability of a cell being infected by n viral particles if the MOI is m is given by the Poisson distribution,

$$P(n) = \frac{m^n \cdot e^{-m}}{n!}$$

The SIP calculation allows to determine the optimal viral volume that results in 30–35% of T cells surviving in puromycin-containing medium or 30–35% BFP+ expressing T cells, ensuring the integration of only one sgRNA per T cell.

This section details the protocol for T cells expansion and selection with puromycin 48 h after the transduction step to enrich in GW-KO T cells.

36. Harvest, pool and count the transduced T cells (take an aliquot to assess the BFP expression in live cells by flow cytometry analysis, [Figure 2](#)).

Note: The viability should range between 40 to 60% and BFP expression should be below 40% to ensure the integration of one sgRNA per cell.

37. Spin down 5 min at 450 g.
38. Prepare 2× fresh T Cell culture medium containing IL-7 [20 ng/mL], IL-15 [10 ng/mL], and puromycin [10 µg/mL].

Note: We recommend to perform a puromycin killing dose response if you are using other than activated T cells.

39. Remove half of the old medium from the centrifuged T cells.
40. Add half fresh medium (2×) to ensure a concentration of 5×10^6 T cells/mL, with 5 mL per well in 6 well plates.
41. Place in an incubator at 37°C with 5% CO₂ for 3 days.

Note: For SIP calculation, seed 10^6 transduced T cells in triplicate in 96 well plate, in a final volume of 200 µL with 100 µL of old medium and 100 µL of fresh medium with IL-7 [20 ng/mL], IL-15 [10 ng/mL] with or without puromycin treatment. Similarly, incubate at 37°C with 5% CO₂ for 3 days.

42. For cells staining, prepare a 50 µL/sample master mix of fluorochrome-labeled antibodies against T cells markers of interest in MACS buffer with Fc block and counting beads (For example: live/dead, CD4, CD8, CD44 activation marker, CD62L homing marker at 1:200, [Figure 2](#)).

To calculate the SIP:

Calculate P_{survival} using the following formula:

$$P_{\text{survival}} = \frac{\text{live cells without puromycin}}{\text{live cells with puromycin}}$$

Then, calculate SIP using the following formula from Chen et al.,⁶:

$$SIP = \frac{(1 - P_{\text{survival}}) \times \ln(1 - P_{\text{survival}})}{P_{\text{survival}}}$$

Note: Expected SIP value should be higher than 75% to be sure only one sgRNA have been transduced per T cells.

PCR amplification of sgRNA region for NGS analysis

⌚ Timing: 8 h

This part details the essential steps to uncover changes in sgRNA diversity from the library amplification time point to the end of the screening process. Here, we assess the library representation after plasmid amplification and after viral integration in 50×10^6 GW-KO engineered murine T cells (corresponding to approximately 500 different sgRNA copies). A first step of genomic DNA (gDNA) extraction from GW-KO T cells is required prior to sgRNA region amplification by PCR 1 and Illumina NGS tails tagging with PCR2. A more detailed protocol on this matter has already been published,⁷ we only report here the specificities related to the mentioned GW-KO library of sgRNA.

43. Genomic DNA (gDNA) from 50×10^6 transduced and puromycin-selected T cells is extracted using DNeasy® Blood and Tissue Kit (Qiagen) on ten columns. [Troubleshooting 3,4](#).

Note: The maximum amount of gDNA to be extracted per column correspond to 5×10^6 cells. The PCR1 of gDNA from transduced T cells has to be performed on all the extracted gDNA to ensure library coverage, typically on 300 μg of gDNA.

44. Perform 200 parallel PCR1 reactions in 50 μL final volume with 1.5 μg of DNA per reaction with Herculase II Fusion Enzyme with dNTPs Combo, in the following conditions:

PCR 1 reaction master mix	
Reagent	Amount
DNA template	1 μL
Buffer	10 μL
GWKO_PCR1_Fw_HiSeq_u1 [5 μM]	1 μL
GWKO_PCR1_Rv_HiSeq_l1 [5 μM]	1 μL
dNTP	0.5 μL
Herculase	0.5 μL
ddH ₂ O	36 μL

Note: Keep Herculase on ice or at -20°C until needed.

45. Carry out the same PCR1 with amplified GWKO library DNA plasmid as control: perform 10 parallel reactions with 10ng of DNA each.
46. Transfer 50 μL of the PCR 1 master mix into each well of a 96-well plate and cover.
47. Spin down at 700 g 5 min at 4°C to get all the liquid to the bottom as well as to remove air bubbles.
48. Run in the thermocycler with the following parameters:

PCR 1 cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min 20 s	1
Denaturation	94°C	30 s	25 cycles
Annealing	60°C	10 s	
Extension	72°C	25 s	
Final extension	68°C	2 min	1
Hold	10°C	∞	

Note: For the PCR amplification of the GWKO library vector, only run 18 cycles of PCR1.

