TECHNICAL MANUAL

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INSPIRED CELL SOLUTIONS

Dharmacon™ Decode™ Pooled Lentiviral shRNA Screening Libraries

Contents

1 Lentiviral Particle Product Safety Level Information

This Lentiviral Particle Product Safety Level Information constitutes Product Documentation according to clause 1 of the Product Terms and Conditions.

Dharmacon™ Decode™ Pooled Lentiviral shRNA Screening Libraries are solely for internal research use (as set forth in the Product Terms and Conditions) in laboratories where the containment measures stated below and in applicable laws and regulations are met. Products may not be used for diagnostic, therapeutic or other commercial purposes and may not to be administered to humans for any purpose or to animals for therapeutic purposes. The Products are replication-incompetent, self-inactivating (SIN) and non-pathogenic (do not cause infectious human disease).

Any investigator who purchases Dharmacon Lentiviral Particle Products is responsible for consulting with their institution's health and biosafety personnel for specific guidelines on the handling of lentiviral vector particles. Furthermore, each investigator is fully responsible for obtaining the required permissions for research usage and the acceptance of replication-incompetent SIN lentiviral vectors and replication-defective lentiviral particles into their local jurisdiction and institution.

For questions concerning the design or production of the Products, please contact:
Technical Support [ts.dharmacon@horizondiscovery.com, 1 800 235 9880 (option 2)].

Biosafety Level 2 containment measures in the US

For US guidance on containment for lentiviral vectors, please refer to the Recombinant DNA Advisory Committee (RAC) guidelines for research with lentiviral vectors here.

The U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition, Dec 2009 downloadable here.

See also the NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines), downloadable here.

Biosafety Level 2 containment measures in the EU

For the EU directives, please consult the following:

1. Council Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified micro-organisms. (revised version of Directive 90/219/EEC of the European Parliament and of the Council of 23 April 1990 on the contained use of genetically modified micro-organisms, amended by Council Directive 98/81/EC of 26 October 1998); and
1. Council Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC.

Required containment measures in Germany

The containment requirements as stated in the German Genetic Safety Ordinance (Gentechnik-Sicherheitsverordnung) of Safety Level 2* or higher have been assigned to the handling of the above-mentioned lentiviral vector particles. Please note that a higher Security Level might be required if the lentiviral vector particles are used for genetic engineering operations with other products which require a higher Security Level. ***Safety Level 2: activities of low risk for human health and the environment by the state of scientific knowledge (Stand der Wissenschaft). For the German regulations, please consult the following:***

1. German Genetic Engineering Act (Gentechnikgesetz - GenTG); and
2. Genetic Engineering Safety Ordinance (Gentechnik-Sicherheitsverordnung - GenTSV).

Safety is a focus of all lentiviral vector technology. For this reason, lentiviral vector packaging systems have divided the essential functions amongst multiple plasmids to reduce the risk of generating replication-competent lentiviral particles (RCL). The split-genome packaging system is designed so that multiple recombination events between the components are required for autonomous replication. Clinical trials using a split-genome packaging system have shown that this strategy effectively eliminates the creation of RCLs (Levine *et al.* 2006). Commercially available 3rd generation lentiviral vector systems separate the lentiviral envelope, env (such as VSV-G), from the gag-pro-pol, which encodes structural and enzymatic functions. The Pooled Lentiviral shRNA Screening Libraries are produced using the Dharmacon Trans-Lentiviral Packaging System. The Trans-Lentiviral Packaging System provides an even higher level of safety over 3rd generation packaging systems by further splitting the viral pol [reverse transcriptase (RT) and integrase (IN) functions] from gag-pro. Because the RT and IN enzymes are provided in trans to gag-pro, additional recombination events are necessary to produce RCLs.

2 Laboratory Protocols and Calculation Tracking Sheets

The Laboratory Protocols provide a brief description of the Decode Pooled shRNA Screening workflow. Before using these Laboratory Protocols, we strongly recommend that new users familiarize themselves with the detailed protocols provided in this manual. An electronic version of the Laboratory Protocol Worksheet can also be downloaded from the Decode Pooled Lentiviral shRNA Screening Libraries under Resources. The downloadable version of these protocols allow users to incorporate specific input related to their screen and performs key calculations to simplify protocol planning.

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: _____ (0.3 recommended)

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 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)

Table 10. PCR cycling parameters.

Component	Reaction Volume (µL)	Final Concentration	Master Mix (µL)
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		

_____# of reactions per sample

2. PCR cycling conditions

Temperature	Time	
98 °C	3 minutes	
98 °C	10 seconds	23 Cycles
57 °C	15 seconds	
72 °C	15 seconds	
72 °C	5 minutes	

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.

3 Introduction

Decode Lentiviral RNAi screening libraries are pools of Dharmacon™ GIPZ™ short hairpin RNAs (shRNAs) that are packaged into high-titer lentiviral particles. Through RNAi-mediated silencing of hundreds or thousands of genes in parallel, a pooled lentiviral shRNA screen can be performed to identify genes that regulate cellular responses and signaling pathways, or to discover novel gene functions. In contrast to the costly automated techniques that are required to screen using individually arrayed RNAi reagents, Decode pooled shRNA screening libraries allow the researcher to transduce and screen a population of cells within a few tissue culture dishes. Table 1 lists the pre-defined Decode pooled lentiviral shRNA libraries targeting genes within the human genome that are available. These screening libraries contain an average of five shRNAs per target gene and are supplied in sufficient volume of lentiviral particles to allow high biological reproducibility in relevant cells that are refractory to transfection. In addition, Custom Decode pooled lentiviral shRNA libraries are available for order. Researchers may choose any available GIPZ clones (human or mouse) for pooling (from 50 to 10,000 clones per pool), any number of pools, and lentiviral volumes from 100 µL to 5 mL. To request a quote go to dharmacon.horizondiscovery.com or contact us at: ts.dharmacon@horizondiscovery.com. Also available from Dharmacon for use with Decode screening libraries are optimized primers and experimentally tested protocols for reliable identification of shRNA hits by high-throughput sequencing on an Illumina platform.

Table 1. Available Decode Pooled Lentiviral shRNA Screening Libraries.

Library	Cat #	Number of genes targeted	Number of pools × number of shRNA constructs per pool	Lentiviral particle volume per pool
Ubiquitin Conjugation	RHS6076	571	1 pool of 3,830 shRNA	2 tubes × 25 µL (50 µL total)
Phosphatase	RHS6077	254	1 pool of 1,561 shRNA	2 tubes × 25 µL (50 µL total)
Protein Kinase	RHS6078	709	1 pool of 4,675 shRNA	2 tubes × 25 µL (50 µL total)
Ion Channel	RHS6079	347	1 pool of 1,884 shRNA	2 tubes × 25 µL (50 µL total)
GPCR	RHS6080	382	1 pool of 2,591 shRNA	2 tubes × 25 µL (50 µL total)
Protease	RHS6081	478	1 pool of 2,559 shRNA	2 tubes × 25 µL (50 µL total)
Druggable Genome	RHS6082	7494	5 pools of 8,490 shRNA	4 tubes × 25 µL (100 µL total)
Human Genome	RHS6083	18205	10 pools of 9,570 shRNA	4 tubes × 25 µL (100 µL total)

The Decode Pooled shRNA screening protocols begin by transducing cells at a low multiplicity of infection (MOI) with a lentiviral pool containing between 50 and 10,000 unique shRNAs (see Figure 1 for a screening workflow diagram). Individual cells in the resulting transduced population will contain unique shRNAs integrated into their genomes. Following transduction, a selective pressure is applied to identify shRNAs that target genes involved in a specific biological response. As a result of the selective pressure, cells expressing shRNA are either enriched or depleted in the cellular population. To identify hits, genomic DNA (gDNA) is isolated from the initial transduced cell population

(reference cells) and from the transduced cell population that remains following the application of selective pressure and phenotypic selection (experimental cells). shRNA sequences within the isolated gDNA are amplified using Illumina-adapted Decode Forward and Reverse Indexed PCR primers that have been designed and optimized to minimize amplification bias. Following amplification, indexed PCR products can be directly loaded onto Illumina flow cells and sequenced using Decode sequencing primers. The differences in shRNA abundance between reference and experimental cell populations can then be determined.

