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# Immune Saturation Genome Editing (Immune SGE): Variant Analysis in T cells

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**Protocol status:** Working
**We use this protocol and it's working**

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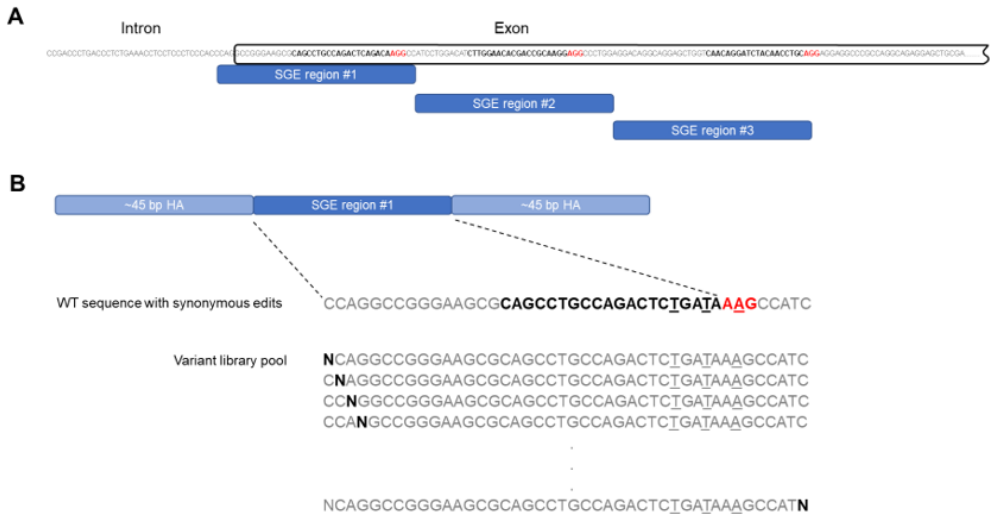
## Abstract

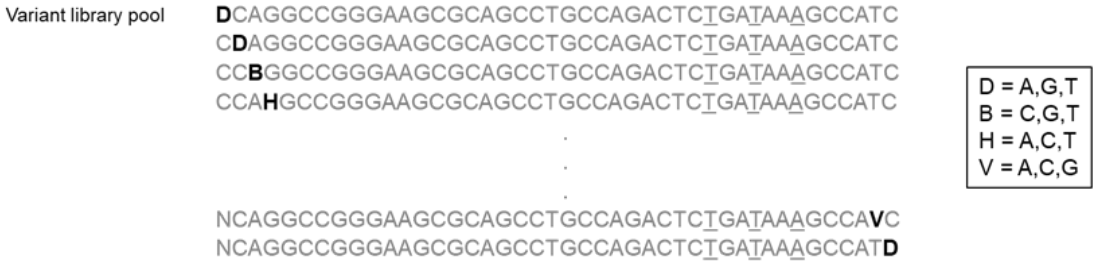
This protocol details immune saturation genome editing (Immune SGE) and variant analysis in T cells. In brief, primary T cells are isolated from donor PBMCs, edited with a variant library centered around a guide cut site, and variants are scored based on how they affect T cell proliferation in response to T cell receptor (TCR) stimulation. This method works well for genes that regulate the response to TCR activation.

## Guidelines




Overview of repair template design of ssODN libraries. A) Example of an intron/exon border. We typically go 4 bp into the introns on either side of the exon. Bold characters indicate the original sgRNA sequence locations. Red characters indicate the original PAM sequence location. B) Our ssDNA repair templates are typically ~140 bp, including 30-50 bp of region to be edited and homology arms on both ends to make up the rest of the 140 bp. Underlined bases indicate synonymous mutations within the guide and PAM sites to discourage recutting of gDNA after repair. We typically go with 3 synonymous mutations with the thinking that some of our variants will revert one of them back to the original. In that case we will still have 2 synonymous mutations to limit recutting of the gDNA after repair. N indicates variant with any of the 4 bases.

