

SMARTvector<sup>TM</sup> Lentiviral  
shRNA Pooled Libraries  
SMARTvector<sup>TM</sup> Inducible Lentiviral  
shRNA Pooled Libraries  
shMIMIC Lentiviral microRNA  
Pooled Libraries  
shMIMIC Inducible Lentiviral  
microRNA Pooled Libraries



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# 1 Introduction

SMARTvector Lentiviral shRNA and shMIMIC Lentiviral microRNA Pooled Libraries are pools of lentiviral constructs expressing short hairpin RNAs (shRNAs) targeting coding genes for knockdown or mature microRNAs mimics for microRNA-overexpression, respectively. Pooled screening can be performed to identify the function of coding or microRNA genes in the regulation of cellular responses and signaling pathways without the need for costly automation and equipment required in arrayed screening.

## A. Available libraries

Table 1 lists the pre-defined pooled lentiviral libraries that are available.

**Table 1.** Available SMARTvector Lentiviral shRNA and shMIMIC Lentiviral microRNA Pooled Libraries.

SMARTvector Lentiviral shRNA Pooled Library <sup>1,2,3,6</sup>	Human		Mouse	
	Number of targeted genes <sup>4</sup>	Number of pools × number of constructs per pool <sup>4,5</sup>	Number of targeted genes <sup>4</sup>	Number of pools × number of constructs per pool <sup>4,5</sup>
Whole Genome	19241	16 pools × 9513 constructs	21745	18 pools × 9502 constructs
Druggable Genome	7341	6 pools × 9735 constructs	9723	8 pools × 9670 constructs
GPCR	377	1 pool × 3012 constructs	494	1 pool × 3909 constructs
Ion Channel	340	1 pool × 2709 constructs	332	1 pool × 2646 constructs
Protein Kinase	702	1 pool × 5602 constructs	697	1 pool × 5558 constructs
Phosphatase	245	1 pool × 1948 constructs	268	1 pool × 2141 constructs
Protease	466	1 pool × 3702 constructs	529	1 pool × 4206 constructs
Ubiquitin Conjugate	557	1 pool × 4431 constructs	511	1 pool × 4069 constructs
shMIMIC Lentiviral microRNA Pooled Library <sup>1,2,6</sup>	Number of mature microRNAs	Number of unique designs	Number of pools × number of constructs per pool <sup>5</sup>	
Human	2580	2555	1 pool × 2665 constructs	
Mouse	1913	1896	1 pool × 2006 constructs	
Human-Mouse Conserved	355 human + 341 mouse	386	1 pool × 496 constructs	

<sup>1</sup>Libraries are available in constitutive promoters (hCMV, mCMV, hEF1α, mEF1α, CAG, PGK, UBC) with fluorescent reporters (TurboGFP, TurboRFP or no reporter) or tetracycline-inducible promoters (TRE3G™) with fluorescent reporters (TurboGFP or TurboRFP). Some promoter options may only be available as custom products or upon request.

<sup>2</sup>Constitutive and inducible lentiviral pooled libraries are provided at  $\geq 5 \times 10^8$  TU/mL ( $\pm 20\%$ ) and  $\geq 5 \times 10^7$  TU/mL ( $\pm 20\%$ ), respectively.

<sup>3</sup>Additional gene families are available on the website and by request ([ts.dharmacon@horizondiscovery.com](mailto:ts.dharmacon@horizondiscovery.com)).

<sup>4</sup>The number of genes and constructs per pool are subject to change at any time without notice.

<sup>5</sup>Number of constructs per pool includes gene-specific and negative control constructs.

<sup>6</sup>Custom libraries targeting rat gene families or researcher-defined human, mouse or rat gene lists are available by request ([ts.dharmacon@horizondiscovery.com](mailto:ts.dharmacon@horizondiscovery.com)).

**SMARTvector Lentiviral shRNA Pooled Libraries include:**

1. shRNAs targeting coding genes in NCBI Reference Sequence Database: Up to eight shRNA designs per gene based on our proprietary algorithm;
2. Gene-specific controls: shRNA constructs targeting 34 protein-coding genes including *ACTB*, *GAPDH*, *LAMB1*, and *PPIB* (eight constructs per gene, 272 shRNAs total) in each pool;
3. Negative controls: 100 Non-targeting shRNA controls in each pool.

