Lentivirus infection and general screening plan—Adherent cells

Prepping for screening

-Ensure you have enough media and plates for screen

-Test small scale (~6-well plate size) transfection and infection to estimate infectivity. Goal infection rate is 20-50% infected cells, as measured by %BFP+ 2-3 days after infection.

-If using a drug/toxin selection, test concentrations and dosing protocols in as close to the screen conditions as possible. See the screening principles document for more information.

-Scale up culture to reach the number of cells you need on infection day.

Calculating number of cells and plates required

Minimum number of cells throughout screen = 1000 x library size / percent infected

Genome-scale 5 sgRNAs/gene = 1000 x 100,000 / 85% = 120x10^6 cells

Plates required for each split = Minimum cells / seeding density per plate

15cm dishes for 120x10^6 cells = 120x10^6 cells / 6x10^6 cells = 20 15cm plates

5 days prior to infection

Seed 15cm plates for mega transfection protocol. Each plate yields ~30mL virus, so calculate the number of plates based on the volume of virus you will need on infection day. The volume will depend on your cell line and packaging efficiency, as tested above. The quantities listed below are typical numbers we use in HEK293T cells. In addition, these quantities are for a single replicate. We typically do screens in duplicate, maintaining two independent cultures starting from the infection. For screens where the number of plates required will be prohibitive, replicates can be done one after another rather than in parallel. Alternately, if you are splitting cells every other day as outlined in this protocol, you can stagger replicates to only split one replicate each day.

Day -1

Seed 5x10^6 cells per 15cm plate in 25mL media.

Day 0 Infection

1. Harvest virus as described in transfection protocol.
2. Add 3.5mL viral supernatant with polybrene to each plate for final concentration of 8ug/mL polybrene.
3. Incubate at 37C overnight.

Day 1 Split cells

1. Aspirate media from each plate, working quickly to avoid drying cells.
2. Wash cells with 5mL PBS without Ca/Mg. Avoid detaching cells.
3. Add 5mL 0.25% Trypsin to coat plate and incubate at 37C until well dissociated.

    a. **Full dissociation is important**—cells that remain attached to the plate or stuck in doublets can increase the stochastic noise of the experiment and confound growth rate measurements. If you find cell viability suffers due to heavy trypsinization, Accutase can also be used to dissociate cells and can be incubated with cells longer with minimal toxicity.
4. Add 10mL to each plate to quench trypsinization, and pool all cell suspensions (e.g. 20 plates*15mL = 300mL total) from one replicate together in a single centrifuge bottle.
5. Measure cell concentration, viability, and %BFP+. BFP% will not accurately represent infection rate until day 2-3.
6. Take the minimum number of cells required for screening, add media to bring volume to 25mL x total number of plates, and seed plates.
    a. You can also take aliquots of the cells to seed one well of a 6-well plate so you can monitor infection rate on non-split days without disturbing the screen plates

<u>Day 2 Start puromycin selection</u>
Monitor cell confluence, and add puromycin to each plate at a final concentration of 0.75ug/mL. If you seeded a 6-well plate, split a well to estimate infection rate.

<u>Day 3</u>
Split cells (as in Day 1) and add puro to 0.75ug/mL. You may want to increase the number of plates you seed on this day to have enough cells for Day 5 (see below). Again seed one well of a 6-well plate, including puro.

<u>Day 4</u>
Split 6-well to monitor infection. Assuming selection is strong enough, exchange media on all plates with fresh puro-free media.

<u>Day 5 “T0”</u>
To start the screen, you will need to harvest cells, with the number of infected cells equal to 1000x the size of the library, for **each** of the following groups:

- The untreated/DMSO-treated control culture
- Each drug-treated condition
- Freezer stocks for sequencing sample prep. At least two stocks are recommended to insure against any issues that may arise in sample prep.

For a 5 sgRNA/gene genome-scale screen with one drug condition, this requires $120x10^6$ cells infected at 85% (for $100x10^6$ infected cells) each for two cultures and two freezer stocks, or almost $0.5x10^7$ cells total.

Split cells as in Day 1, seed plates for untreated and treated conditions, and add drug to the treated cells.

For freezer stocks, centrifuge enough cells for at least 2 1000x stocks, aspirate media, and resuspend in freezing media (FBS with 10% DMSO). Aliquot cells

into cryotubes (either multiple 1mL tubes or one 5mL tube per stock) and slow-freeze at -80C.

Days 6+

Every other day, measure cell concentration, viability (just for your reference, not used for growth calculations), and BFP% for each culture, and split to maintain the minimum seeding density. Remember to pool all cell suspensions from each plate within individual replicates/conditions together. Use these daily measurements to populate the accompanying spreadsheet “screening measurements.xlsx”. You can freeze the remaining cells from splits as midpoint samples if you want to measure cell responses at multiple points of the screen or to protect against contaminations arising late in the screen.

With drug-treated cells, the day-to-day culturing depends on your dosing protocol and cell health—recently treated cells may not require any splitting, while cell populations that have recovered from treatment will return to the untreated growth rate and can be then treated again or harvested depending on the selection pressure exerted by preceding treatments (see below).

Ending the screen

There is no one optimal time for ending a screen. As the screen progresses, there is a trade-off between increasing the selective pressure on the population (thus improving signal-to-noise) and saturating the dynamic range. Each day of course also increases reagent cost. Our general rule of thumb is to measure cell doublings as above, and aim for at least 6 doubling differences between treated and untreated cells, or 10 cell doublings if only culturing an untreated sample (i.e. for essential gene screens). Treated cells should be allowed to recover to almost untreated growth rate before harvesting.

Freeze at least 2 1000x library cell stocks as above, expanding the culture prior to the endpoint day if necessary to have enough cells.