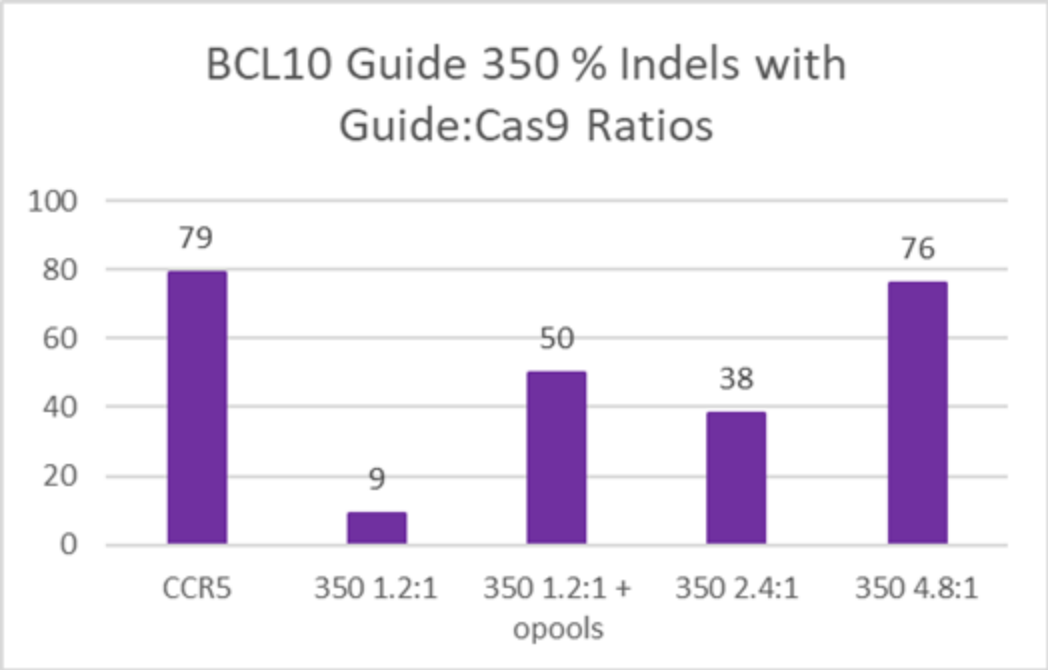
Of note, using N as a designation for different variants when ordering oPool libraries results in ~50% of the edits containing the WT sequence with synonymous edits, thus reducing the fraction of your variants of interest. We are currently looking into how variant coverage changes when we use the other 3 bases in each location and completely limit the possibility of the WT base at that position:




**Important considerations:**

- We identify and test guides using ICE analysis before ordering oPools. We typically test guides alone and any guide that cuts >10% on its own should suffice. Although we prefer guides to cut with efficiency levels >50%, inclusion of

repair templates and increasing guide to Cas9 ratios should significantly increase cutting efficiency.




- It is best to keep editing regions centered around the cut site and within 20bp of the cut site. We see significant dropout of variants that are >20 bp from the cut site. If possible, we will keep our editing regions no larger than 40 bp with 20 bp on either side of the cut site. Sometimes we don't have a choice, so we transfect and sample more cells to ensure reasonable sequencing coverage.
- Occasionally we will have guides and/or PAM sites that cross into the intron. We recommend that the 3 synonymous variants within the "WT sequence with synonymous edits" do not alter the bases involved in splicing as these will most likely result in loss of variants within your analysis.

## Materials

**Reagents:**

- T cell culture media

1. RPMI
2. 20% FBS
3. 1% Glutamax
4. 10 mM HEPES
5. 0.1% BME
6. Add fresh IL-2 on passage days (Peprotech AF200-02, 1x = 50 ng/mL)

- CD4+ T cell isolation kit (stemcell)
- Dynabeads™ Human T-Expander CD3/CD28 **Thermo Fisher Catalog #**11141D
- ImmunoCult Human CD2/3/28 **STEMCELL Technologies Inc. Catalog #**10970
- CellTrace™ CFSE Cell Proliferation Kit, for flow cytometry **Thermo Fisher Catalog #**C34570
- TRIzol Reagent **Thermo Fisher Scientific Catalog #**15596026 )
- Quick-DNA miniprep plus kit **Zymo Research Catalog #**D4069

## **OVERVIEW**: ssODN repair template and oPool design notes

1 Determine your editing region of interest, trying to stay within 20bp on either side of the cut site.

2 Add respective homology arms to both the 5’ and 3’ ends of the editing region such that ssODN is at least 140 bp long.

3 Introduce 3 synonymous mutations in the editing region within the guide homology region and the PAM. Ideally you have one mutation in the PAM site.