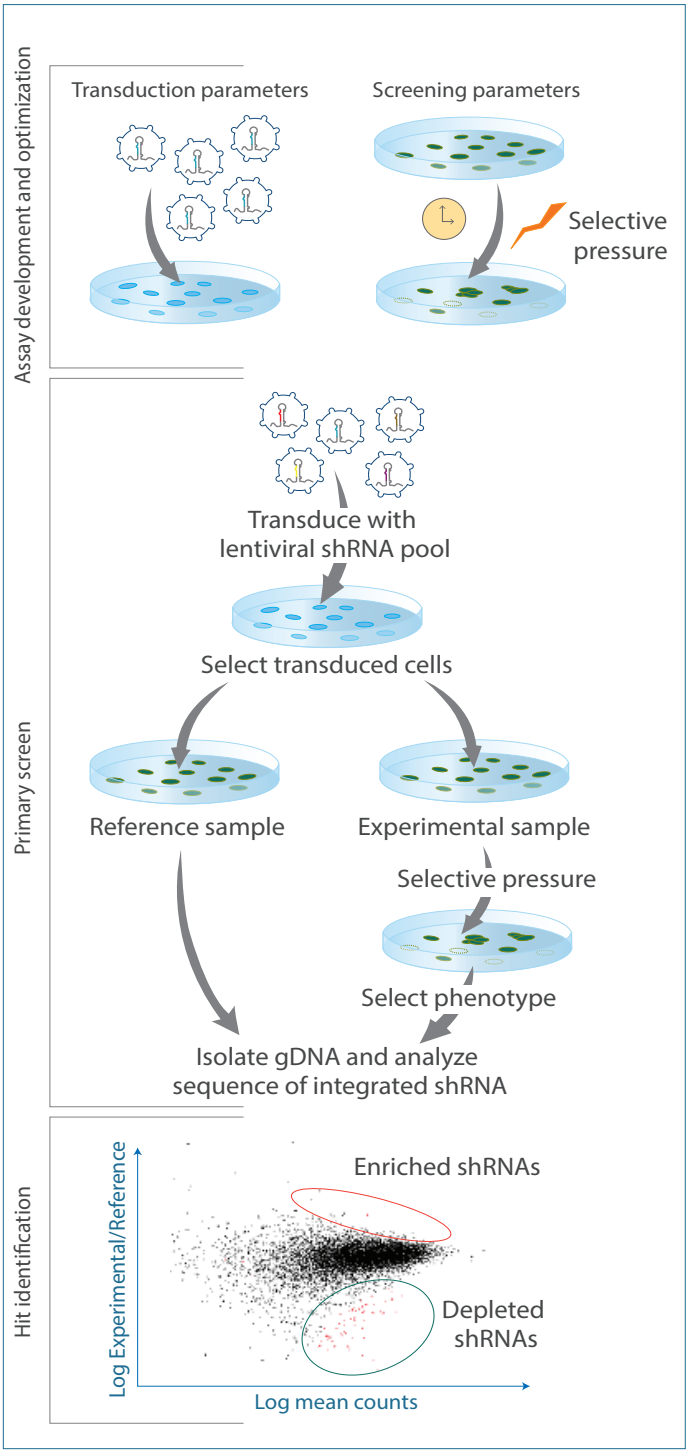

Figure 1. Pooled shRNA screening workflow.

Assay Development and Optimization: Establish optimal experimental conditions, including those for lentiviral transduction and screening parameters, such as selective pressure and time between collection of reference and experimental samples.

Primary Screen: A stable population of cells expressing single integrants of shRNAs is created by transducing Decode lentiviral pools at low MOI. Transduced cells are then split into reference and experimental populations for application of a selective pressure and phenotypic selection. gDNA is then isolated from reference and experimental populations of transduced cells. Illumina-adapted primers and Phusion™ Hot Start II High-Fidelity DNA Polymerase are used to PCR-amplify integrated shRNA sequences and add Illumina flow-cell binding sequences. The resulting amplicons are sequenced on Illumina platform sequencers, using the sequencing primers provided.

Hit Identification and Follow Up: shRNA sequences are identified in reference and experimental libraries. shRNAs that are enriched or depleted during the screen are identified as hits, and the genes that they target are identified. Hits can be confirmed and studied further using individual shRNA constructs that can be ordered from the GIPZ lentiviral shRNA collection.

Decode Pooled Lentiviral shRNA Screening Libraries are comprised of the GIPZ lentiviral shRNA, in which the gene-specific silencing sequence is embedded in a primary microRNA transcript, thus creating an RNAi trigger that silences genes with increased specificity and minimal cellular toxicity (Figures 2 and 3). The Decode Pooled Lentiviral shRNA Screening Libraries combine the advantages of the microRNA-adapted GIPZ shRNA design with the convenience of high-titer lentiviral delivery to create a powerful multiplexed RNAi screening resource capable of producing loss-of-function phenotypes in many dividing and non-dividing cells.

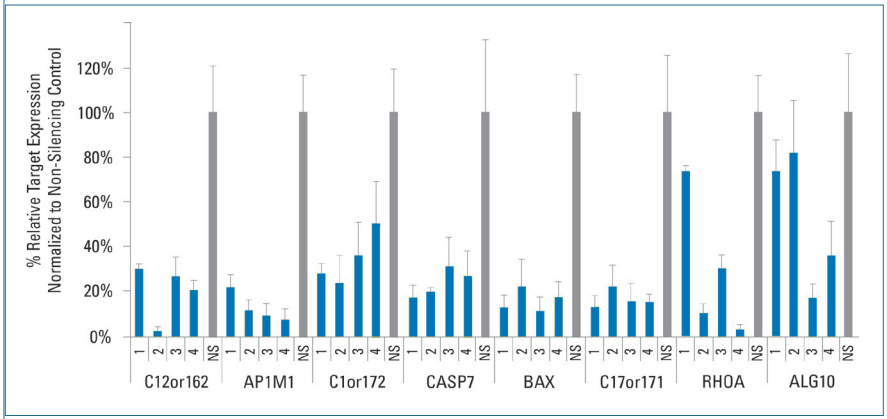

Figure 2. GIPZ shRNAs effectively knockdown genes of interest. HEK293T cells were transduced with lentiviral particles containing shRNA to the indicated genes and a non-silencing (NS) control, at an average MOI of ten. Forty-eight hours after transduction, cells were selected with puromycin for four days prior to RNA isolation. Expression of indicated genes was determined by RT-qPCR and normalized to non-silencing control.

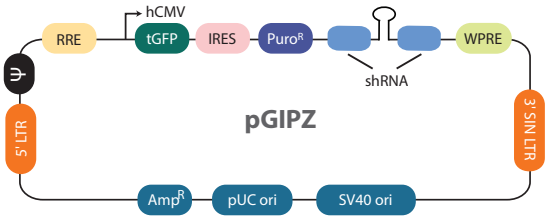

Figure 3. pGIPZ lentiviral vector.

Vector Element	Utility
hCMV	Human cytomegalovirus promoter drives strong transgene expression
tGFP	TurboGFP reporter for visual tracking of transduction and expression
Puro^R	Puromycin resistance permits antibiotic-selective pressure and propagation of stable integrants
IRES	Internal ribosomal entry site allows expression of TurboGFP and puromycin resistance genes in a single transcript
shRNA	microRNA-adapted shRNA (based on miR-30) for gene knockdown
5' LTR	5' long terminal repeat
3' SIN LTR	3' self-inactivating long terminal repeat for increased safety
Ψ	Psi packaging sequence allows lentiviral genome packaging using lentiviral packaging systems
RRE	Rev response element enhances titer by increasing packaging efficiency of full-length lentiviral genomes
WPRE	Woodchuck hepatitis posttranscriptional regulatory element enhances transgene expression in the target cells

Decode shRNA pools are created using experimentally tested methods that ensure uniform representation of shRNA constructs in every lentiviral pool. Specifically, individual shRNA clones are transferred from 96-well plate collections onto agar plates and allowed to grow overnight. Colonies are then combined into a slurry and grown for a defined period of time. High quality DNA is prepared from cultures of *E. coli* and analyzed by high-throughput sequencing to examine shRNA representation and identity. This quality control allows us to verify that the abundance of 70% of the shRNAs is less than 5-fold different from each other and the abundance of 90% of the shRNAs is less than 25-fold different from each other. During this quality control process, the relative representation and identity of every shRNA in the plasmid pool is determined and provided with the product to ensure high quality screening materials and confidence in Decode lentiviral pool starting materials.