**shMIMIC Lentiviral microRNA Pooled Libraries include:**

1. shMIMIC microRNAs (lentiviral microRNA expression constructs) representing all human or mouse mature microRNAs as annotated in miRBase database;
2. Gene-specific controls: shRNA constructs targeting 10 protein-coding genes including *ACTB*, *GAPDH*, *LAMB1*, and *PPIB* (eight constructs per gene, 80 shRNAs total) in each pool;
3. Negative controls: 30 Non-targeting shRNA controls in each pool.

**shMIMIC Lentiviral Human-Mouse Conserved microRNA Pooled Libraries include:**

1. shMIMIC Lentiviral microRNAs representing human and mouse mature microRNAs that are broadly conserved across most vertebrates ([www.targetscan.org](http://www.targetscan.org)), plus all matures that are identical between human and mouse.
2. Gene-specific controls: shRNA constructs targeting five human and five mouse protein-coding genes including human and mouse *GAPDH* and *PPIB* (eight constructs per gene, 80 shRNAs total) in each pool;
3. Negative controls: 30 Non-targeting shRNA controls in each pool.

All libraries are delivered with a data file containing complete library information, including: gene IDs, construct sequences, complete list of controls, and counts per millions of mapped reads.

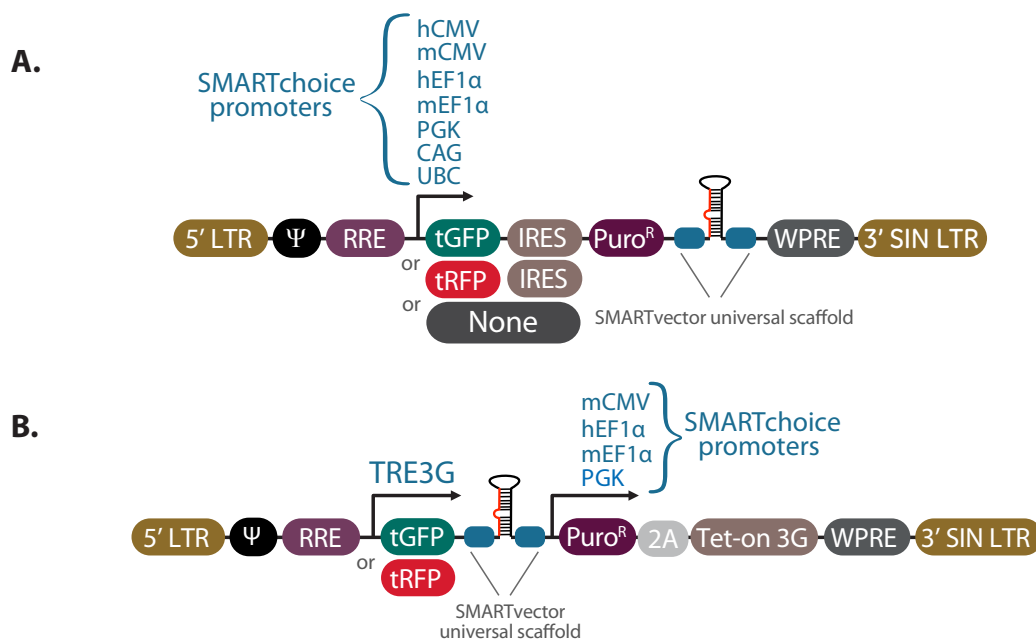
Also available for use with screening libraries are optimized primers and experimentally tested protocols for reliable identification of shRNA and microRNA hits by high-throughput sequencing on an Illumina platform (see Section 3 for details on additional recommended materials).

## B. SMARTchoice™ configuration platform

The SMARTvector Lentiviral shRNA and shMIMIC Lentiviral constructs are part of the SMARTchoice configuration platform, an innovative system ideally suited for RNA interference (RNAi). The purpose is to provide the researcher with the most effective tools for delivering and expressing genetic content in their cells of interest.

The SMARTchoice platform provides a choice of constitutive (Figure 1A) or tetracycline-inducible (TRE3G™; Clontech, Mountain View, CA) promoters (Figure 1B), and presence or absence of reporter genes (TurboGFP or TurboRFP; Evrogen, Moscow, Russia).





