Last Modified: September 2020; hygromycin addition
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Protocol: Puromycin, Blasticidin and Hygromycin Titration

Brief Description:

A lentiviral construct carrying an antibiotic resistance gene will allow selection of the cell population that has integrated that transgene. This protocol describes a method to establish a dose of puromycin, blasticidin or hygromycin to select the transgenic cells. The pRosetta (aka pLKO_TRC060) (PuroR, BlastR, GFP) or pRosettav2 (PuroR, BlastR, HygroR, GFP) lentiviruses are available from GPP (gpp-reagents@broadinstitute.org).

Prior to this assay, the appropriate concentration of polybrene must be optimized for the cells:

☐ Polybrene sensitivity curve (see **Protocol: Optimization of lentiviral transduction using spinfection)**

Materials and reagents required:

- ~20 million cells
- 6-well and 12-well plates
- Polybrene
- 1 mL pRosetta or pRosettav2 lentivirus
- V-bottomed 96-well plate
- Flow buffer (PBS, 2% FBS, 5μM EDTA)
- Flow cytometer
- 10 mg/mL puromycin (Sigma Aldrich, catalog # P9620)
- 10 mg/mL blasticidin (Life Technologies, catalog # A1113903)
- 50 mg/mL hygromycin (Life Technologies, catalog # 10687010)

Procedure

Day 0 AM: Infection

1. Check cells under the microscope to ensure they are in good health and around 80-90% confluent.
2. Aspirate media, wash cells with PBS, trypsinize, quench, and count.
3. Thaw the virus on ice or at room temperature, **do not thaw in the water bath.**
4. Make a suspension of cells at a concentration of 3E6 cells/mL in a total of 5 mL and add a 2X dose of polybrene.
5. Seed 1 mL of cell suspension as an NIC (no-infection control) into one 12-well.

6. Seed 1 mL of cell suspension into 3 other wells, one for a low, medium and high virus volume. Suggested pRosetta virus volumes are 20, 100 and 500 uL. The objective is to be able to choose a virus volume that yields an infection efficiency of ~30%.
7. Add the designated volumes of virus to the wells.
8. Bring each well up to 2 mL total with media.
9. Spin plates 930g for 2 hours at 30°C.
10. When centrifugation is complete, add 2 mL of media to each well dropwise and place in a 37°C incubator overnight.

Day 1 AM: Flow Cytometry and 6-well Plating

1. From each well, aspirate media, trypsinize cells (200 uL trypsin) and quench in 800 uL of media.
2. Take 200 uL of resuspended cells from each well and seed into a V-bottomed 96-well plate for flow cytometry. Add 100 uL flow buffer (PBS, 2% FBS, 5μM EDTA) to each well and mix.
3. Use the NIC cells to draw appropriate gates for EGFP-negative cells, then assay the range of virus volumes via flow cytometry. **Continue the experiment with the cells from the virus volume that yields closest to 30% GFP+ cells.**
4. Add 4 mL of media to each well of 3x 6-well plates (one each for puromycin, blasticidin and hygromycin), then seed 150,000 cells per well. A NIC is no longer needed.
5. Incubate plates at 37˚C.

Day 1 PM: Drug Addition

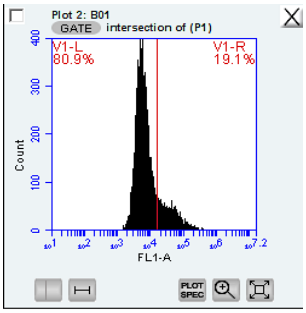
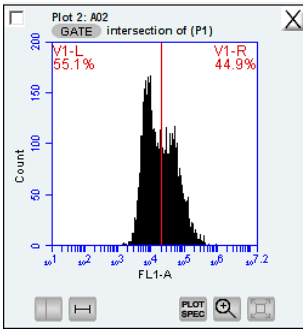
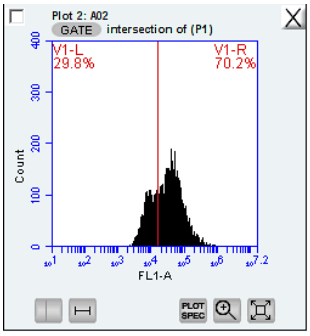
1. Choose a range of six doses for each drug and label the 6-well plates. Recommended doses:
    a. Puromycin: 0, 0.25, 0.5, 1, 2, 4 ug/mL
    b. Blasticidin: 0, 2, 4, 8, 16, 32 ug/mL
    c. Hygromycin: 0, 25, 50, 100, 200, 400 ug/mL
2. Perform serial dilutions to obtain the chosen concentrations at **5X working stocks**. If using the recommended doses, do the following:
    a. Label five conicals with 4, 2, 1, 0.5 and 0.25 ug/mL puromycin.
    b. Add 4 mL of media to the '4 ug/mL' conical and 2 mL of media to the remaining conicals.
    c. Add 8 uL of 10 mg/mL puromycin to the '4 ug/mL' conical and mix thoroughly.
    d. Take 2 mL from the '4 ug/mL' conical and add it to the '2 ug/mL' conical. Mix thoroughly.
    e. Repeat step d. for the subsequent tubes to serially dilute the working stocks by half.
    f. Repeat the serial dilution with blasticidin and hygromycin, initially adding 64 uL and 160 uL to the first conical, respectively.
3. Add 1 mL of each 5X working stock media to its corresponding labeled 6-well (total volume now 5 mL). **Be sure to include a no-drug control on each plate.**

Day 3-15: Flow Cytometry and Passaging

1. Passage cells every couple of days, or when they reach ~90% confluence, continuing to dose the cells with the drug doses chosen on Day 1. Each well might need to be passaged at different times.
2. Set aside 200 uL to assay each condition by flow cytometry. Use the no-drug control wells to draw appropriate gates for EGFP-negative cells.

3. Choose the lowest dose that achieves 95% GFP+ in the majority of wells within ~day 7 for puromycin ~day 14 for blasticidin/hygromycin. Various cell lines will take different amount of time to reach this point, and the experiment may be continued as long as needed.

Here are a few representative flow plots (live cell population gated using forward and side scatter to exclude debris) from an experiment with the G402 cell line:

Day 1 (before drug addition) Depending on the infection efficiency, at this point a small fraction of cells are GFP+ before drug selection begins. The red vertical line of separation is determined by comparison to uninfected cells.	
Day 5 on blasticidin At this time point, cells begin to clearly separate into two populations, as the selection drug has begun enriching for a GFP+ population.	
Day 8 on blasticidin At this point, drug selection should be progressing, with a distinct shift towards a growing GFP+ population.	
Day 15 on blasticidin Drug selection at most doses should be nearly complete at this point, thus showing >90% GFP+. There may still be a small fraction of GFP- cells, most likely at lower doses, but at least some doses in the range chosen should show nearly complete selection (>95% GFP).