Decode plasmid pools are packaged into lentiviral particles with a minimum titer specification of ≥ 5×10^8 TU/mL. The titer of each Decode lentiviral shRNA pool is determined by transducing HEK293T cells with production batches of lentiviral particles and quantifying the number of transduced cells expressing TurboGFP™ (Evrogen™, Moscow, Russia) 72 hours after transduction. Lentiviral particles are examined to confirm lack of mold and bacterial contamination. Concentrated lentiviral particles are supplied to enable screening in cells that are not easily transduced and inaccessible by standard lipid-mediated transfection of synthetic siRNA reagents. Enough lentiviral particle volume is provided such that each individual shRNA can be transduced into multiple cells, thus maintaining a high average shRNA fold-representation during the pooled screen. This results in improved screen reproducibility and more reliable hit calling (Strezoska *et al*. 2012).

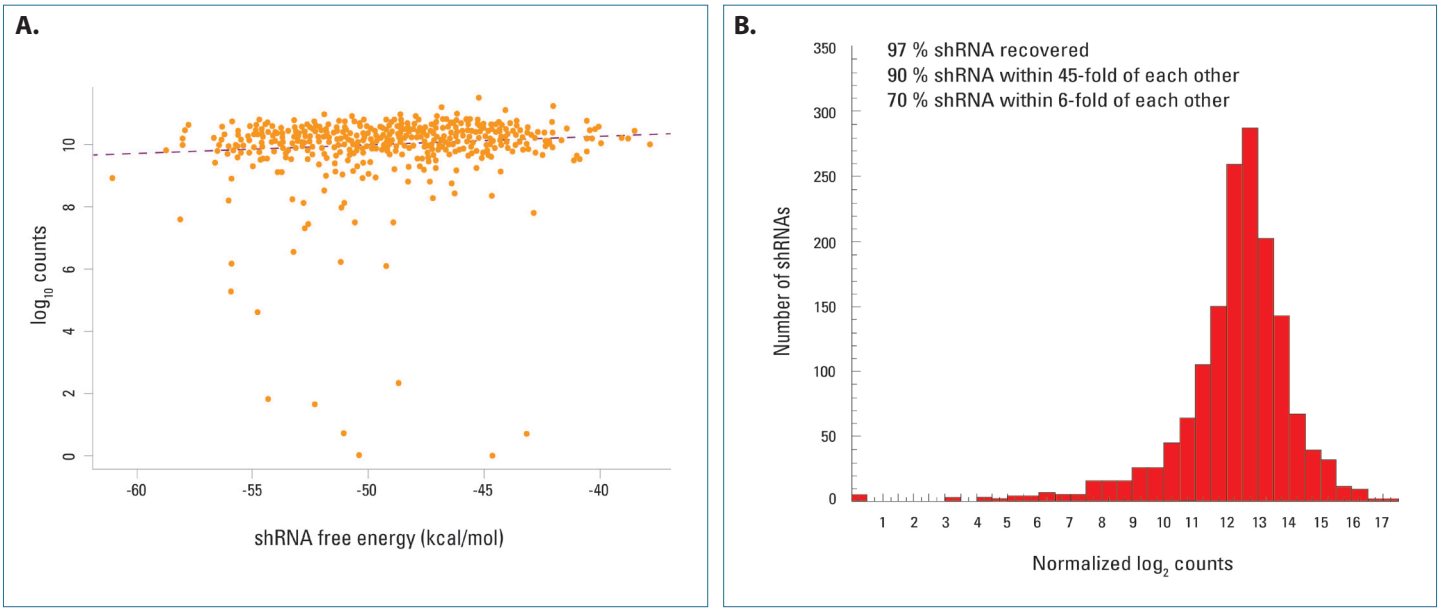

Figure 4. PCR amplification of shRNA libraries from gDNA does not introduce biased representation. **A.** Free energy of shRNA hairpins does not affect their ability to be amplified from gDNA with Decode PCR primers and sequenced on an Illumina platform. Free energy of each shRNA hairpin is plotted against $\log^{10}$ sequencing counts. **B.** Decode PCR amplification and high-throughput sequencing of shRNA pools results in a tight distribution of shRNA counts. The Decode Phosphatase library was transduced into HEK293T cells at 1000-fold representation and gDNA was isolated. Decode PCR and Sequencing primers were used to amplify and sequence shRNA. The histogram of normalized $\log_2$ counts demonstrates that shRNAs are recovered with a tight distribution. This ensures ample representation of each shRNA in the screening experiment.

4 Decode Pooled Lentiviral shRNA screening required materials

The Decode shRNA screening workflow requires:

1. Pools of concentrated lentiviral particles (Table 1).
1. 2 × 25 µL tubes of GIPZ non-silencing shRNA control lentiviral particles (Dharmacon Cat #RHS4348).
1. Decode Indexing PCR and Sequencing Primer Kit (Dharmacon Cat #PRM6178).
   Note: We recommend purchasing two Decode Indexing PCR and Sequencing Kits for use with the Decode Human Genome Library.
1. Phusion™ Hot Start II High-Fidelity DNA Polymerase and 5x Phusion™ HF Buffer (Thermo Scientific Cat #F-549S, F-549L).
   Note: Please see section 7 to determine the amount of DNA polymerase required for a screening workflow.

Additional recommended materials but not supplied include:

1. Betaine Solution 5M (Sigma-Aldrich Cat #B0300)
1. Qiagen™ QIAquick™ PCR Purification Kit (Cat #28104)
1. Thermo Scientific™ GeneRuler™ Low Range DNA Ladder, ready-to-use, 25-700 bp (Cat #SM1193)
1. Qiagen™ Blood and Cell Culture DNA Maxi Kit (Cat #13362)
1. Thermo Scientific™ dNTP™ Mix, 10 mM each (Cat #R0191)
1. HyClone™ Puromycin 2 HCl (Cat #SV30075.01)

5 Assay development and optimization: transduction parameters

A. Optimization of Lentiviral transduction

While GIPZ shRNA lentiviral particles exhibit broad cell tropism, the conditions for successful and efficient delivery can vary significantly. It is essential to determine the optimal lentiviral transduction conditions in each cell line or type of interest. Please keep in mind that the conditions you select during these optimization steps must be compatible with your primary screening protocols and conditions. GIPZ non-silencing control lentiviral particles can be used for optimization of transduction conditions. Parameters that may influence the efficiency of lentiviral transduction include, but are not limited to:

Transduction medium: When possible, the transduction of cells with lentiviral particles should be performed in a small volume of low-serum (0.5-2%) or serum-free medium. For cells sensitive to low serum conditions, transduction optimization can be performed in complete medium.

Transduction duration: Incubation time can vary between 4 and 24 hours and will depend on your cell line.

Transduction medium additives: Cationic polymers such as hexadimethrine bromide (Polybrene) may be added to enhance lentiviral particle binding to the cell surface. We recommend testing a range of concentrations, from 0-10 µg mL, for identification of optimal transduction efficiency with minimal or no cell toxicity.

Cell density at transduction: The density at which cells are seeded may also influence transduction efficiency. We recommend seeding cells at a range of densities for optimization of transduction efficiency. Plate sizes for screening should be chosen accordingly.

GIPZ shRNA constructs express the Evrogen TurboGFP reporter gene, facilitating determination of transduction efficiency using fluorescence microscopy.

Please note that conditions can vary between batches and passages of cells. We recommend banking enough cells for optimization, primary screen and follow up work.

A detailed guide to optimization of lentiviral transduction conditions can be found under Resources for shRNA reagents

B. Determination of functional titer

Pooled shRNA libraries and GIPZ non-silencing shRNA controls are supplied as concentrated lentiviral particles. Specifications can be found on the Certificate of Analysis (C of A) provided with lentiviral particles. Functional titer can be determined either by counting GFP-positive colonies using fluorescence microscopy or by FACS analysis of GFP-positive cells. The following protocol describes how to estimate functional titer of each Decode lentiviral pool by using GIPZ non-silencing control lentiviral particles and determining titer by fluorescence microscopy.