4 Use custom scripts to create your list of variants within the editing region.

5 Determine number of stop codons in library. Add up to 10 stop codons total if there aren’t enough.

6 Take all variants and add the homology arms (we do this in excel).

7 Add asterisks after the first 2 bases and before the last 2 bases if incorporating phosphorothioate bonds (this greatly improves stability as most cells contain nucleases that will degrade nonmodified ssDNA).

8 Get quote from IDT. Phosphorothiate and 3bp codes are not currently supported on IDT’s website when ordering oPools.

## **OVERVIEW:** T Cell Culture method

9 Step 1 - Isolate CD4+ T cells from PBMCs

10 Step 2 - Activate CD4+ T cells with CD3/28 beads for 72 hours in Complete media + IL-2

11 Step 3 - Remove CD3/28 beads and allow cells to proliferate o/n

12 Step 4 - Transfect cells with Maxcyte

13 Step 5 - Collect day 2/3 samples for gDNA and RNA

14 Step 6 - Replace media with IL-2 every 2-3 days (M/W/F)

15 Step 7 - Take off IL-2 on day 9

16 Step 8 - Take 2-4e6 cells for gDNA and RNA

17 Step 9 - Stain cells with CFSE and stim with CD2/3/28 (no IL-2) for 72 hours

18 Step 10 - Sort cells on flow cytometer

## **Step 1**: Isolate CD4+ T cells from PBMCs (starting from ~3.5 mL)

22m 33s

19 Pipette 5 mL of filtered FBS into a 50mL conical tube.

20 Thaw a vial of frozen cells in a 37 °C water bath until cells are completely thawed.

21 Use a 5ml serological pipette to take cells from vial and slowly add the thawed cells dropwise into the FBS in the 50mL conical while swirling.

22 Add PBS 2% FBS (or EasySep buffer) dropwise to the 50 mL conical until the volume reaches 20 mL .

23 Spin down cells and aspirate sup.

24 Optional step: If cell pellets appear red, add in 5 mL of 1x ACK buffer. Let sit for 00:02:00 Room temperature. Dilute out ACK with 20 mL PBS before centrifuge. 2m *

25 Resuspend cells in 20 mL CD4+ T cell isolation buffer.

26 At this point you can take 10 µL of the cells for counting. While counting, spin down cells.

27 If you use hemocytometer to count total PBMC, make 1:10 dilution with Trypan blue before counting: If you use cell counter, follow manufacturer's protocol. Record cell concentration (cells/ml) and calculate total PBMC counts.

28 Aspirate the supernatant and resuspend the cells to 5.0E+07cells/mL in EasySep buffer (PBS containing 2% FBS and [M] 1 millimolar (mM) EDTA).

29 Transfer cells to a 14 mL Falcon round bottom polystyrene tube.

30 Add the EasySep Human CD4+ T Cell enrichment cocktail at 50 uL/mL cells (e.g. add 100 µL of cocktail to 2 mL of cells). Mix well and incubate at Room temperature ( 15 °C - 25 °C ) for 00:10:00. 10m

31 Vortex the EasySep™ D Magnetic Particles for 00:00:30. Ensure that the particles are in a uniform suspension with no visible aggregates. (Solution should be uniformly brown). 30s

32 Add the EasySep™ D Magnetic Particles at 100 µL/mL cells (e.g. for 2 mL of cells, add 200 µL of magnetic particles). Mix well and incubate at Room temperature ( 15 °C - 25 °C ) for 00:05:00. 5m

33 Bring the cell suspension up to a total volume of 2.5 mL (for 5ml tube), 5 mL (for samples ≤ 2 mL), 10 mL (for samples ≥ 2 mL) by adding EasySep buffer. Mix the cells in the tube by gently pipetting up and down 2 - 3 times. Place the tube (without cap) into the magnet. Set aside for 00:05:00. 5m

34 Pick up the EasySep™ Magnet, and in one continuous motion invert the magnet and tube, pouring off the desired fraction into a new 14 mL polystyrene tube. 3s

- The magnetically labeled unwanted cells will remain bound inside the original tube, held by the magnetic field of the EasySep™ Magnet.
- Leave the magnet and tube in inverted position for 00:00:02 - 00:00:03, and then return to an upright position.

