Decode Pooled Lentiviral shRNA Screening Laboratory Protocols & Calculation Tracking

These laboratory protocols and calculation tracking sheets are provided as a benchtop guide for using this product. We strongly recommend that you thoroughly read through the technical manual before using this guide.

Section V. Assay Development and Optimization: Transduction Parameters

A. Optimization of Lentiviral Transduction

Transduction conditions should be determined for your cell line and screening conditions and can be noted here:

Transduction medium: _____ % FBS (0.5-2% recommended)

Transduction duration: _____ hours (4-24 hours recommended)

Transduction medium additives: _____ µg/mL Polybrene (0-10 µg/mL recommended)

Cell density at transduction: _____ cells/mm^2

B. Determination of Functional Titer

Decode lentiviral shRNA pool titer as provided in Certificate of Analysis (C of A): _____ TU/mL

GIPZ non-silencing control titer as provided in Certificate of Analysis (C of A): _____ TU/mL

1. The day before transduction seed a 96-well cell culture plate (Destination Plate) with your cells at _____ cells/mm^2.
2. Make dilution medium using _____ % FBS and _____ µg/mL Polybrene.
   Make dilutions of GIPZ non-silencing control lentiviral particles in a round-bottom 96-well plate (Dilution Plate).
   Use one row of the plate for each replicate of the dilution series of the lentiviral stock.
   a. Add 40 µL of dilution medium to wells A1 and B1. Add 80 µL of dilution medium to each well A2-A8 and B2-B8.
   b. Thaw GIPZ non-silencing control lentiviral particles on ice and then add 10 µL each to wells A1 and B1.
      Mix contents of each well by pipetting 10-15 times. Discard pipette tip.
   c. Transfer 20 µL from wells A1 and B1 to the corresponding wells in column 2.
      Mix contents of each well by pipetting 10-15 times. Discard pipette tip.
   d. Repeat transfer of 20 µL for columns 2 through 8, mixing 10-15 times for each dilution.
   e Allow lentiviral particle-Polybrene complexes to form for 3-5 minutes at room temperature.
4. Remove culture medium from the cells in the 96-well plate.
5. Transfer 25 µL of each dilution of virus from the 96-well dilution plate to the corresponding wells in the Destination Plate.
   Be careful to not create bubbles.
6. Incubate the cells for _____ hours.
7. Add 75 µL of normal growth media to cells.
8. Culture cells for 48-72 hours.
9. Choose one well in the transduction plate for counting TurboGFP-expressing colonies of cells.
   Count each multi-cell colony as one transduction event.
   Calculate the average number of TurboGFP-positive colonies from the same destination well of each replicate.

Functional titer of non-silencing control in your cell line:

TurboGFP-positive colonies **X** Dilution factor **X** Volume of lentiviral particles **=** Functional titer

_____ TurboGFP-positive colonies **X** _____ Dilution factor **÷** 0.025 mL **=** _____ TU/mL functional titer

Relative transduction efficiency of your cell line:

Functional titer of non-silencing control in your cell line	÷	Titer of non-silencing control lentiviral particles stock, as calculated by Thermo Scientific in HEK293T	=	Relative transduction efficiency of your cell line

_____ TU/mL functional **÷** _____ TU/mL **=** _____ relative transduction efficiency

Functional titer in your cell line (calculate for every Decode pool):

Relative transduction efficiency of your cell line	X	Titer of the lentiviral pool, as calculated by Thermo Scientific in HEK293T cells	=	Anticipated functional titer in your cell line

_____ Relative transduction efficiency **X** _____ TU/mL **=** _____ TU/mL anticipated functional titer

C. Optimization of Puromycin Selection

1. On day 0, plate cells at a density appropriate for your cell type. Incubate overnight.
2. On day 1 change to fresh medium supplemented with puromycin at a range of concentrations (0-15 µg/mL).
   Incubate for 3-6 days.
3. Approximately every 2-3 days replace with freshly prepared puromycin medium.
4. Monitor the cells daily and visually observe the percentage of surviving cells.
   Optimum effectiveness should be reached in 3-6 days under puromycin selection.
5. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 3-6 days from the start of antibiotic selection:

   Puromycin concentration: _____ µg/mL puromycin (0.1-15 µg/mL recommended)

   Days of antibiotic selection: _____ days (3-6 days recommended)

Section VI. Assay Development and Optimization: Screening Parameters

A. Assay-specific Screening Conditions

Assay-specific conditions, such as application of selective pressure and phenotypic selection, should be determined and optimized before beginning the screen. Wherever possible, optimize your assay using a positive control shRNA against a known gene target.

B. Average shRNA Fold Representation During Transduction and Number of Biological Replicates

Critical parameters to decide upon include average shRNA fold representation and the number of biological replicates. The technical manual provides details on how to determine these factors.

Average shRNA fold representation during transduction: _____ fold representation (> 500 recommended)

Number of biological replicates: _____ replicates (> 2 recommended)

C. Number of Cells Needed for Transduction

Desired number of cells with lentiviral integrants (calculate for every Decode pool):

Number of shRNA constructs in the lentiviral pool **X** shRNA fold representation **=** Desired number of cells with lentiviral integrants

_____ shRNAs **X** _____ Fold representation **=** _____ Cells with lentiviral integrants

Desired MOI: _____

Number of cells required at the time of transduction (calculate for every Decode pool):

Desired number of cells with lentiviral integrants **÷** Proportion of cells with lentiviral integrants **=** Required number of cells at the time of transduction

_____ cells with lentiviral integrants **÷** _____ proportion of cells with integrants **=** _____ Cells required at transduction

Number of plates required per sample (calculate for every Decode pool):

Cells required at transduction **÷** Cell density at transduction **÷** Size of plate (mm^2) **=** Number of plates required per sample

_____ cells required at transduction **÷** _____ cells/mm^2 **÷** _____ mm^2 per plate **=** _____ plates per sample

D. Volume of Lentiviral Particles Needed for Transduction

Transducing units of lentiviral particles (calculate for every Decode pool):

Desired MOI **X** Number of cells at the time of transduction **=** Required number of transducing units

_____ MOI **X** _____ Cells required at transduction **=** _____ TU

Volume of lentiviral particles per sample (calculate for every Decode pool):

Number of transducing units (TU) **÷** Functional titer in your cell line (TU/mL) **=** Volume of lentiviral particles per sample (mL)

_____ TU **÷** _____ TU/mL functional titer **=** _____ mL lentiviral particles

Volume of lentiviral particles per pool (calculate for every Decode pool):

Volume of lentiviral particles per sample (mL) **X** Number of biological replicates **=** Volume of lentiviral particles per pool (mL)

_____ mL lentiviral particles **X** _____ Biological replicates **=** _____ mL lentiviral particles per pool

Section VII. Primary Screen

A. Cell Transduction and Selection Screening

1. On day 0, seed cells in normal growth medium. Incubate overnight.
2. On day 1, remove the growth medium and add medium with _____ % FBS, _____ µg/mL Polybrene and the appropriate volume of lentiviral particles so that the cells are just covered.
   If a single lentiviral shRNA pool will be added to multiple plates, divide the volume of lentiviral particles evenly between plates.
3. _____ hours post-transduction, add additional normal growth medium to your cells such that the cells can be incubated for 48-72 hours.
4. At 48-72 hours post-transduction, examine the cells microscopically for the presence of TurboGFP reporter expression.
5. Begin puromycin selection to remove non-transduced cells. Monitor the cells daily.
   Every 2-3 days, replace with fresh medium containing puromycin.
6. Once a pure population of transduced cells has been obtained, begin selection screening.
   Split cells into at least two populations: one as a reference and another for application of selective pressure and phenotypic selection.
   Maintain your desired shRNA fold representation in the library at each cell passage.