1. The day before transduction, seed a 96-well cell culture plate (Destination Plate) with your cells at the density determined during transduction optimization. Grow cells overnight.
2. Make dilution medium using serum and Polybrene conditions determined during transduction optimization. Make dilutions of GIPZ non-silencing control lentiviral particles in a round-bottom 96-well plate (Dilution Plate). As shown in Figure 5 and Table 2, use one row of the plate for each replicate of the dilution series of the lentiviral stock. We recommend performing two replicates. The procedure for dilution of the lentiviral stock is described below and results in a series of five-fold dilutions to reach a final dilution of 390,625-fold.
   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 up and down 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 up and down 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-Polybrene complexes to form for 3-5 minutes at room temperature.
3. Remove culture medium from the cells in the 96-well plate Destination Plate.
4. Transfer 25 µL of each dilution of lentiviral particles from the Dilution Plate to the corresponding wells in the Destination Plate, being careful to not create bubbles.
5. Incubate the cells for 4-24 hours (as determined during transduction optimization).
6. Add 75 µL of normal growth medium to cells.
7. Culture cells for 48-72 hours (as determined during transduction optimization).
8. Choose one well in the Destination Plate for counting TurboGFP-expressing colonies of cells. This should be a well in which individual colonies of cells can be visualized and counted. Count each multi-cell colony as one transduction event, as the cells have been dividing over the culture period (Figure 6). Calculate the average number of TurboGFP-positive colonies from the same destination well of each replicate.

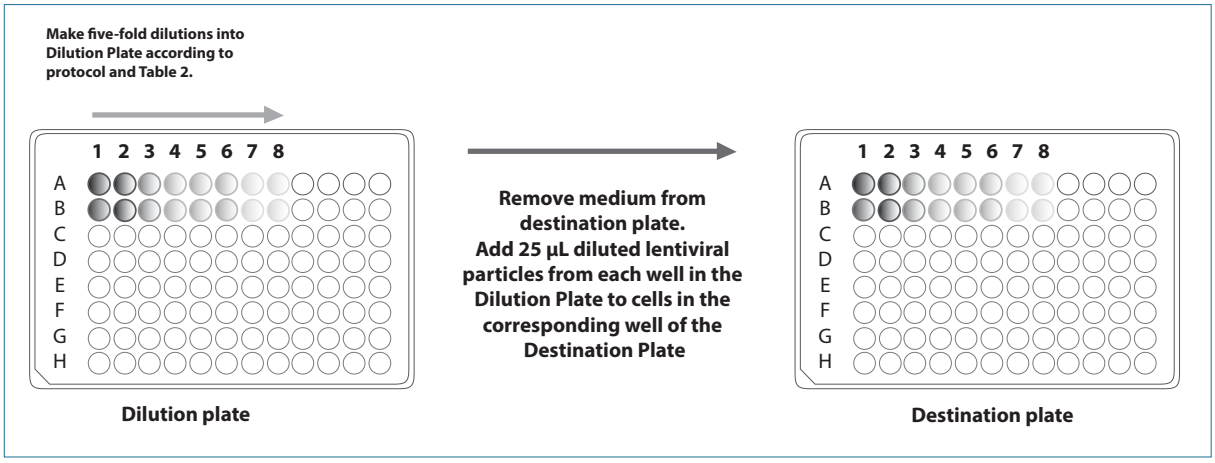

Figure 5. Diagram for dilution series of lentiviral particles (Dilution Plate) and addition to cells (Destination Plate).

Table 2. Example setup for lentiviral particle dilution series.

	Dilution Plate		Dilution factor	Volume of diluted lentiviral particles used in transduction (Destination Plate)
Well	Lentiviral particle serial dilution volume	Volume of dilution medium		
A1	10 µL (*control)	40 µL	5	25 µL
A2	20 µL (from A1)	80 µL	25	25 µL
A3	20 µL (from A2)	80 µL	125	25 µL
A4	20 µL (from A3)	80 µL	625	25 µL
A5	20 µL (from A4)	80 µL	3125	25 µL
A6	20 µL (from A5)	80 µL	15625	25 µL
A7	20 µL (from A6)	80 µL	78125	25 µL
A8	20 µL (from A7)	80 µL	390625	25 µL

*Control (GIPZ non-silencing shRNA control lentiviral particles). Repeat identical dilution series in wells B1 to B8.

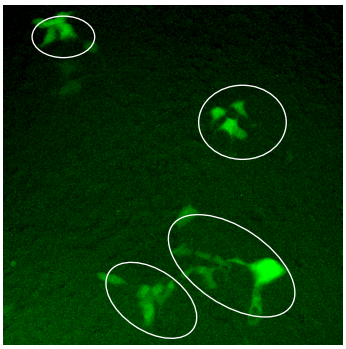

Figure 6. Example of individual colonies in HEK293T cells 72 hour post-transduction. Four colonies are circled. Imaged at 40x magnification.

Functional titer in transducing units per mL (TU/mL) can be determined using the following formula:

Number of TurboGFP-positive colonies × Dilution factor (Table 2) ÷ 0.025 mL (Volume of diluted lentiviral particles used) = Functional titer of GIPZ non-silencing control lentiviral particles stock in your cell line (TU/mL)

Relative transduction efficiency of your cell type can be determined by using the following formula:

Functional titer of non-silencing control in your cell line (TU/mL) ÷ Titer of non-silencing control lentiviral particles stock as calculated by Dharmacon in HEK293T (TU/mL) (reported on the C of A) = Relative transduction efficiency of your cell line

Use the calculated relative transduction efficiency of your cell line to calculate the anticipated functional titer for each Decode lentiviral pool using the following formula:

Relative transduction efficiency of your cell line × Titer of the lentiviral pool as calculated by in HEK293T cells (TU/mL) = Anticipated functional titer of the pool in your cell line (TU/mL)

Calculation Examples

If you counted 58 Evrogen TurboGFP-positive colonies in well A7 of the destination plate, the titer of the GIPZ non-silencing control lentiviral particles in your cell line would be calculated as follows:

58 (TurboGFP positive colonies) × 78,125 (dilution factor) ÷ 0.025 mL (volume of diluted lentiviral particles used) = 1.8×10^8 TU/mL functional titer of GIPZ non-silencing control in your cell line

If the titer for the GIPZ non-silencing control lentiviral particles on the product insert was listed as 9.0×10^8 TU/mL, the relative transduction efficiency of your cell type would be determined as follows:

1.8×10^8 TU/mL (functional titer in your cell line) ÷ 9.0×10^8 TU/mL (titer as indicated on product insert) = 0.2 relative transduction efficiency

If the relative transduction efficiency of your cell line is 0.2 and the titer of a Decode Lentiviral Pool, as indicated on the C of A, is 5.0×10^8 TU/mL, the anticipated functional titer of the pool in your cell line would be determined as follows:

0.2 (relative transduction efficiency) × 5.0×10^8 TU/mL (titer as indicated on product insert) = 1.0×10^8 TU/mL anticipated functional titer in your cell line

C. Optimization of puromycin selection

The GIPZ shRNA vector contains the puromycin selectable marker and TurboGFP reporter, which allow for selection of cells that have integrated the GIPZ shRNA construct. Selection can be performed using an appropriate concentration of puromycin or by fluorescence-activated cell sorting (FACS) to isolate cells expressing TurboGFP. If using puromycin selection to generate a purely transduced population of cells, it is important to determine the optimal concentration of puromycin required to kill non-transduced cells. This concentration can be identified by generating a puromycin kill curve.

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. We recommend using HyClone™ Puromycin 2 HCl (Cat #SV30075.01).
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.

6 Assay development and optimization: screening parameters

A. Assay-specific screening conditions

The pooled shRNA screening workflow described here facilitates identification of genetic regulators of a range of biological processes. Hundreds or thousands of different shRNAs are introduced into a population of cells such that each cell expresses a single shRNA. These cells are subsequently subjected to selective pressure and phenotypic selection. Cells expressing shRNAs targeting genes involved in the phenotype of interest are enriched or depleted relative to the reference population of transduced cells. These shRNAs are identified by comparing the relative shRNA abundance between the experimental and reference population of cells. Screening approaches can be designed to identify genes involved in a phenotype of interest. shRNA-mediated knockdown screens can be used to identify genes that affect cell proliferation and/or survival, cause changes in cellular behavior (such as migration or adhesion), modulate response to different treatments (such as drugs or radiation) or change reporter or surface marker expression. Variables to consider when planning screening conditions include, but are not limited to, assay duration, conditions of selective pressure (such as concentration or duration), and method of phenotypic selection (such as viability, surface marker expression or migration). We recommend that you optimize all assay conditions prior to beginning a pooled shRNA screen, if possible, using an shRNA for a target known to be involved in the phenotype(s) of interest.