35 Take an aliquot for counting, and spin down.

36 Count and resuspend in cell culture media + IL-2 at 0.5e6 cells/mL.

37 Record the PBMC count and T cells count in the PBMC tracking file at the proper place.

## **Step 2**: Activate CD4+ T cells with CD3/28 beads

3d 0h 0m 45s

38 Calculate volume of beads needed. We use 30 µL beads/1e6 cells.

39 Vortex CD3/CD28 beads for 00:00:10 - 00:00:15 to fully resuspend beads. Add desired volume of beads to 1.5 mL Eppendorf.

15s

40 Add 500 µL media into each Eppendorf tube and place in DynaMag™-2 magnet for 00:00:30.

30s

41 Remove media avoiding beads (should be visible held to the side of the tube).

42 Resuspend beads with media (same volume of beads suspension) and add to each well containing T cells.

43 Gently mix T-cells and CD3/CD28 beads with a P1000 pipette or 5ml serological pipette. Place cells in 37 °C incubator for 72:00:00.

3d

## **Step 3**: Remove CD3/28 beads

8h 2m

44 Observe cells under microscope before removing beads. Cells should be clumping in the well and most of cells should be visibly larger prior to removing beads.

45 Use a P1000 pipette or 5ml serological pipettes to gently pipet up and down 10 times to separate cells from beads.

46 Collect cells in 15 mL tube, at no more than 10 mL per tube (multiple tubes if culture volume is greater than 10 mL).

47 Place on magnet for at least 00:01:00 . 1m

48 Carefully transfer cells in culture to a new tube without touching the beads.

49 Wash culture wells with 0.5 mL - 1 mL fresh media and add to tube containing only the beads. Take the tube off the magnet to re-suspend the beads well.

50 Place on magnet for at least 00:01:00 to separate the beads and media, and then collect media to the tube containing cells. 1m

51 Discard tube containing only beads.

52 Repeat steps 36 – 37 at least once more to ensure all beads are removed.

53 Count cells and record cell concentration as well as total cell numbers. Resuspend cells at a concentration to $0.5x10^6$ cells/mL in fresh media + IL-2.

54 Culture at 37 °C for ~16 hours ( Overnight ) before transfection. 8h

**Note**

Cells should double or triple overnight. Transfection will most likely fail if cells do not expand.

## **Step 4**: Transfect cells with Maxcyte 1h 20m

55 1h

Note

We typically make a 125 µL transfection mix containing 5e6 cells, sgRNA, Cas9, and oPool library and pipette 100 µL into a single well of a 100x2 maxcyte assembly or 50 µL each into 2 wells of a 50x8 assembly.

If using crRNA, complex with trRNA. crRNA and trRNA are resuspended at [M] 100 micromolar (µM) and mixed 1:1. Add 15 µL of crRNA + 15 µL trRNA in sterile PCR tube, mix well. Heat to 95 °C 5 minutes and allow to cool slowly to Room temperature , 00:30:00 - 01:00:00 .

56 Add 7.5 µL of recombinant Cas9 (Cas9 stock is [M] 40 micromolar (µM) ). You now have a guide:Cas9 ratio of 4:1. Allow mix to rest at Room temperature for 00:15:00 - 00:20:00 . 20m

57 While Cas9 guide complex is forming, spin down cells, resuspend in PBS and count. Spin down enough cells for transfection, then wash 1x with Maxcyte buffer.

58 Resuspend 5e6 cells in 82.5 µL of Maxcyte buffer.

59 Add 5 µL of oPool HDR library (oPool library is at 40 pmol/uL) to the Cas9 guide complex, then add cells.

60 Add cells to Maxcyte assembly and transfect with protocol “Expanded T Cell-1”.

61 Transfer cells to 4 mL - 5 mL media + IL-2.

## **Step 5**: Collect day 2/3 samples for gDNA and RNA

62 If there are enough cells (>10e6), collect 2 samples, each containing 2-3e6 cells. Spin down and aspirate supernatant.