Note: To avoid cross-contaminations with the generated amplicons, we recommend to perform the following steps in a dedicated post-PCR room with a specific cabinet.

49. After sgRNA region amplification, pool the 200 parallel reactions and the 10 reactions in two different 15 mL tubes and mix. Expected PCR1 product size is 246 bp.
50. Perform PCR1 products clean-up using AMPure XP beads and DynaMagTM-2 magnet from 150 μL of each pooled tube [according to the manufacturer's instructions](#) with a 1:1.8 ratio.
51. Elute and concentrate in 50 μL of ddH₂O.
52. Set up the PCR2 mix accordingly:
53. Prepare a master mix for 8 reactions (4 reactions for transduced T cells gDNA PCR2, 4 reactions for the amplified library plasmid PCR2).

PCR 2 reaction master mix/reaction	
Reagent	Amount
Purified PCR 1 product	10 μ L
Buffer	10 μ L
GWKO_PCR2_Rv_iPCRtagT1 or GWKO_PCR2_Rv_iPCRtagT2...	1 μ L
GWKO_PCR2_Fw_PE1.0	1 μ L
dNTP	0.5 μ L
Herculase	0.5 μ L
ddH ₂ O	μ L

54. Transfer 50 μ L of the PCR 2 master mix into each well of a 96-well plate and cover.
55. Centrifuge at 2,000 rpm for 5 min at 4°C.
56. Run in the thermocycler with the following parameters:

PCR 2 cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	10 cycles
Annealing	66°C	15 s	
Extension	72°C	20 s	
Final extension	72°C	5 min	1
Hold	10°C	∞	

57. After running PCR2, pool the 4 reactions in two microcentrifuge tubes.
58. Perform a PCR2 products clean-up by using AMPure XP beads from 150 μ L of each pooled tube with a 1:1 ratio according to the manufacturer's instructions and elute in 50 μ L of ddH₂O.
59. Quantify the purified samples with the Qubit dsDNA HS Assay Kit using manufacturer's protocol (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_HS_Assay_UG.pdf). Expected PCR2 product size is 313 bp.
60. Adjust the DNA concentration to 10 pg/ μ L - 500 pg/ μ L of DNA.

Note: The maximum total DNA concentration for this sensitive chip is 500 pg/ μ L per fragment. https://resources.perkinelmer.com/labsolutions/resources/docs/GDE_DNA_High_Sens_User_Guide.pdf?_ga=2.180854887.1582170491.1662388256-1531866421.1662388256.

61. Precisely evaluate the purified PCR2 product size and purity using LabChip GX Touch HT with DNA 1K / 12K / Hi Sensitivity Assay LabChip and DNA High Sensitivity Reagent Kit prior to pooling the two samples in equimolar concentration and sending for sequencing.
62. Send the samples for sequencing together with an aliquot of U6-Illumina-seq2 and iPCR-tagseq primers.

Note: If heteroduplex are observed, you should run PCR1 or PCR2 with less cycles.

EXPECTED OUTCOMES

This protocol allows to generate a pool of genome-wide inactivated primary murine T cells from Cas9-expressing mice. It is applicable to any difficult to transduce lymphocytes, including Tregs, CD4 or CD8 TCR-Tg and NK cells with an adapted activation/expansion protocol. Starting from 200×10^6 activated T cells, we expect to obtain around 50×10^6 GW-KO T cells, which corresponds to 500 copies of each sgRNA, e.g., more than 2,500 sgRNA per gene. Of note, for *in vivo* screening approach, we have injected 10 to $12 \times 10^6/100 \mu$ L GW-KO T cells per mouse intravenously.⁸ We strongly recommend to have at least 100 copies of each sgRNA per mouse.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sequencing is performed with a 10% PhiX control, using the 25–base pair (bp) single-end sequencing protocol. FASTQ files obtained after sequencing are demultiplexed using the HiSeq Analysis software (Illumina). MAGeCK⁹ count command is used to generate per-sgRNA read count table by matching single-end reads with sgRNA sequences from the genome-scale sgRNA Yusa library.¹ MAGeCK,⁹ MAGeCK-VISPR⁹ and MAGeCKFlute¹⁰ allow users to perform quality control, read count generation, normalization and calculate beta score to evaluate differential expression of each sgRNA and gene selection performance. [Troubleshooting 5](#).

LIMITATIONS

Even if we and others have successfully engineered murine T cells using lentiviruses CRISPR construct,^{8,11} the delivery of genes into murine T cells is much easier using retroviral constructs. This protocol allows to efficiently perform GW-KO CRISPR screens in murine T cells but is limited by (I) the delivery challenge of Cas9, which is overcome using Cas-expressing mice, (II) the required activation of T cells for retroviral transduction,¹² (III) the requirement of selection/enrichment (puro-mycin or congenic marker) for transduced T cells at low MOI.