B. Average shRNA fold representation during transduction and number of biological replicates

A critical and necessary consideration of pooled lentiviral shRNA screening is the extent to which any given shRNA construct in a pooled library will be represented in the screen; in other words, the number of cells that contain an independent genomic integration of any given shRNA or the number of biological replicates of each shRNA integration event and subsequent phenotypic selection. High shRNA representation results in high reproducibility between biological replicates and ensures that there is a sufficient window for detection of changes in shRNAs representation after phenotypic selection (Strezoska et al. 2012). A high shRNA fold representation is desirable, if technically feasible, for your assay. We recommend between 500 and 1,000 independent integrations per shRNA, particularly if you are interested in observing shRNA depletion hits. Increasing the number of biological replicates in a screen can also improve the ability to identify hits; however, we have observed that shRNA fold representation has a greater impact on the ability to identify hits than the number of biological replicates. Therefore, we recommend at least two biological replicates, while maintaining an average shRNA fold representation as high as is practical for your screen.

C. Number of cells needed for transduction

A critical and necessary consideration of pooled lentiviral shRNA screening is the extent to which any given shRNA construct in a pooled library will be represented in the screen; in other words, the number of cells that contain an independent genomic integration of any given shRNA or the number of biological replicates of each shRNA integration event and subsequent phenotypic selection. High shRNA representation results in high reproducibility between biological replicates and ensures that there is a sufficient window for detection of changes in shRNAs representation after phenotypic selection (Strezoska *et al*. 2012). A high shRNA fold representation is desirable, if technically feasible, for your assay. We recommend between 500 and 1,000 independent integrations per shRNA, particularly if you are interested in observing shRNA depletion hits. Increasing the number of biological replicates in a screen can also improve the ability to identify hits; however, we have observed that shRNA fold representation has a greater impact on the ability to identify hits than the number of biological replicates. Therefore, we recommend at least two biological replicates, while maintaining an average shRNA fold representation as high as is practical for your screen.

	Number of lentiviral integrants per cell				
MOI	0	1	2	3	4
0.1	0.90	0.09	0.00	0.00	0.00
0.2	0.82	0.16	0.02	0.00	0.00
0.3	**0.74**	**0.22**	**0.03**	**0.00**	**0.00**
0.4	0.67	0.27	0.05	0.01	0.00
0.5	0.61	0.30	0.08	0.01	0.00
0.6	0.55	0.33	0.10	0.02	0.00
0.7	0.50	0.35	0.12	0.03	0.00
0.8	0.45	0.36	0.14	0.04	0.01
0.9	0.41	0.37	0.16	0.05	0.01
1.0	0.37	0.37	0.18	0.06	0.02

Table 3. Poisson distribution showing the distribution of cells with the indicated number of lentiviral integrants at various MOIs. At each MOI, the portion of cells having 0, 1, 2, 3, or 4 lentiviral integrants is indicated. For example, at an MOI of 0.3, only 3% of cells are predicted to contain more than 1 lentiviral integrant.

To calculate the number of lentiviral integration events required to achieve a given shRNA representation, use the following formula:
Number of shRNA constructs in the lentiviral pool × shRNA fold representation = Desired number of cells with lentiviral integrants

Table 4 provides examples of the number of cells with lentiviral integrants required for various shRNA fold representations with a pool.

shRNA fold representation	Number of lentiviral integrants
100	1×10^5
500	5×10^5
1,000	1×10^6

Table 4. Number of lentiviral integrants required for indicated average shRNA fold representation at transduction with libraries of 1,000 shRNAs.

To calculate the number of cells you will need per sample at the time of transduction, first determine the proportion of cells with lentiviral integrants (Table 4), then use the following formula:
Desired number of cells with lentiviral integrants ÷ Proportion of cells with lentiviral integrants = Required number of cells at the time of transduction

Calculation Examples

If you have a pool of 1,000 shRNAs and you wish to transduce with 500-fold average shRNA representation, the desired number of lentiviral integration events would be calculated as follows:

500 (average shRNA representation) × 1,000 (number of shRNAs) = 5.0×10^5 lentiviral integration events

At an MOI of 0.3, approximately 25% of seeded cells will receive at least one lentiviral shRNA integrant. Therefore, calculate the number of cells needed at the time of transduction as follows:

5.0×10^5 (lentiviral integration events) ÷ 0.25 (proportion of cells with lentiviral integrants) = 2.0×10^6 cells at the time of transduction

Plate sizes should be chosen such that the required number of cells can reach the optimum density for transduction, as determined above, at the time of transduction. Prepare additional plates of cells for each pool and biological replicate that you wish to transduce. The calculations below outline how to determine cell plating requirements.

shRNA fold representation	Number of cells required at transduction	Recommended cell plating
100	4.0×10^5	1 × 100 mm
500	2.0×10^6	1 × 100 mm
1,000	4.0×10^6	2 × 100 mm

Table 5. Number of cells required at the time of transduction and cell plating recommendations for indicated average shRNA fold representation with shRNA libraries of 1,000 shRNAs. This assumes optimal transduction at 250 cells/mm^2 and an effective surface area on 100 mm plates of 7800 mm^2.

To determine the number of plates required for each biological replicate, use the following formula:

Required number of cells at the time of transduction ÷ Cell density at transduction ÷ Size of plate (mm^2) = Number of plates required per sample

To determine the total number of plates to seed per shRNA pool, use the following formula:

Number of plates required per sample × Number of biological replicates = Number of plates required per pool

Table 5 gives examples of cell and plate numbers and plate sizes for various shRNA fold representations with a pool of 1,000 shRNAs and a cell line with optimal transduction at 250 cells/mm^2.

D. Volume of Lentiviral particles needed for transduction

Before proceeding with the Decode pooled shRNA screen, confirm that you have enough lentiviral particles for your experimental design. Follow the steps below to calculate the volume of lentiviral particles required for each shRNA pool. Lentiviral particles come in tubes containing 25 µL.

First, identify the number of transducing units of lentiviral particles required based on the following formula:

MOI × Number of cells = Number of transducing units of lentiviral particles (TU)

Next, determine the volume of lentiviral particles required for the desired number of transducing units based on the following formula:

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

To determine the total volume of lentiviral particles required for each pool, use the following formula:

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

Calculation Examples

From the example above, at an MOI of 0.3 and a 500-fold average shRNA representation at transduction, you will have prepared 2.0×10^6 cells at the time of transduction. Based on the above formula calculate the number of lentiviral particles needed:

0.3 (MOI) × 2.0×10^6 cells = 6.0×10^5 TU of lentiviral particles are required for transduction

If you have determined that you need 6.0×10^5 TU and you have a functional titer of 1.0×10^8 TU/mL in your cell line, you can calculate the volume of lentiviral particles needed as follows:

6.0×10^5 TU ÷ 1.0×10^8 TU/mL = 0.006 mL of lentiviral particles

If you are performing two biological replicates you can determine the total volume of lentiviral particles required per pool as follows:

0.006 mL of lentiviral particles × 2 biological replicates = 0.012 mL of lentiviral particles

Before proceeding with a Decode Pooled shRNA screen, carefully consider whether you have obtained a sufficient amount of lentiviral particles to perform a screen at the desired fold coverage, given the relative transduction efficiency of your cell line and the number of biological replicates you wish to perform. Table 6 outlines the volume of lentiviral particles needed for various shRNA fold representations and relative transduction efficiencies. Additional lentiviral particles can be ordered separately.

	Relative Transduction Efficiency			
shRNA fold representation	1	0.5	0.2	0.1
100	0.2 µL	0.5 µL	1.2 µL	2.4 µL
500	1.2 µL	2.4 µL	6.0 µL	12 µL
1,000	2.4 µL	4.8 µL	12 µL	24 µL

Table 6. Volume of lentiviral particles at 5.0×10^8 TU/mL in HEK293T cells required for the indicated fold representation and relative transduction efficiencies. The volume of lentiviral particles is indicated for one pool of 1,000 shRNAs with one biological replicate at an MOI of 0.3.

7 Primary Screen

The following sections describe the primary screening workflow (Figure 7).

