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Protocol: OOF-EGFP Gene Editing Reporter Assay

This protocol is to determine whether your cell line is amenable to CRISPR gene editing. The virus introduced expresses an Out-of-Frame (OOF) eGFP. Upon co-expression of Cas9 and an sgRNA for an in-frame eGFP, the Out-of-Frame (OOF) EGFP DNA will be cleaved and repaired to express the correct eGFP. Therefore cells with a functional Cas9 will appear green.

Prior to gene editing experiments, various volumes of puro-2A-OOF-eGFP virus should be introduced into your cells of interest to achieve an infection efficiency of about 30-50% (MOI ~ 1, one integrant per cell). This protocol assumes you already know the correct polybrene and puromycin concentrations for your cells.

Materials:

- Puro-2A-OOF-eGFP virus: Expresses PuroR – 2A – Out-of-Frame eGFP (1 mL aliquot)
- pXPR_201 with sgRNA: sgRNA expression plasmid for transfection (10 μ L of 10 ng/ μ L aliquot – ampicillin resistance for bacterial growth)
OOF sgRNA: CTCAGGGCGGAATAACCACG
- pXPR_203: Cas9-expression plasmid for transfection (10 μ L of 10 ng/ μ L aliquot – ampicillin resistance for bacterial growth)
- Repair template oligonucleotide (not supplied):
5'CGACGGCCCCGTGCTGCTGCCGACAACCACTACCTGAGCACCCAGTCC
GCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCAC3'

DAY 1

1. Trypsinize and count cells. Resuspend 1.25 million cells in a final volume of 10 mL.

2. Add 10 μ 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 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 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.

DAY 4 - 6

1. When the no-infection control well *with* selection is completely dead and the no-infection control well *without* selection reaches 100% confluence, trypsinize and count the cells.¹ **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. Continue to passage the cell population with the virus volume that gave 15 – 30% infection efficiency. These cells will *not* be EGFP+ as the EGFP is still out-of-frame.

Gene Editing Homology-Directed Repair Transfection Assay: What follows are the conditions we have optimized for 293T cells with the transfection reagent LT1. Every cell line / reagent combination will be different, and the purpose of this reporter assay is to determine what transfection conditions give rise to the best gene editing conditions. Our optimal conditions for 293T cells are provided for guidance, but there is no guarantee that other cell lines will perform the same. *Transfection reagent, pDNA amount, and oligonucleotide repair template amount all need to be optimized.*

DAY -1 (17-24 hours prior to transfection):

1. Seed Puro-2A-OOF-eGFP positive cells from the previous step at the appropriate density for your preferred transfection reagent. For HEK-293T cells and the transfection reagent LT1, this is 100,000 cells per well in a 24-well plate. Be sure to include one well that will remain untransfected as a control.

DAY 0 (Transfection preparation for a single well):

¹ 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 correct concentration of puromycin, and this should be re-optimized.

1. Prepare Transfection Mix: Add 5 μ L of transfection agent (LT1) to 45 μ L of OptiMEM. Incubate Transfection Mix for 5 minutes at room temperature.
2. Prepare DNA Mix: Use 50 ng pXPR_203, 50 ng of pXPR_201, and 50 pmol repair template oligo, to a final volume of 50 μ L (dilute in Opti-MEM, if necessary).
These DNA amounts are highly dependent upon cell type – trying multiple DNA amounts is recommended.
3. Add 50 μ L of Transfection Mix to 50 μ L of DNA Mix. Incubate at room temperature for 25 minutes.
4. During incubation period, remove 0.5 mL of media from cells and add 0.5 mL fresh media.
5. After 25 minute incubation period, add the DNA + Transfection mixture (100 μ L) dropwise to cells.
6. Centrifuge cells at 1000 x g for 30 minutes at 37°C.
7. 4 – 8 hours post-transfection, replace media on cells.

DAY 1 - 6

1. Maintain cells, passaging as necessary.

DAY 3 - 6

1. Flow cells for GFP expression. For 293T cells, Day 3 is sufficient, but other cell lines may take longer.