B. Genomic DNA isolation

1. Collect cells for gDNA isolation by trypsinizing and counting.
   Use at least the number of cells that corresponds to the desired number of viral integrants.
   Follow manufacturer's protocol for gDNA isolation (Qiagen Blood and Cell Culture DNA Maxi Kit Cat #13362 recommended).
   Combine gDNA isolations after elution, as needed.
2. Quantify the isolated gDNA using a spectrophotometer and assess the DNA purity by spectrophotometry.

C. PCR Amplification of shRNA Hairpins from gDNA

i. Number of PCR reactions

Grams of gDNA required to maintain shRNA fold representation (calculate for every Decode pool):

Number of cells with viral integrants **X** Nanograms per genome **=** Mass of gDNA required to maintain representation of each shRNA

_____ cells with lentiviral integrants **X** 6.58 x 10^{-3} ng/genome* = _____ ng gDNA *diploid

Number of PCR reactions per sample (calculate for every Decode pool):

Mass of gDNA required to maintain representation of each shRNA **÷** ng per PCR reaction **=** Number of PCR reactions required to maintain representation of each shRNA

_____ ng gDNA **÷** 825 ng/reaction **=** _____ PCR reactions per sample

Number of PCR reactions per pool (calculate for every Decode pool):

Number of PCR reactions per sample **X** Number of samples per pool **=** Number of PCR reactions per pool

_____ PCR reactions per sample **X** _____ Samples per pool **=** _____ PCR reactions per pool

Units of Phusion HotStart II DNA Polymerase required per pool (calculate for every Decode pool):

Number of PCR reactions per pool **X** Units of polymerase per PCR reaction **=** Units of polymerase per pool

_____ PCR reactions per pool **X** 4 Units/PCR reaction **=** _____ Units Phusion HSII

ii. Multiplexing

Sequencing reads per sample

Number of shRNA constructs in lentiviral pool **X** Read coverage per shRNA **=** Output reads required per sample

_____ shRNAs **X** 1,000 reads/shRNA **=** _____ Output reads required per sample

Sample indices per sequencing lane

Expected deep sequencing read output **÷** Output reads required per sample = Sample indices per lane

_____ Reads **÷** _____ Output reads per sample **=** _____ Indexes per lane

iii. PCR from genomic DNA

1. PCR components for shRNA amplification (calculate Master Mix for every sample)

Component	Reaction Volume (µL)	Final Concentration	Master Mix (µL) _____ # of reactions per sample
5x Phusion HF Buffer	10	1x	
10 mM dNTPs	1.0	200 µM each	
Decode Forward PCR Primer (50 µM)	0.5	0.5 µM	
Decode Reverse Indexed PCR Primer (50 µM)	0.5	0.5 µM	
5 M Betaine	5	0.5 M	
gDNA (825 ng) + PCR grade H_2O	31	16.5 ng/µL	
Phusion Hot Start II DNA polymerase (2 U/µL)	2	0.08 U/µL	
Total	50 µL		

2. PCR cycling conditions

Cycles	Temperature	Time
1	98 °C	3 minutes
23	98 °C	10 seconds
	57 °C	15 seconds
	72 °C	15 seconds

3. Combine reactions amplifying the same gDNA sample into a single 1.5 mL tube.
   Confirm that a 660-base pair amplicon is achieved from each sample by running 10 µL of PCR product on a 2% agarose gel.
4. Purify PCR-amplified gDNA.
5. Evaluate purified gDNA using the quality standards recommended by your Illumina platform.

Section VIII. Illumina Platform Sequencing

Follow the manufacturer's instructions for Illumina platform sequencing.
Load Illumina flow cell with gDNA sample (we recommend 5-10 pM using standard loading volumes).
Obtain at least 22 single-end reads with the provided **Decode Read 1 Sequencing** primer.
Perform index read with **Decode Index Read Sequencing** primer.

Section IX. Hit Identification and Follow Up

Bin each index tag.
Trim sequences to 22 base pairs.
Align sequence reads with FASTA files provided with your Decode pool.
Count the number of alignments for each shRNA.
Perform differential expression analysis to determine primary hits.