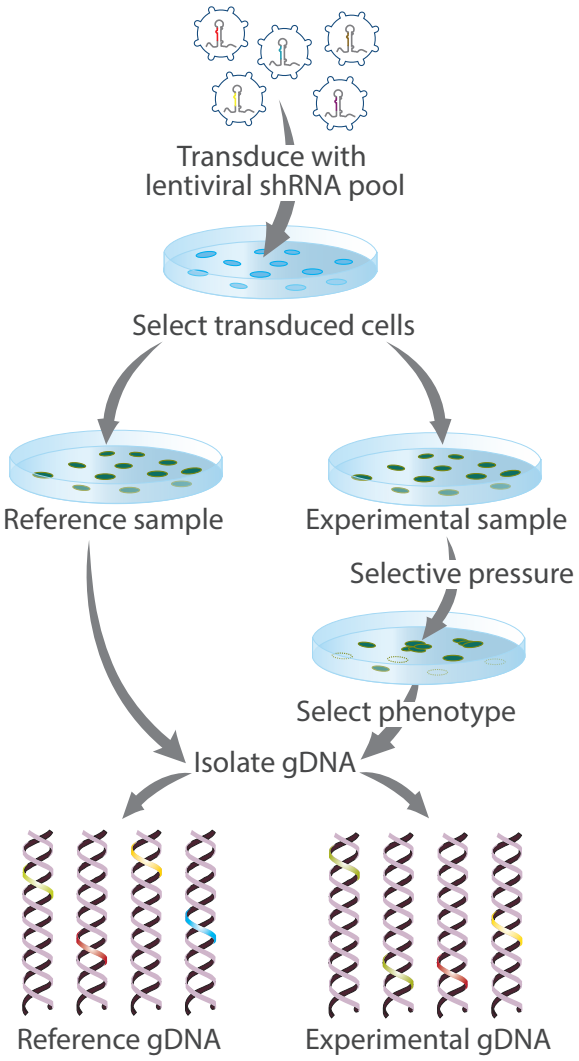

Figure 7. Primary screen workflow.

A. Cell Transduction and Selection Screening

The experimental conditions described here, and in Figure 8, serve as a guide for performing lentiviral transductions. However, the precise cell number and volume of lentiviral particles necessary to achieve the desired MOI and average shRNA fold representation in the library should be determined specifically for each cell line of interest and each intended screening experiment, as outlined above. Similarly, conditions should be clearly defined prior to starting the screen for application of selective pressure and selection of phenotype of interest. For libraries comprised of multiple pools, we recommend that cells be transduced with individual shRNA pools in separate plates.

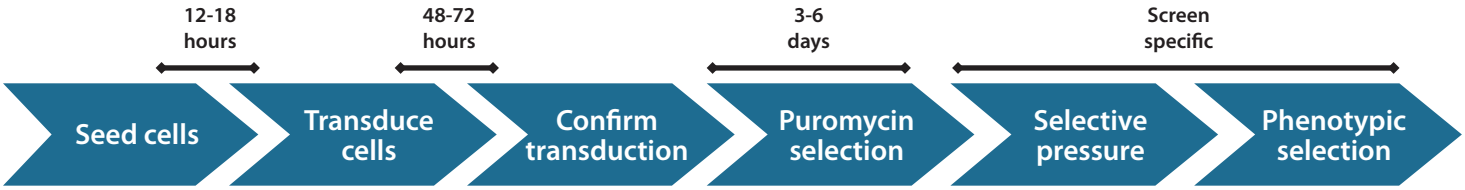

Figure 8. Timeline of primary screen.

1. On day 0, seed cells in normal growth medium in the number of plates determined in section 6. The number of cells seeded should be determined by extrapolating from the number of cells needed at the time of transduction and the doubling time of your cell type. Incubate overnight.
2. The next day (day 1), remove the medium and add optimized transduction medium (section 5) with the appropriate amount of lentiviral particles (section 6) so that the cells are just covered. If a single lentiviral pool will be added to multiple plates, as determined in section 6, divide volume of lentiviral particles evenly between plates.
3. After the appropriate transduction time (section 5), 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; this will be the first indication as to the efficiency of transduction.
   Note: When using a MOI = 0.3 you should expect approximately 25% of the cells to express TurboGFP.
5. Begin puromycin selection to remove non-transduced cells. Use the appropriate concentration of puromycin, as determined in section 5. Monitor the cells daily and observe the percentage of TurboGFP-positive cells. Every 48-72 hours, replace with fresh medium containing puromycin and passage cells as needed. Selection should be performed for the number of days determined in section 5.
6. Once a pure population of transduced cells has been obtained (3-6 days), begin selection screening. Split cells into at least two populations: one as a reference and another for application of selective pressure and phenotypic selection. To maintain your desired shRNA fold representation in the library at each cell passage, always retain at least the number of cells that corresponds to the desired number of lentiviral integrants.

B. Genomic DNA Isolation

Following selection, gDNA should be isolated from control and experimental cell populations.
Note: Isolation of gDNA from cells transduced with Decode pools has been optimized in the protocol below using Qiagen Blood and Cell Culture DNA Maxi Kit (Cat #13362); however, kits from other manufacturers may also be suitable.

7. Collect cells for gDNA isolation by trypsinizing and counting. To maintain your desired fold representation during gDNA isolation, use at least the number of cells that corresponds to the desired number of lentiviral integrants. The most accurate results can be obtained by counting cell number prior to gDNA isolation. Follow the manufacturer's protocol for purification of gDNA from cell cultures.

Note: It is important that you elute gDNA samples in EDTA-free buffer to prevent inhibition of subsequent PCR reactions. To ensure good DNA quality and yield, do not use more than the manufacturer's recommended cell number and be sure that your gDNA is fully solubilized. If multiple purification columns are required to maintain representation of your sample, combine gDNA isolations after elution.

8. Quantify the isolated gDNA using a spectrophotometer and assess the DNA purity by measuring the ratio of the absorbance at
260 and 280 nm ($A_{260/280}$) and at A230 ($A_{260/230}$). High-quality gDNA samples should have an A_{260}/A_{280} ratio of 1.8 to 2.0, indicating the absence of contaminating proteins, and an A_{260}/A_{230} ratio of > 2.0, indicating the absence of other organic contaminants.

C. PCR Amplification of shRNA from Genomic DNA

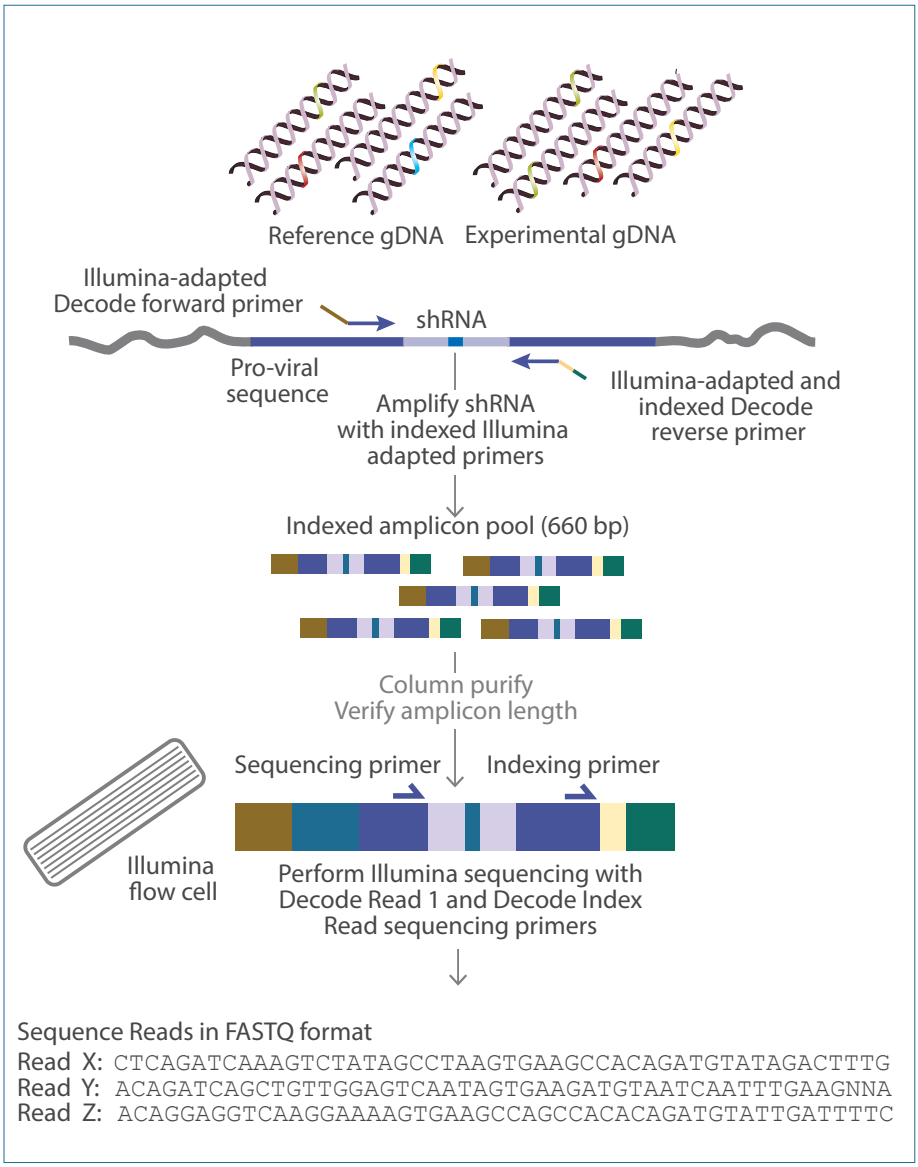

Figure 9. PCR amplification and Illumina high-throughput sequencing workflow.