TROUBLESHOOTING

Problem 1

Poor transfection efficiency resulting in low virus concentration in the supernatant (related to steps 4–13).

Potential solution

For optimal viral production, Plate-E cells must be at 80% confluence for the transfection (24 h after seeding). To have appropriate expansion, use Plat-E that has been thawed and cultivated for at least one week but still at a low passage (≤ 10).

Problem 2

If you're facing uneven viral production or difficulties to synchronized viral production with T cell activation (related to steps 27–35).

Potential solution

Frozen virus can be used although the resulting transduction efficiency would be lower and the diversity might be slightly compromised. To do so, freeze the virus right after purification of the supernatant at -80°C in small aliquots. Once thawed, the virus shall be used immediately and shall not be freeze again. Before using the frozen virus, make sure to titrate it on a small sample of T cells to obtain a sufficient number of effectively transduced T cells while performing your experiment.

Problem 3

If performing an *ex vivo* screen or with a limited number of cells ($<100\,000$ cells), we recommend you to avoid using a column and propose an alternative DNA extraction protocol (related to step 43).

Potential solution

- Use a cell sorter to retrieve the cells of interest in PBS + RPMI medium in a 1.5 mL microcentrifuge tube.
- Spin down at 450 g for 5 min.
- Gently remove the supernatant with a pipet (as the pellet is not visible, we recommend you to keep 40 μL).

▮▮ **Pause point:** you can freeze your pellet at -20°C for several months.

- Thaw your cells on ice if frozen or proceed directly to the next step.
- Add 1 volume of lysis buffer from DNeasy® Blood and Tissue Kit (Qiagen) (around 40 μ L).
- Add 1/10 volume of proteinase K (20 mg/mL) from the kit.
- Incubate at 56°C for 30 min in an Eppendorf ThermoMixer® C or equivalent shaking heat block.
- Denature at 95°C for 30 min.
- Add 2 volumes (80 μ L) of ddH₂O and maintain on ice while preparing the PCR or freeze.

Problem 4

If you have to isolate gDNA from fixed cells (related to step 43), we recommend you to perform this protocol.³

Potential solution

- Resuspend cell pellets in lysis buffer (0.5% SDS, 50 mM Tris, pH 8, 10 mM EDTA) with 1:25 v/v of 5 M NaCl to reverse crosslinking and incubated at 66°C for 16 h.
- Add RNase A (10 mg/mL) at 1:50 v/v and incubate at 37°C for 1 h.
- Add Proteinase K (20 mg/mL) at 1:50 v/v and incubate at 45°C for 1 h.
- Add Phenol:Chloroform:Isoamyl Alcohol (25:24:1) to the sample 1:1 v/v and transfer to a phase lock gel light tube, invert and centrifuge at 20,000 g for 5 min.
- Transfer the aqueous phase into a clean tube.
- Add NaAc at 1:10 v/v, 1 μ L of GeneElute-LPA and isopropanol at 2.5:1 v/v.
- Vortex the sample and incubate at –80°C until frozen solid.
- After thawing, centrifuge at 20,000 g for 30 min.
- Wash the cell pellet with 500 μ L of 75% EtOH, gently invert and centrifuge at 20,000 g for 5 min, aspirate, dry and resuspend in 20 μ L TE buffer.

Problem 5

The analysis revealed a high number of targets rendering the validation step tedious (related to quantification and analysis).

Potential solution

We recommend you to set a cutoff for the adjusted *p* value at 0.1 (False Discovery Rate, FDR) and *p* value at 0.001 when using MAGeCK Analysis.¹³ Alternatively, you could design a secondary validation library and combine it with transcriptomic perturbation analysis.¹⁴

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Laurie Menger (laurie.menger@gustaveroussy.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This protocol did not generate/need datasets.

