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## Protocol: Assaying Cas9 activity with an EGFP reporter assay

After having established a Cas9-expressing cell line (see Protocol: Introducing Cas9 lentivirus into cell lines and determining infection efficiency), this assay is to determine the level of Cas9 activity. An sgRNA for EGFP is introduced using a lentivirus (pXPR\_011 or pXPR\_011v2) that also contains EGFP as a target. pXPR\_011v2 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.

Prior to this assay, you should already know the appropriate concentration of polybrene and puromycin for your Cas9-expressing cells. 1 million cells and about 945  $\mu$ L of pXPR\_011 virus are required.

Material required:

pXPR\_011 or pXPR\_011v2 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  $\mu$ L 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  $\mu$ L, 30  $\mu$ L, 60  $\mu$ L, 120  $\mu$ L, 240  $\mu$ L, and 480  $\mu$ L. To the remaining 2 wells add no virus (no-infection control).
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 one of the no-infection control wells, 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* selection reaches 100% confluence, trypsinize and count the cells.<sup>1</sup> Do not throw out the remaining cells at this step!
2. In real-time, determine infection efficiency: the number of cells in the with-infection/with-selection well divided by the number of cells in the no-infection/no-selection well.
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.

### Determine percentage of GFP-negative cells:

DAY 5, if using pXPR\_011v2.

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.

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<sup>1</sup> If the no-selection well of the uninfected well reaches 100% confluence before the with-selection well is completely dead, split again. It is also possible that you are not using the right concentration of puromycin, and this should be re-optimized.