When referring to this protocol, please cite: Wang L, Brugge JS, Janes KA. (2011) Intersection of FOXO and RUNX1 gene-expression programs in single breast epithelial cells during morphogenesis and tumor progression. *Proc Natl Acad Sci*, 108, E803-12.

1. Seed 50,000 cells per well on a 6-well dish overnight
   - *The low seeding density ensures that cells will be actively proliferating during the entire infection protocol and increases the effective MOI for the same amount of virus*
   - *Alternatively, 25,000 cells can be seeded for two days before the first infection*
2. Aspirate the culture medium, wash cells with PBS, and add 0.5 ml culture medium containing 16 μg/ml (2×) polybrene
   - *Polybrene increases the efficacy of transduction by several orders of magnitude; thus, polybrene and virus are mixed together only on the plate*
   - *Be sure to include an infection with an appropriate control virus; this can be an empty vector for overexpression or a control shRNA for knockdown*
   - *Also, be sure to include a mock infection where cells simply receive 1× polybrene in culture medium; this sample will be used to ensure that cells tolerate the polybrene treatment (some cell types do not) and also to verify that the subsequent antibiotic selection has worked*
3. Add 0.5 ml retroviral or lentiviral supernatants dropwise to the well, agitate gently, and incubate overnight
   - *Addition of 0.5 ml virus dilutes the polybrene to 1× final concentration*
   - *If adding less than 0.5 ml virus, add culture medium to a total volume of 1 ml per well*
4. In the morning, aspirate the virus mixture and add 2 ml culture medium
   - *There is no need to wash cells with PBS at this step*
5. If performing serial infections, repeat Steps 2–4 up to two additional times
6. 24 or 48 hr after the last infection, trypsinize the cells and plate the entire well on a 10-cm plate in the presence of the appropriate selection antibiotic
   - *Wait 24 hr if 2-3 serial infections were done in total or 48 hr if only one infection was done so that cells have enough time to express the selection antibiotic*
   - *Be sure to similarly plate out the mock-infected cells; these cells should be clearly dying after ~2 days in the presence of the selection antibiotic*
7. Surviving cells can be considered stably transduced after the mock-infected plate is completely clear of cells
   - *Even though expression of the viral transgene is theoretically stable, in practice expression can fade with time in culture; therefore, expand the surviving cells to ~three 10-cm plates and freeze ~10 vials for future experiments (expand and freeze more if the cells are likely to be used extensively)*
   - *It not uncommon for the control virus to grow faster than the other infections; simply freeze down the control cells earlier (do not passage them until the other infections "catch up")*
8. Verify expression or knockdown of the intended protein by Western blotting (see Janes_Westernblotting.pdf or Janes_WesternblottingLicor.pdf)

Buffer recipes

- **1000× polybrene** Store in 1 ml aliquots at –20°C
  Dissolve 8 mg/ml polybrene (Sigma #H9268) in water and sterile filter

- **1000× puromycin** Store in 1 ml aliquots at –20°C
  Dissolve 2 mg/ml puromycin (MP Biomedicals #100552, available through Fisher) in water and sterile filter

- **100× G418** Store in 1 ml or 5 ml aliquots at –20°C
  Dissolve 30 mg/ml G418 (Sigma #A1720) in water and sterile filter

- **500× hygromycin** Store at 4°C
  Aliquot ~50 mg/ml hygromycin B solution (Sigma #H0654)