

Cas9 activity assay using flowcytometry

The Cas9 efficiency will be determined by introducing an sgRNA targeting the B2M gene. Disruption of this gene will lead to loss of surface MHC Class I (assayed by flowcytometry)

Generation sgRNA B2M lentivirus:

1. Seed 293T cells at $0.75/1.0/1.5 \times 10^6$ per 6 well and grow O/N at 37 °C in total 2 ml IMDM with 10% FCS and P/S (complete IMDM)
2. For transfection choose well where confluency of the 293T cells is about 70%
3. Prepare transfection mix (amounts for one 6-well):
 - a. 100 µl Optimem containing:
 - i. 0.44 µg pMD.G
 - ii. 0.67 µg pCMV dR8.91
 - iii. 0.89 µg pKLV-sgRNA B2M-BFP-puro
 - b. 100 µl Optimem containing:
 - i. 4 µl TransIT293 transfection reagent
4. Mix solution a and c together
5. Incubate 20 min at RT
6. Add transfection mix dropwise to 293T cells
7. Remove supernatant from cells containing the virus 48 hours later
 - a. Note: 293T cells should look very sick (membranes bubbly, etc)
8. Filter supernatant with 0.45 µm filter to remove 293T cells
 - a. Virus can be stored at -80 °C
9. Add 50 µl of virus to 5×10^4 HAP1 Cas9 cells in a 24-well in 1 ml complete IMDM
10. Spinfect:
 - a. Centrifuge plate 1,800 RPM for 1 hour at 37 °C
11. Transfer cells to incubator and grow for 48 hours at 37 °C
12. Puromycin selection:
 - a. Add 3.5 µg/ml puromycin
13. Expand cells for one week post transfection with puromycin, freeze and measure surface MHC Class I by flow cytometry

Flowcytometry analysis surface MHC Class I:

1. Harvest cells from 24-well and use 50 % of them for flowcytometry
2. Transfer cells to 4 ml FACS tube and wash with PBS + 2 % FCS
3. Resuspend cells in 100 µl PBS + 2 % FCS including 1 µl W6/32-APC antibody
4. Incubate 30 minutes at 4 °C in the dark
5. Wash cells with PBS + 2 % FCS
6. Resuspend in 500 µl PBS + 2 % FCS
7. Flowcytometry:
 - a. Measure BFP and APC
 - b. Determine % of BFP and APC double negative cells
 - i. This population contains all surface MHC Class I negative cells (read-out for Cas9 activity)
 - ii. This population should be ≥ 80 %