

Last Modified: June 2020; Cas12a addition

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Protocol: Assaying Cas9 or Cas12a activity with an EGFP reporter

Brief description:

After having established a Cas9 or Cas12a-expressing cell line (see **Protocol: Optimization of lentiviral transduction using spinfection**), this assay is to determine the level of Cas9 or Cas12a activity. Either one or two sgRNAs for EGFP are introduced using a lentivirus that also contains EGFP as a target.

The Cas9 activity assay vectors are either pXPR_047 or pXPR_011; the Cas12a activity assay vector is pRDA_221. pXPR_047 and pRDA_221 express a destabilized GFP that allows readout at 5 days post-infection, as opposed to 8-10 days with pXPR_011. pRDA_221 contains two Cas12a-specific sgRNAs linked by a direct repeat sequence and can be used to test activity in either AsCas12a or EnAsCas12a cells.

In a Cas9 or Cas12a-expressing cell line, the EGFP sequence will be cleaved and the cells will not fluoresce. Additionally, the pXPR_011 or pXPR_047 and pRDA_221 vectors serve as controls for one another in this assay. The pXPR_011, pXPR_047 and pRDA_221 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 parental and Cas9 or Cas12a-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:

- ~3 million Cas9 or Cas12a-expressing cells
- 1 mL pXPR_011 or pXPR_047 (Cas9) lentivirus
- 1 mL pRDA_221 (Cas12a) lentivirus
- Polybrene
- Puromycin
- 12-well plates
- Flow cytometer

STEP 1: Spinfection and selection

Day 1 AM

1. Trypsinize and count the Cas9 or Cas12a-expressing cells. Resuspend 2.5 million cells in a final volume of 20 mL.
2. Add 20 μ L of 1000x polybrene.
3. Add 1 mL of cell suspension to 8 wells of a 12-well plate, then repeat with a second 12-well plate.
4. To the first 12-well plate, add varying volumes of pXPR_011 or pXPR_047 virus to 6 of the wells: 15 μ L, 30 μ L, 60 μ L, 120 μ L, 240 μ L, and 480 μ L. To the remaining 2 wells add no virus (no-infection controls).
5. Repeat step 4, adding the pRDA_221 virus to the second 12-well plate.
6. Spin the plates for 2 hours at 1,000 x g at 30°C then move plates to an incubator.

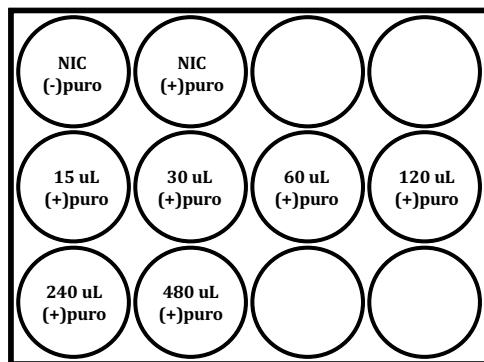
Day 1 PM

7. 4 - 6 hours after the spin is complete, carefully remove the media and replace with 1 mL per well of fresh media.

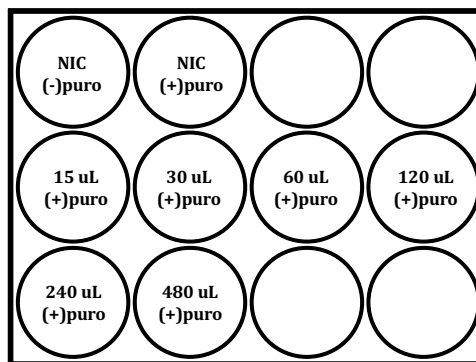
Day 2

1. For each of the cell plates: >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.

pXPR_011 or pXPR_047 plate:



pRDA_221 plate:



STEP 2: Assessing infection efficiencies

Days 3 - 5

1. When the no-infection control wells *with* puromycin selection are completely dead and the no-infection control wells *without* puromycin selection reach 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:

15 uL (+)puro	30 uL (+)puro	60 uL (+)puro	120 uL (+)puro	240 uL (+)puro	480 uL (+)puro
NIC (-)puro	NIC (-)puro	NIC (-)puro	NIC (-)puro	NIC (-)puro	NIC (-)puro

3. Three cell populations are needed for the remainder of the experiment:
 - a. No-infection/no-puromycin cells from either plate
 - b. The with-infection/with-puromycin cells from the pXPR_011 or pXPR_047 virus volume well that gave a 15 – 30% infection efficiency
 - c. The with-infection/with-puromycin cells from the pRDA_221 virus volume well that gave a 15 – 30% infection efficiency
4. If using pXPR_011, continue to passage these three populations for another 5 days. If using pXPR_047, proceed directly to Step 3.

STEP 3: Determine percentage of GFP-negative cells

Day 5, if using pXPR_047 and pRDA_221

Days 8-10, if using pXPR_011 and pRDA_221

1. Assay the four populations of cells by flow cytometry.
2. Use the no-infection/no-puromycin cells (a) to draw appropriate gates for EGFP-negative cells.
3. Cells infected with the alternative technology's vector (b or c) should appear >95% EGFP-positive (i.e. pRDA_221-infected cells if using pXPR_047 to assess Cas9 activity). If they are not, this may indicate that the puromycin dose is too low and should be re-determined.
4. Cells without active Cas9 or Cas12a will appear green, while cells with active Cas9 or Cas12a will not fluoresce. The activity of Cas9 or Cas12a in the cells, then, is the fraction of the with-infection/with-puromycin cells (b or c) that are EGFP-negative.

While we have observed wide ranges of activity, >75% EGFP-negative is typical and 'screenable' for most experimental purposes.