Vector element	Utility
hCMV	Human cytomegalovirus immediate early promoter
mCMV	Mouse cytomegalovirus immediate early promoter
hEF1α	Human elongation factor 1 alpha promoter
mEF1α	Mouse elongation factor 1 alpha promoter
PGK	Mouse phosphoglycerate kinase promoter
CAG	Human cytomegalovirus, chicken β-actin hybrid promoter
UBC	Human ubiquitin C promoter
TRE3G	Inducible promoter with Tetracycline Response Elements which is activated by the Tet-On 3G protein in the presence of doxycycline
tGFP or tRFP	TurboGFP or TurboRFP reporter for visual tracking of transduction and expression
None	No reporter option for use in applications where fluorescence is not required
IRES	Internal Ribosomal Entry Site allows for translation initiation in the middle of a mRNA sequence
Puro <sup>R</sup>	Puromycin resistance permits antibiotic-selective pressure and propagation of stable integrants
SMARTvector universal scaffold	Universal primary microRNA context into which an shRNA or mature microRNA is embedded
5' LTR	5' Long Terminal Repeat necessary for lentiviral particle production and integration of the construct into the host cell genome
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 Post-transcriptional Regulatory Element enhances transgene expression in target cells
3' SIN LTR	3' Self-inactivating Long Terminal Repeat for generation of replication-incompetent lentiviral particles

**Figure 1.** Maps of the SMARTvector constitutive (A) and inducible (B) lentiviral vectors with promoters available from the SMARTchoice configuration platform.

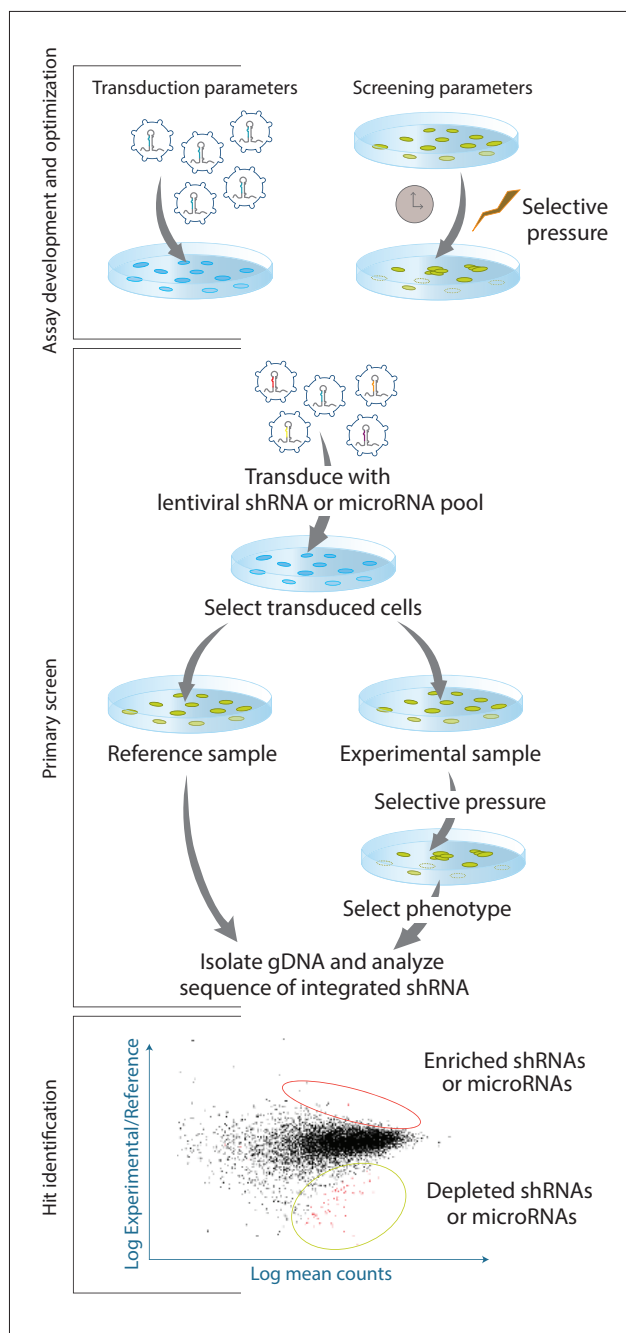
### C. Optimized pooled screening workflow

