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 (pXPR_BRD109), this assay is to determine the level of dCas9 activity. An sgRNA for CD4 and an sgRNA for CD45 are introduced using lentiviruses (XPR_502). When CD4 and CD45 sgRNAs are introduced into a dCas9-expresssing cell line, expression of the corresponding target should increase in comparison to the baseline expression. The pXPR_BRD109, sgCD4, and sgCD45 lentiviruses are available from GPP (gpp-reagents@broadinstitute.org). Please note the guide sequences are for the human taxon only.

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

☐ Polybrene sensitivity curve (see **Protocol: Optimization of lentiviral transduction using spinfection)**

☐ Puromycin dosing (see **Protocol: Puromycin, blasticidin and hygromycin titration**)

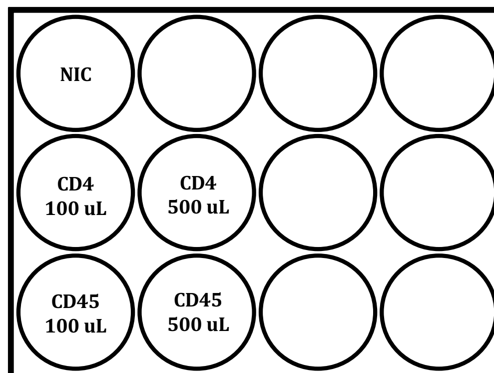
Materials and reagents required:

- 9 million dCas9-expressing cells (pXPR_BRD109)
- 1 mL sgCD4 in XPR_502 lentivirus (human, clone BRDN0002435409, available from Addgene plasmid #158704)
- 1 mL sgCD45 in XPR_502 lentivirus (human, clone BRDN0002435411, available from Addgene plasmid #158705)
- 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 (ex. Accuri)

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:

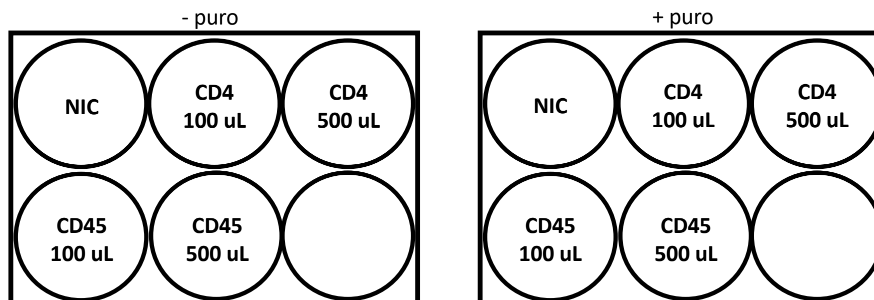


5. Spin plate for 2 hours at 930 x g at 30°C.
6. After spin is complete, add 2 mL of media dropwise per well (for a total volume of 4 mL per well) and return plate to incubator.
7. 4-6 hours after the spin is complete, transfer the cells from each 12-well into a 6-well plate or T75 (depending on optimal seeding density).

DAY 3

1. Trypsinize and count cells.
2. Seed an in-line titer into two 6-well plates. From 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). **Save the remaining cells!**

    Example of in-line titer plates:



3. Seed remaining NIC cells in media without puromycin in a T75 or 10 cm dish at standard confluence.
4. Seed the remaining sgCD4- and sgCD45-infected cells 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 plates and flasks. Make sure that the (-)puro wells of the in-line titer plates do not reach confluence.

DAY 8

A: Read out in-line titer

1. Visually confirm that the all cells in the (+)puro NIC 6-well are dead. If they are not, continue passaging titer plates and puromycin-selection flasks until they are.
   - *This may indicate that the puromycin dose is too low and should be re-determined.*
2. Trypsinize and count the cells in each 6-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 sgCD4 and sgCD45, select the virus volume that gives the closest to a 15-40% infection efficiency to stain and flow in the following step.

B: 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 carefully remove carefully supernatant.
4. Turn off hood and room light. Resuspend each well in 90 uL of flow buffer. Then add:
   a. 10 uL of flow buffer to the NIC unstained control well.
   b. 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 carefully remove supernatant.
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.