Last modified: April 2019
Last reviewed: April 2019

Protocol: Assaying dCas9 activity for CRISPRi screening with adherent cells

After having established a KRAB-dCas9-expressing cell line (pXPR_BRD121), this assay is to determine the level of dCas9 activity. An sgRNA for CD81, a cell surface protein, is introduced using lentivirus (pRDA_054). When the CD81 sgRNA is introduced into a dCas9-expresssing cell line, expression of the corresponding target should decrease in comparison to the baseline expression. The pXPR_BRD121 and sgCD81 lentiviruses are available from GPP (gpp-reagents@broadinstitute.org).

The APC anti-human CD81 antibody concentration and the timepoint for flow cytometry readout of the assay can be optimized for the chosen cell line.

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 and blasticidin titration**)
- ☐ Test for CD81 expression in parental cells – low expression at baseline may hinder the interpretation of the assay

Materials and reagents required:

- ~2 million KRAB-dCas9-expressing (pXPR_BRD121) cells
- 1 mL sgCD81 lentivirus (pRDA_054; GCCTGGCAGGATGCGCGGTG)
- Polybrene
- Puromycin
- 96-v clear bottom plate (Corning #3357)
- 6-well plates
- 12-well plates
- APC anti-human CD81 antibody (Biolegend #349509)
- Flow buffer (PBS, 2% FBS, 5µM EDTA)
- Flow cytometer (ex. Accuri)

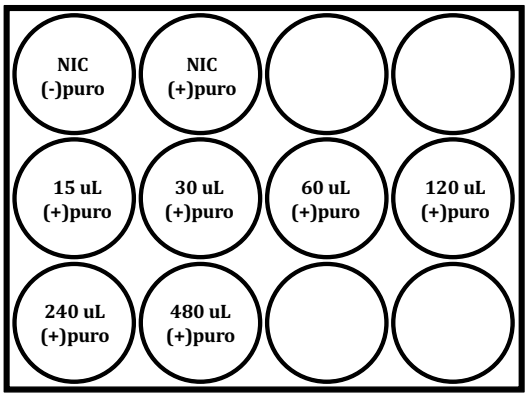
DAY 1

1. Trypsinize and count cells. Resuspend 1.25 million cells in a final volume of 10 mL.
2. Add 10 µL of 1000x polybrene.
3. Add 1 mL of cell suspension to 8 wells of a 12-well plate.

4. Add varying volumes of sgCD81 virus to 6 of the wells: 15 µL, 30 µL, 60 µL, 120 µL, 240 µL, and 480 µL. To the remaining 2 wells add no virus (no-infection controls).
5. Spin plates for 2 hours at 1,000 x g at 30˚C. Move cells to incubator.
6. 4 - 6 hours after the spin is complete, carefully remove the media and replace with 1 mL of fresh media.

DAY 2

1. >24 hours after the infection, add 1 mL of fresh media to one of the no-infection control wells. To the remaining 7 wells, including the other no-infection control well, add 1 mL of 2x puromycin.



DAYS 3 – 5

1. When the no-infection control well *with* puromycin selection is completely dead and the no-infection control well *without* puromycin selection reaches 100% confluency, trypsinize and count the cells. **Do not throw out the remaining cells at this step!**
2. In real-time, determine the infection efficiencies: the number of cells in the with-infection/with-selection well divided by the number of cells in the no-infection/no-selection well:

$$\frac{\text{15 uL (+)puro}}{\text{NIC (-)puro}} \quad \frac{\text{30 uL (+)puro}}{\text{NIC (-)puro}} \quad \frac{\text{60 uL (+)puro}}{\text{NIC (-)puro}} \quad \frac{\text{120 uL (+)puro}}{\text{NIC (-)puro}} \quad \frac{\text{240 uL (+)puro}}{\text{NIC (-)puro}} \quad \frac{\text{480 uL (+)puro}}{\text{NIC (-)puro}}$$

3. Two cell populations are needed for the remainder of the experiment: the no-infection/no-puro control well, and the with-infection/with-puro well with the virus volume that gave 15 – 40% infection efficiency. Re-seed these two populations into appropriately-sized flasks, maintaining the sgCD81-infected cells with puromycin.

DAYS 5 – 10

1. Continue to passage these two populations, maintaining the sgCD81-infected cells with puromycin.

DAY 10

1. Trypsinize and count cells from the unselected no-infection control flask and the sgCD81-infected flask.
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 sgCD81-infected cells
3. Spin plate (5 min @ 1,000 x g) and carefully remove supernatant.
4. Turn off hood and room light. Resuspend each well in 99 uL of flow buffer. Then add:
    a. 1 uL of flow buffer to the NIC unstained control well.
    b. 1 uL of APC anti-CD81 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 FL4-A for APC-anti-CD81.
    a. Gate live cells using FSC/SSC.
    b. Use the NIC unstained control well to set gates on FL4-A. CD81 knockdown should be assessed relative to the NIC stained control, using both the % positive cells and the mean fluorescence.