63 For sample 1, isolate gDNA with favorite kit. We use the Quick-DNA miniprep plus kit from Zymo, cat. No. D4069.

64 For sample 2, resuspend in 600 µL of Trizol reagent and store at -20 °C or -80 °C until processing.

## **Step 6**: Replace media with IL-2 every 2-3 days (M/W/F)

65 Replace media with IL-2 every 2-3 days. If cell counts are <40e6, we plate cells at 1e6/mL. If cell counts are >40e6/mL (typically after 7 days post transfection) we plate at 2e6/mL.

## **Step 7**: Take off IL-2 on day 9

66 1 day prior to CFSE labeling and restimulation with CD3/28, spin down cells and resuspend at 1-2e6/mL of media without IL-2.

## **Step 8**: Collect day 10 samples for gDNA and RNA

67 Wash day 10 cells 1x with PBS. Resuspend in 10 mL PBS and count cells.

68 Like section "Collect day 2/3 samples for gDNA and RNA" above, collect 2 samples, each containing 2-3e6 cells. Spin down and aspirate supernatant.

69 For sample 1, isolate gDNA with favorite kit.

70 For sample 2, resuspend in 600 µL of Trizol reagent and store at -20 °C or -80 °C until processing.

71 Stain 80e6 cells with CFSE and stim with CD2/3/28 (below).

## **Step 9**: Stain cells with CFSE and stimulate with CD2/3/28 (no IL-2) for 72 hours 1h 46m

72 Resuspend each vial of CFSE (Thermo, cat. No. C34570) in 18 µL DMSO.

73 Resuspend 80e6 cells in 40 mL PBS. Add 20 µL of CFSE, mix well and incubate at Room temperature for 00:05:00 . 5m

74 Add 5 mL of FBS, mix well and incubate at Room temperature for 00:05:00 . 5m

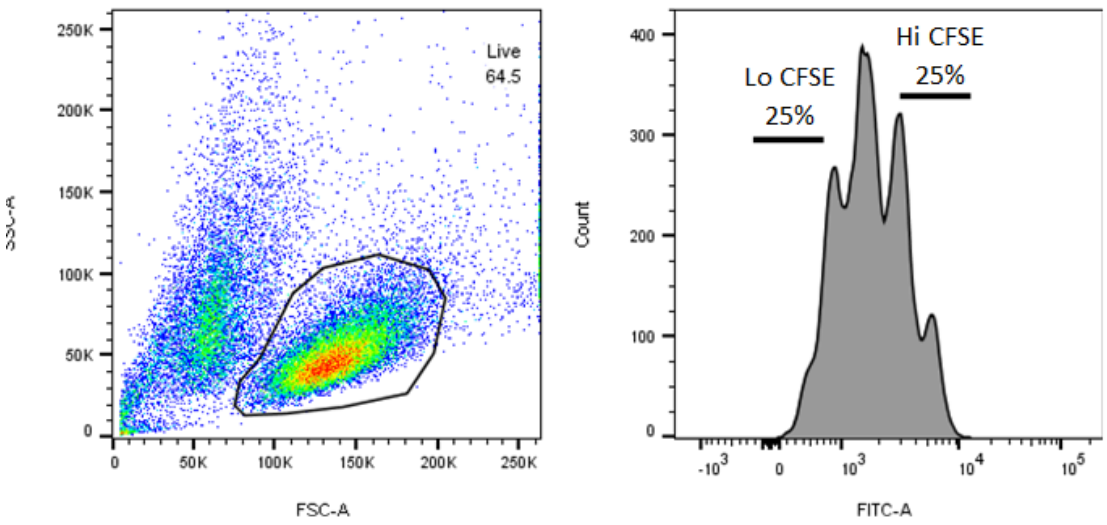
75 Spin down cells, wash 1x in 10 mL media without IL-2.

76 Resuspend cells in 40 mL media without IL-2 and add 125 µL of ImmunoCult Human CD2/3/28 (Stemcell, cat. No. 10970). Place in incubator and wait 72:00:00 - 96:00:00 before sorting. 1h 36m

## **Step 10**: Sort cells on flow cytometer 4d

77 72- 96:00:00 post stimulation, spin down cells and resuspend in PBS at 5-10e6 cells/mL. 4d

78 Pipette through strain cap into FACS tube and sort 2-4e6 cells based on the lowest and highest CFSE staining:




79 Isolate gDNA from both CFSE Lo and CFSE Hi fractions.