Last Modified: February 2019; vector name change
Last Reviewed: February 2019

Protocol: Assaying Cas9 activity with an EGFP reporter

After having established a Cas9-expressing cell line (see **Protocol: Optimization of lentiviral transduction using spinfection**), this assay is to determine the level of Cas9 activity. An sgRNA for EGFP is introduced using a lentivirus (pXPR_011 or pXPR_047) that also contains EGFP as a target. pXPR_047 expresses a destabilized GFP that allows readout at 5 days post-infection, as opposed to 8-10 days with pXPR_011. In a Cas9-expressing cell line, the EGFP sequence will be cleaved and the cells will not fluoresce. The pXPR_011 or pXPR_047 lentiviruses are available from GPP (gpp-reagents@broadinstitute.org).

Prior to this assay, the appropriate concentration of polybrene and puromycin must be optimized for the Cas9-expressing cells:

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

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

Materials and reagents required:

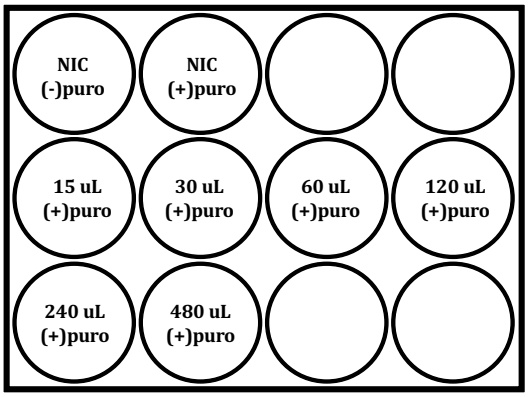
- ~2 million Cas9-expressing cells
- 1 mL pXPR_011 or pXPR_047 lentivirus
- Polybrene
- Puromycin
- 12-well plates
- Flow cytometer

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 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-selection control well, and the with-infection/with-selection well with the virus volume that gave 15 – 30% infection efficiency. If using pXPR_011, continue to passage these two populations.

<u>Determine percentage of GFP-negative cells</u>:

DAY 5, if using pXPR_047
DAY 8-10, if using pXPR_011

1. Assay the two populations of cells by flow cytometry. Use the no-infection, no-selection cells to draw appropriate gates for EGFP-negative cells.
2. Cells that don't express Cas9 will appear green, while the Cas9-expressing cells will not fluoresce. The activity of Cas9 in the cells, then, is the fraction of the with-infection, with-selection cells that are EGFP-negative. While we have observed wide ranges of activity, 75 – 85% GFP-negative is most typical and 'screenable' for most experimental purposes.