The PCR amplification and high-throughput sequencing workflow described here (Figure 9) has been designed to amplify shRNAs from gDNA without bias, such that differences in shRNA representation after sequencing are due to enrichment or depletion that occurs during the primary screen. It is important to use adequate template copies per shRNA in the PCR amplification step such that the average shRNA fold representation at transduction is maintained. This ensures assay reproducibility and facilitates hit identification. Details about optimization of PCR conditions and amplifying pools of shRNA without bias can be found in Strezoska *et al*. (2012).

Note: The Decode PCR primers provided have been designed for use with Illumina sequencing platforms. They are not compatible with other high-throughput sequencing systems.

i. Number of PCR Reactions

Calculate the amount of gDNA required to maintain shRNA fold representation, assuming a single lentiviral integration event per cell (genome), using the following formula:

Desired number of cells with lentiviral integrants × 6.58 × 10^{-3} ng/genome (mass of a single human genome) = mass of gDNA required to maintain representation of each shRNA

Note: The calculation above assumes a diploid human genome. Some cell lines are not diploid and calculations must be adjusted accordingly.

We have optimized the PCR conditions to remain in the linear phase of log amplification. We recommend a maximum of 825 ng of gDNA per PCR reaction (using more gDNA per PCR reaction will inhibit the efficiency of the reaction and will result in either failure of the PCR reaction or biased shRNA amplification). Calculate the number of PCR reactions required for each sample using the following formula:

Mass of gDNA required to maintain representation of each shRNA (in ng) ÷ 825 ng per reaction = Number of PCR reactions required to maintain representation of each shRNA (round up to the nearest whole number)

Table 7 gives an example of the input gDNA and number of PCR reactions required for various shRNA fold representations with a pool of 1,000 shRNAs.

shRNA fold representation	Input gDNA (µg)	Number of PCR reactions
100	0.7	1
500	3.3	4
1,000	6.6	8

Table 7. Input gDNA for PCR amplification and number of PCR reactions at indicated fold representation for a pool of 1,000 shRNAs.

The calculations above are used to determine the number of PCR reactions required per sample. To determine the total number of PCR reactions required for your screen use the following formula:

Number of PCR reactions per sample × Number of samples per pool = Number of PCR reactions per pool

The PCR reaction conditions outlined below have been optimized using Phusion™ Hot Start II DNA Polymerase (Cat #F-549S/L). To determine how much Phusion™ polymerase is required for each Decode pool use the following formula:

Number of PCR reactions per pool × 4 Units Phusion™ per PCR reaction = Units of Phusion™ required per pool

Calculation Examples

If you anticipate 5×10^5 cells with lentiviral integrants, the mass of gDNA required would be calculated as follows:

5×10^5 lentiviral integrants $\times$ 6.58×10^{-3} ng/genome = 3.3×10^3 ng

The maximum amount of gDNA per PCR reaction is 825 ng, therefore the number of PCR reactions necessary can be calculated as follows:

3.3×10^3 ng gDNA ÷ 825 ng/PCR reaction = 4 PCR reactions

If you have four samples (reference and experimental samples in biological duplicates), then you would need the following total number of PCR reactions:

4 PCR reactions × 4 samples = 16 PCR reactions

To perform 16 PCR reactions you need the following amount of Phusion™ Hot Start II DNA Polymerase:

16 PCR reactions × 4 Units Phusion™ /PCR reaction = 64 Units of Phusion™ Hot Start II required per pool

ii. Multiplexing of High-throughput Sequencing Samples

Most high-throughput sequencing platforms provide a high enough number of sequence reads to allow multiplexing of samples in sequencing runs (running multiple samples in one lane). To ensure accurate hit identification, we recommend obtaining a minimum of 1,000 reads per shRNA. If the number of reads required per sample is less than the expected output of your sequencing platform, it may be possible to multiplex samples. Sequencing of the PCR amplicon library is a non-standard application and yields approximately 50% of the maximum anticipated reads per lane for standard Illumina libraries. Therefore, sequencing read output can be estimated from the manufacturer's specifications by dividing by two. From this information the number of samples you can run per lane can be determined using the following calculations:

Number of shRNA constructs in your lentiviral pool × 1,000 reads per shRNA = Output reads required per sample

Expected number of reads per sequencing lane (manufacturer's specifications ÷ 2) ÷ Output reads required per sample = Number of sample indices per lane

Note: We provide 12 indexed PCR primers, limiting the number of samples per lane to 12.

Calculation Examples

If your pool contains 1,000 shRNAs, then the number of output reads required per sample can be calculated as follows:

1,000 shRNAs × 1,000 reads/shRNA = 1 million output reads required per sample

If you are using a sequencing platform with a projected output of 40 million reads per lane then the number of samples per lane can be calculated as:

4.0×10^7 reads per lane × 0.50 (reduction in reads for non-standard library) ÷ 1.0×10^6 output reads required per sample = 20 samples per lane

Note: We provide 12 indexed PCR primers, limiting the number of samples per lane to 12.

Table 8 gives examples of the number of sequencing reads required per sample for various pool sizes and the degree to which these samples can be multiplexed using the Decode™ indexed PCR primers based upon the calculation above.

	Decode indices per lane			
Number of shRNA Constructs	Output Required (Reads)	100 million read expected output	20 million read expected output	5 million read expected output
500	0.5 million	12	12	5
1,000	1 million	12	10	2
5,000	5 million	10	2	N/A
10,000	10 million	5	1	N/A

Table 8. Multiplexing indices per lane with indicated pool sizes.

Illumina-adapted PCR primers for shRNA amplification from gDNA are provided as 50 µM forward and reverse indexed primers. The index tags include:

Index 1) ATCACG **Index 2)** CGATGT **Index 3)** TTAGGC **Index 4)** TGACCA **Index 5)** ACAGTG **Index 6)** GCCAAT
Index 7) CAGATC **Index 8)** ACTTGA **Index 9)** GATCAG **Index 10)** TAGCTT **Index 11)** GGCTAC **Index 12)** CTTGTA

iii. PCR from Genomic DNA

Once you have determined the number of PCR reactions you need per sample and your multiplexing capabilities, PCR can be performed as outlined below:

Note: shRNA sequence amplification from gDNA has been optimized using Phusion™ Hot Start II DNA Polymerase (Cat #F-549S/L). The amplification conditions recommended below have been determined to provide maximum yields of PCR product while staying within the linear phase of log amplification.

1. Perform PCR in 50 µL reactions using 96-well PCR plates. PCR conditions have been optimized to amplify 825 ng of gDNA per 50 µL reaction. As described above, calculate the number of reactions needed for the amplification of total gDNA necessary to maintain the shRNA fold representation used at transduction. Table 9 provides the components and volumes needed for one 50 µL PCR amplification reaction.

We recommend using:

- Betaine Solution 5M (Sigma-Aldrich Cat #B0300)
- dNTP Mix, 10 mM each (Cat #R0191)

Component	Volume per reaction	Final concentration
5x Phusion™ HF Buffer	10 µL	1x
10 mM dNTPs	1 µL	200 µM each
Decode Forward PCR Primer (50 µM)	0.5 µL	0.5 µM
Decode Reverse Indexed PCR Primer (50 µM)	0.5 µL	0.5 µM
5 M betaine	5 µL	0.5 M
gDNA + PCR grade H_2O	31 µL	~ 825 ng/reaction
Phusion™ Hot Start II DNA Polymerase (2 U/µL)	2 µL	0.08 U/µL
Total	**50 µL**	

Table 9. PCR components for shRNA amplification.

