BROAD INSTITUTE | GENETIC PERTURBATION PLATFORM

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Protocol: Assaying dCas9 Activity for CRISPRa screening with Adherent Cells

After having established a dCas9-expressing cell line, this assay is to determine the level of dCas9 activity. A sgRNA for CD4 and a sgRNA for CD45 is introduced using a lentivirus (XPR_502). When CD4 and CD45 sgRNA's are introduced into a dCas9-expresssing cell line, expression of the corresponding target should increase in comparison to the baseline expression.

Before starting, it is recommended you complete the following steps:

- [ ] Puromycin or Blasticidin dosing (see **Protocol: Puromycin and Blasticidin titration**)

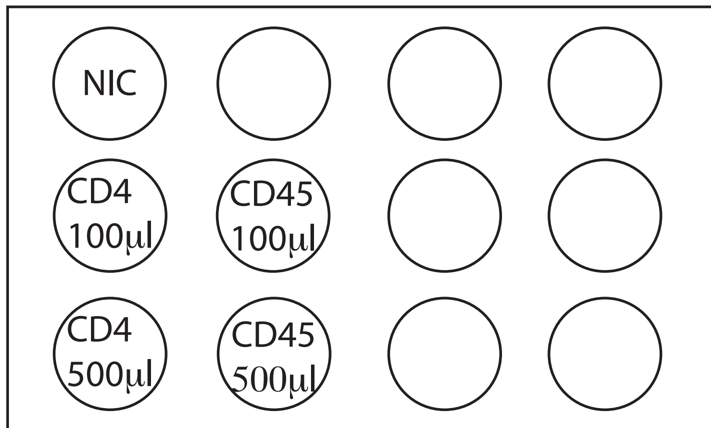
Materials and Reagents required:
9 million dCas9 (pXPR_BRD109) cells
sgCD4 in XPR_502 lentivirus (1x1 mL aliquot)*
sgCD45 in XPR_502 lentivirus (1x1 mL aliquot)*
Polybrene
Puromycin
96-v clear bottom plate (Corning, #3357)
6-well plates
12-well plates
APC anti-human CD4 antibody (Biolegend, #357408)
FITC anti-human CD45 antibody (Biolegend, #368508)
Flow buffer (PBS, 2% FBS, 5µM EDTA)
Flow cytometer (Accuri)

*The pXPR_BRD109, sgCD4, and sgCD45 lentiviruses are available from GPP (gpp-reagents@broadinstitute.org).

DAY 1

1. Trypsinize and count cells. Resuspend 9 million cells in a final volume of 9 mL of media.
2. Add 12 uL of 1000x polybrene.
3. Add 1.5 mL of cell suspension per well into 5 wells of a 12-well plate.
4. Add virus to wells.
    a. Add 500 uL of media to 1 well [no-infection control (NIC)].
    b. Add 100 uL of sgCD4 virus and 400 uL of media to 1 well.
    c. Add 500 uL of sgCD4 virus to 1 well.
    d. Add 100 uL of sgCD45 virus and 400 uL of media to 1 well.
    e. Add 500 uL of sgCD45 virus to 1 well.

Example of infection plate:

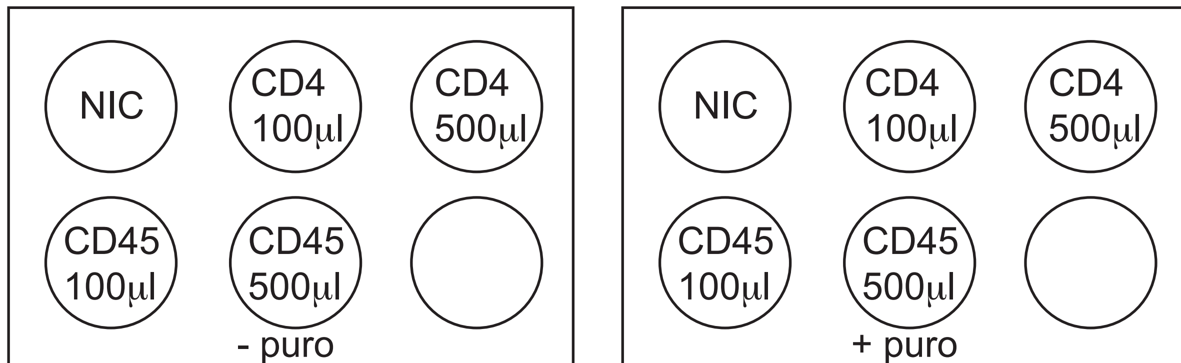


5. Spin plate for 2 hours at 1,000 x g at 30°C. After spin is complete, add dropwise 2 mL of media per well (for a total volume of 4 mL per well) and return plate to incubator.
6. 4-6 hours after the spin is complete, transfer the cells from each well into a T75 or 6-well dish (depending on optimal seeding density).

DAY 3

1. Trypsinize and count cells.
2. Seed an in-line titer into 6-well plates. For each cell population, seed 2 wells with 1.5E5 cells per well (for a total of 10 wells). Add puromycin to half of the wells (one for each population).

Example of in-line titer plate:



3. For the NIC cells, seed remaining cells in media without puromycin in a T75 or 10 cm dish at standard confluence.
4. For the sgCD4- and sgCD45-infected cells, seed remaining cells in puromycin-containing media in a T75 or 10 cm dish at approximately 50% confluence (to account for selection).

DAY 4-8

1. Passage and/or maintain the in-line titer and flasks. Make sure that the - puro wells in the in-line titer do not reach confluence.

DAY 8

Read out in-line titer

1. Visually confirm that the all cells in the + puro NIC well are dead. If they are not, continue passaging titer and puromycin-selection flasks until they are.
    a. This may also indicate that the puromycin dose is too low and should be re-determined.
2. Trypsinize and count the cells in each well. Obtain the infection efficiency for each virus volume by dividing the number of cells in the + puro well by the number of cells in the - puro well. For each virus, select the volume that gave the closest to 15-40% infection efficiency to stain and flow in the following step.

Stain and flow

1. Trypsinize and count cells from the unselected NIC flask and the chosen virus volumes of sgCD4-infected and sgCD45-infected cells.
2. In a 96 well V-bottom plate, seed approximately 2.5E5 cells/well of the following:
    a. 2 wells of NIC cells (1 unstained control and 1 stained control)
    b. 1 well of sgCD4-infected cells

    c. 1 well of sgCD45-infected cells
3. Spin plate (5 min @ 1,000 x g) and remove supernatant with aspirating wand.
4. Turn off hood and room light. Resuspend each well in 90 uL of flow buffer.
    a. Add 10 uL of flow buffer to the NIC unstained control well.
    b. Add 5 uL of anti-CD4 antibody and 5 uL of anti-CD45 antibody to each remaining well, for a total volume of 100 uL per well.
    c. Resuspend each well.
5. Cover the plate with foil and incubate on ice for 20-30 min.
6. Spin (5 min @ 1,000 x g) and remove supernatant with aspirating wand.
7. Add 200 uL flow buffer/well and resuspend.
8. Repeat steps 6 – 7 two more times.
9. Flow plate using a flow cytometer. If using the Accuri use: FL1-A for FITC-anti-CD45 and FL4-A for APC-anti-CD4.
    a. Gate live cells using FSC/SSC.
    b. Use the NIC unstained control well to set gates on FL1-A and FL4-A. CD4 and CD45 activation should be assessed with both the % positive cells and the mean fluorescence.