**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 x 106 per 6 well and grow O/N at 37 ℃ in total 2 ml IMDM with 10% FCS and P/S (complete IMDM)
2. For transfection choose well were confluency of the 293T cells is about 70%
3. Prepare transfection mix (amounts for one 6-well):
   1. 100 μl Optimem containing:
      1. 0.44 μg pMD.G
      2. 0.67 μg pCMV dR8.91
      3. 0.89 μg pKLV-sgRNA B2M-BFP-puro
   2. 100 μl Optimem containing:
      1. 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
   1. Note: 293T cells should look very sick (membranes bubbly, etc)
8. Filter supernatant with 0.45 μm filter to remove 293T cells
   1. Virus can be stored at -80 ℃
9. Add 50 μl of virus to 5 x104 HAP1 Cas9 cells in a 24-well in 1 ml complete IMDM
10. Spinfect:
    1. Centrifuge plate 1,800 RPM for 1 hour at 37 ℃
11. Transfer cells to incubator and grow for 48 hours at 37 ℃
12. Puromycin selection:
    1. 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 ℃ in the dark
5. Wash cells with PBS + 2 % FCS
6. Resuspend in 500 μl PBS + 2 % FCS
7. Flowcytometry:
   1. Measure BFP and APC
   2. Determine % of BFP and APC double negative cells
      1. This population contains all surface MHC Class I negative cells (read-out for Cas9 activity)
      2. This population should be ≥ 80 %