TRC Assay Development for HTS Guidelines

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One goal of the RNAi Platform at Broad is to enable high-throughput screening collaborations with other members of the TRC, the Broad community, Broad associates, and the wider biological research community. Successful, high quality HT screens require a period of assay development to test and optimize the screening conditions. This document provides a guideline to assay development experiments specific to arrayed RNAi screening with the TRC lentivirus library.

Assay Development Experiments

Stage I: Proof-of-concept

- Lentivirus transduction: Infect cells with GFP-expressing lentivirus, for example 0 µL, 1 µL, 2 µL, 5 µL, 10 µL, 20 µL PGK-GFP in 24-well plates. Starting two days post-infection, estimate infection efficiency by visual inspection (or by FACS). Increasing amounts of virus should result in increasing numbers of GFP-positive cells, and at high MOI, in increasing GFP signal per cell. Test whether virus is tolerated by the cells overnight or must be removed immediately following spin infection.

- Polybrene toxicity: Expose cells to media containing 8 µg/mL polybrene to determine polybrene toxicity. At a minimum, assess cell growth and cell morphology for several days following 24 hour polybrene exposure (compared to no polybrene exposure); ideally, compare actual screening assay results +/- polybrene exposure. If polybrene is toxic, test shorter exposure times (minimum ~2 hours) or substitute protamine sulfate.

- RNA interference: Co-infect cells with GFP-expressing lentivirus and with shGFP-expressing knockdown lentivirus. Use a control shRNA (non-GFP targeting) lentivirus as a control in parallel. Starting two days post-infection, compare the level of GFP-expressing in cells infected with shGFP virus compared to the control (non-GFP targeting shRNA). If available, a stable GFP-expressing derivative cell line may be used – compare GFP levels following infection with shGFP virus vs. infection with a control shRNA virus. Cells infected with shGFP virus should have lower GFP levels than the control shRNA.

- Test phenotypic assay(s) under high throughput conditions: scale down assays to small numbers of cells and small assay volumes to mimic 96 well or 384 well conditions (divide a large sample into many small samples or test directly in plates). The assay should be reproducible across large numbers of replicates. Ideally, include a positive control (drug, siRNA, shRNA, etc) to demonstrate the phenotype of interest.
  The RNAi Platform is familiar with several types of high-throughput phenotypic assays, check with Screening Project Manager for details.

Stage II: Validation and Optimization of Screening Conditions

- Test assay in 96 well and/or 384 well plates.

The following tests may require a few iterations to optimize conditions. Different assay timelines will have different optimal seeding densities, and different seeding densities may have different optimal puromycin selection.

- Cell seeding density: seed at several densities (e.g. 96 well plates: 1,000 – 10,000 cells per well; 384 well plates: 100-1,000 cells per well), infect with test virus (e.g. shGFP), select +/- puromycin, inspect over several days, assay if needed. Some assays are optimized for the highest density wells to be nearing confluency at the time of the assay, while others require lower confluency (e.g. high content imaging).

- Puromycin selection: seed cells, infect with titration series of test virus (e.g. shGFP) covering low to high MOI conditions and mock-infected control, select with range of puromycin (0, 1, 2, 3, 4, 5 μg/mL), assay. Determine optimal puromycin concentration for selection such that mock-infected samples are dead while samples infected at high MOI have good survival and growth. Low MOI samples should have a mix of live and dead cells under visual inspection, and should have a cell count or cell viability in between high MOI and mock-infected wells.

- Effect of viral titer on assay: seed cells, infect with a titration series of virus from a library test plate, assay. Include duplicate infection plates for each virus volume, treat one plate with puro selection and one without to have paired +/- puro wells for analysis. Analyze data to assess whether increasing amounts of virus affect the screening results. For example, does increased virus lead to decreased cell counts/cell viability?

- Assay timeline: seed cells, infect with test virus (e.g. shGFP), select +/- puromycin, assay at different timepoints.

When screening protocol is optimized

- Assay reproducibility: seed cells, infect at least 2 plates with one volume of 1-5 viral stocks (e.g. 1 uL of shGFP in 50 wells per plate), grow under screening conditions (e.g. +/- puromycin), assay. Include uninfected and/or mock-infected cells in parallel. Analyze data for distribution and variation – test various forms of the data for normal distribution (e.g. raw data, background-subtracted, log transformed); compute standard deviation,

Stage III: Prescreen

- Prescreen using a sampling of screening set virus plates with optimized HTS protocol. Test ~4 virus infection volumes for 2-4 library (96 well) plates in the screening set, which are selected to represent the range of titer in the screening set. The idea of the prescreen is to run a small number of plates in the exact HTS conditions before beginning the large scale screen; this also determines the amount of virus to use in the final screen.

Stage IV: Screen!