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AUTHOR CONTRIBUTIONS

M.L.S. and C.C.R. performed the experiments. M.L.S., C.C.R., and L.M. wrote the manuscript. All authors read, revised, and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Tzelepis, K., Koike-Yusa, H., De Braekeleer, E., Li, Y., Metzakopian, E., Dovey, O.M., Mupo, A., Grinkevich, V., Li, M., Mazan, M., et al. (2016). A CRISPR dropout screen identifies genetic vulnerabilities and therapeutic targets in acute myeloid leukemia. *Cell Rep.* *17*, 1193–1205. <https://doi.org/10.1016/j.celrep.2016.09.079>.
2. Henriksson, J., Chen, X., Gomes, T., Ullah, U., Meyer, K.B., Miragaia, R., Duddy, G., Pramanik, J., Yusa, K., Lahesmaa, R., and Teichmann, S.A. (2019). Genome-wide CRISPR screens in T helper cells reveal pervasive crosstalk between activation and differentiation. *Cell* *176*, 882–896.e18. <https://doi.org/10.1016/j.cell.2018.11.044>.
3. Cortez, J.T., Montauti, E., Shifrut, E., Gatchalian, J., Zhang, Y., Shaked, O., Xu, Y., Roth, T.L., Simeonov, D.R., Zhang, Y., et al. (2020). CRISPR screen in regulatory T cells reveals modulators of Foxp3. *Nature* *582*, 416–420. <https://doi.org/10.1038/s41586-020-2246-4>.
4. Loo, C.-S., Gatchalian, J., Liang, Y., Leblanc, M., Xie, M., Ho, J., Venkatraghavan, B., Hargreaves, D.C., and Zheng, Y. (2020). A genome-wide CRISPR screen reveals a role for the non-canonical nucleosome-remodeling BAF complex in Foxp3 expression and regulatory T cell function. *Immunity* *53*, 143–157.e8. <https://doi.org/10.1016/j.immuni.2020.06.011>.
5. Eremenko, E., Taylor, Z.V., Khand, B., Zaccai, S., Porgador, A., and Monsonogo, A. (2021). An optimized protocol for the retroviral transduction of mouse CD4 T cells. *STAR Protoc.* *2*, 100719. <https://doi.org/10.1016/j.xpro.2021.100719>.
6. Chen, S., Sanjana, N.E., Zheng, K., Shalem, O., Lee, K., Shi, X., Scott, D.A., Song, J., Pan, J.Q., Weissleder, R., et al. (2015). Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis. *Cell* *160*, 1246–1260. <https://doi.org/10.1016/j.cell.2015.02.038>.
7. Wohlhieter, C.A., Uddin, F., Quintanal-Villalonga, A., Poirier, J.T., Sen, T., and Rudin, C.M. (2021). An optimized NGS sample preparation protocol for in vitro CRISPR screens. *STAR Protoc.* *2*, 100390. <https://doi.org/10.1016/j.xpro.2021.100390>.
8. Sutra Del Galy, A., Menegatti, S., Fuentealba, J., Lucibello, F., Perrin, L., Helft, J., Darbois, A., Saitakis, M., Tosello, J., Rookhuizen, D., et al. (2021). In vivo genome-wide CRISPR screens identify SOCS1 as intrinsic checkpoint of CD4⁺ T_H1 cell response. *Sci. Immunol.* *6*, eabe8219. <https://doi.org/10.1126/sciimmunol.abe8219>.
9. Li, W., Köster, J., Xu, H., Chen, C.-H., Xiao, T., Liu, J.S., Brown, M., and Liu, X.S. (2015). Quality control, modeling, and visualization of CRISPR screens with MAGeCK-VISPR. *Genome Biol.* *16*, 281. <https://doi.org/10.1186/s13059-015-0843-6>.
10. Wang, B., Wang, M., Zhang, W., Xiao, T., Chen, C.-H., Wu, A., Wu, F., Traugh, N., Wang, X., Li, Z., et al. (2019). Integrative analysis of pooled CRISPR genetic screens using MAGeCKFlute. *Nat. Protoc.* *14*, 756–780. <https://doi.org/10.1038/s41596-018-0113-7>.
11. Ye, L., Park, J.J., Peng, L., Yang, Q., Chow, R.D., Dong, M.B., Lam, S.Z., Guo, J., Tang, E., Zhang, Y., et al. (2022). A genome-scale gain-of-function CRISPR screen in CD8 T cells identifies proline metabolism as a means to enhance CAR-T therapy. *Cell Metab.* *34*, 595–614.e14. <https://doi.org/10.1016/j.cmet.2022.02.009>.
12. Cepko, C., and Pear, W. (2001). Retrovirus infection of cells in vitro and in vivo. *Curr Protoc Mol Biol. Curr. Protoc. Mol. Biol. Chapter 9. Unit9.14.* <https://doi.org/10.1002/0471142727.mb0914s36>.
13. Bodapati, S., Daley, T.P., Lin, X., Zou, J., and Qi, L.S. (2020). A benchmark of algorithms for the analysis of pooled CRISPR screens. *Genome Biol.* *21*, 62. <https://doi.org/10.1186/s13059-020-01972-x>.
14. Datlinger, P., Rendeiro, A.F., Schmidl, C., Krausgruber, T., Traxler, P., Klughammer, J., Schuster, L.C., Kuchler, A., Alpar, D., and Bock, C. (2017). Pooled CRISPR screening with single-cell transcriptome readout. *Nat. Methods* *14*, 297–301. <https://doi.org/10.1038/nmeth.4177>.