2. To PCR-amplify the shRNA constructs, use the cycling conditions outlined in Table 10. The conditions indicated in this protocol ensure that amplification is in the exponential phase when using Phusion™ Hot Start II DNA Polymerase; if using another polymerase, the optimal cycle conditions should be determined empirically.

1. Combine reactions amplifying the same gDNA sample into a single 1.5 mL tube. Confirm that a 660 bp amplicon is achieved from each sample by running 10 µL of PCR product on a 2% agarose gel.
   Note: Due to the low number of PCR cycles, the product will be a faint band on a gel before purification.

We recommend using:

- 2% agarose
- GeneRuler™ Low Range DNA Ladder, ready-to-use, 25-700 bp (Cat #SM1193)

4. Purify the remaining PCR-amplified gDNA for each sample to remove excess reaction components using the GeneJET™ PCR Purification Kit (Cat #K0701) or a similar kit for purification of PCR products.
   Note: 100-300 ng can be expected from each 50 µL PCR reaction.

2. Evaluate purified amplicons using the quality standards that are recommended for your Illumina Sequencing platform, such as analysis on an Agilent™ Bioanalyzer™ and qPCR.

8 Illumina Platform Sequencing

Please follow the manufacturer's instructions for Illumina platform sequencing, with the following modifications. Quantify the library using a Qubit™ and then follow Illumina's instructions for dilution and denaturation of the library. We recommend spiking the library with 10% PhiX Control to serve as an internal control and loading the library onto the Illumina flow cells at 5-10 pM using standard loading volumes. For sequencing on the Illumina HiSeq system prepare the Decode Read 1 Sequencing primer by adding 5 µL of the 100 µM primer stock to 995 µL HT1 buffer. Use this Read 1 primer/HT1 buffer for cluster generation. At least 22 single-end reads are required to sequence the passenger strand of the shRNA, as outlined in Figure 10. To prepare the Decode Index Read Sequencing primer, add 5 µL of the 100 µM primer stock to tube HP8 in the Illumina Multiplexing Reagents Kit. Use this Index Read/HP8 buffer for the Index read. For sequencing on the Illumina MiSeq Desktop Sequencer, after loading your sample intro the Illumina MiSeq Reagent Cartridge, add 5 µL of the 100 µM Decode Read 1 Sequencing primer stock to the "Read1 Primer Mix" reservoir and 5 µL of the 100 µM Decode Index Read Sequencing primer stock to the "Index Primer Mix" reservoir on the Illumina MiSeq reagent cartridge (please check the MiSeq System User Guide to confirm the inlet positions).

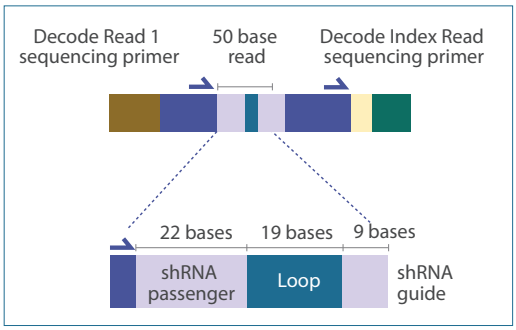

Figure 10. Position of Decode Sequencing primers and contents of 50 base sequencing reads, including shRNA passenger, loop and guide.

Note: The Decode primers will not interfere with the standard Illumina primers present in the loading buffers. The Illumina primers will allow sequencing of the PhiX Control. The PhiX Control contains a balanced representation of A,T,G and C nucleotides helping with sequencing of low diversity libraries.

9 Hit Identification and Follow Up

Alignment of sequence files with shRNA sequences and hit identification can be performed efficiently using open source programs that require basic bioinformatics knowledge. However, we recommend that you consult with an expert in bioinformatics for data analysis and hit identification. A more detailed protocol can be downloaded from the Decode Pooled Lentiviral shRNA Screening Libraries under Resources.

Sort the FASTQ file on the sequencer to bin each index tag with the appropriate sample. The first 22 bases of every read is the shRNA passenger strand (the variable region) and is sufficient to differentiate the shRNAs. The remaining bases in the read include the shRNA and a partial guide strand sequence and can be ignored in the subsequent analysis (Figure 10). Using an alignment tool, such as Bowtie (Langmead *et al.* 2009), align the 22 base passenger strand reads to the reference file provided with your Decode pool. Bowtie has an option to ignore bases on the 3' end of the read, use this option to ignore all the bases up to the 22 base passenger strand. Take the output of the aligner and count the

number of alignments for each shRNA. Next, use a differential expression tool built for discrete count data, such as DeSeq (Gentleman *et al*. 2004), to determine primary hits. Hits can be confirmed and studied further using individual shRNA constructs that can be ordered from the GIPZ lentiviral shRNA collection.

10 Frequently Asked Questions (FAQs)

Questions	Answers
Where can I find titer information for my pools?	The titer of your Lentiviral shRNA pools will be indicated on the Certificate of Analysis (C of A). C of As for purchased Decode Lentiviral shRNA pools can also be requested from Technical Support.
Where can I find the sequence of an individual shRNA included in my pool(s)?	The full insert sequences are in the Decode Data Files CD sent with your shipment. Please contact Technical Support with your Purchase Order number if you did not receive this CD in your shipment.
How many screens can I perform with my pools?	The number of screens which can be performed per lentiviral pool will depend on: 1. The transducibility of your specific cell line/cell type. 2. Your target MOI. 3. The shRNA fold representation you choose to maintain throughout the screen. 4. The number of biological replicates you intend to include.
Can I run my PCR samples on a Life Technologies™ Ion Torrent Sequencing Platform?	No. The adaptors that are integrated into our Decode PCR primers are specific to the Illumina sequencing platforms and are not compatible with other sequencing platforms.
Can I use Sanger sequencing instead of high-throughput sequencing to deconvolute my screen with the Decode primers?	No. Decode PCR Primers, Read 1 Sequencing and Index Read Primers are designed for high-throughput sequencing on an Illumina instrument only. If you wish to perform Sanger sequencing, you can design your own primers.
How is gDNA input calculated to maintain desired shRNA fold representation?	The quantity of gDNA required for maintaining a desired shRNA fold representation can be calculated using the formulas and values (Table 11) below. ***Note: this calculation is for a diploid genome. Some cell lines are not diploid and calculations should be adjusted accordingly.***

Constants	Value
Mass of a base pair	660 g/mol/bp
Base pairs per diploid human genome	6×10^9 bp
Avogadro's number	6.02×10^{23} mol

Calculate the mass of human genome:
$(6 \times 10^9 \text{ bp/genome}) \times (660 \text{ g/mol/bp}) = 3.96 \times 10^{12} \text{ g/mol/genome}$
$(3.96 \times 10^{12} \text{ g/mol/genome}) \div (6.02 \times 10^{23} \text{ mol}) = 6.58 \times 10^{-12} \text{ g/genome}$

Table 11. Constants needed for calculating gDNA input.

11 Troubleshooting

For answers to questions that are not addressed here, please email Technical Support at ts.dharmacon@horizondiscovery.com with the answers to the questions below, your sales order or purchase order number and the Cat # or Clone ID of the construct or pooled library with which you are having trouble.

1. What was the relative transduction efficiency between your cell line and that reported by Dharmacon?
2. What was your lentiviral titer for your cell line?
3. At what MOI did you use the pools?
4. What is your cell line?

5. Did you maintain the cells on puromycin after transduction?
6. How much time elapsed from transduction to puromycin selection?
7. How much puromycin did you use?
8. How long did your cells stay on puromycin selection?
9. In brief, what is your assay?

12 References

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13 Label Licenses

The shRNA and gene expression Products, use and applications, are covered by pending and issued patents. Certain Label licenses govern the use of the products, these can be found at dharmacon.horizondiscovery.com/uploadedFiles/dharmacon-licensing-statements.pdf. It is each Buyer's responsibility to determine which intellectual property rights held by third parties may restrict the use of Products for a particular application. Please review the Label Licenses governing all use of the shRNA and gene expression Products.

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If you have any questions, contact

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