Once vector configuration options and basic transduction and assay conditions have been optimized, screening begins by transducing cells at a low multiplicity of infection (MOI) with a lentiviral pooled library (see Figure 2 for a screening workflow diagram). Individual cells in the resulting transduced population will contain single constructs integrated into their genomes. Following transduction, a selective pressure is applied such that those constructs involved in a specific biological response can be identified. As a result of the selective pressure, cells expressing the shRNA or mature microRNA construct are either enriched or depleted in the cell 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/or phenotypic selection (experimental cells). shRNA and microRNA constructs within the isolated gDNA are amplified using SMARTvector Forward and Reverse Index PCR primers that have been designed and optimized to minimize amplification bias. Following amplification, indexed reaction products can be directly loaded onto Illumina flow cells and sequenced using SMARTvector Illumina Sequencing Primers. The differences in shRNA or microRNA construct abundance between reference and experimental cell populations can then be determined to identify hits from the primary screen.

Each step of the lentiviral pooled screening workflow, from transduction to hit identification, has been empirically tested. Optimal PCR conditions were identified to ensure uniform amplification and high reproducibility. The screening system for SMARTvector Lentiviral shRNA and shMIMIC Lentiviral microRNA Pooled Libraries utilizes universal Illumina-adapted PCR primers for identification of shRNA or shMIMIC microRNA sequences from gDNA by high-throughput sequencing on

Illumina instrumentation. SMARTvector primers are designed for efficient amplification of constructs while minimizing thermodynamic bias and variation in representation. In addition, SMARTvector PCR primers have built-in adaptor and index sequences that allow the researcher to easily move from PCR amplification to Illumina high-throughput sequencing. Direct identification of construct guide strands facilitates data analysis and ensures accurate target gene identification.



**Figure 2.** Pooled lentiviral shRNA or microRNA 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 shRNA or microRNA constructs is created by transducing a lentiviral pooled library at low MOI. Transduced cells are split into reference and experimental populations for application of a selective pressure and/or phenotypic selection. gDNA is then isolated from reference and experimental populations of transduced cells. Illumina-adapted SMARTvector PCR primers and Phusion™ Hot Start II High-Fidelity DNA Polymerase are used to PCR amplify integrated constructs including various control 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:** Sequences in reference and experimental samples are identified and compared. Constructs that are enriched or depleted are identified as hits to be confirmed and studied further using individual SMARTvector Lentiviral shRNA or shMIMIC Lentiviral microRNA constructs in additional phenotypic and/or biochemical assays.



## 2 Laboratory protocols and calculation tracking worksheet

The **Laboratory protocols and calculation tracking worksheet** is a tool for the recording of key calculations and experimental parameters (see Appendix A). Please review all of the detailed protocols provided in this manual prior to using the worksheet. An electronic version of the [Laboratory protocols and calculation tracking worksheet](#) can also be downloaded from the Dharmacon web page. The downloadable Excel worksheet allows users to incorporate specific inputs related to their screen and performs key calculations to simplify protocol planning.

## 3 Required materials for lentiviral pooled screening

### A. Materials required

- Lentiviral pooled library (Table 1)
- Positive and/or negative controls for optimization with matched promoter/reporter options
  - » Catalog numbers for gene-specific and negative controls with a constitutive promoter may be found here: [dharmacon.horizondiscovery.com/rnai/controls/shrna/smartvector-lentiviral-controls](https://dharmacon.horizondiscovery.com/rnai/controls/shrna/smartvector-lentiviral-controls)  
Catalog numbers for gene-specific and negative controls with an inducible promoter may be found here: [dharmacon.horizondiscovery.com/rnai/controls/shrna/smartvector-inducible-lentiviral-controls](https://dharmacon.horizondiscovery.com/rnai/controls/shrna/smartvector-inducible-lentiviral-controls)
- SMARTvector Indexing PCR and Sequencing Primer Kit Set A (Illumina platform) (Dharmacon, Cat #PRM7668)
- SMARTvector Indexing PCR and Sequencing Primer Kit Set B (Illumina platform) (Dharmacon, Cat #PRM10186) for additional indexed primers (if experimental design requires)
- Phusion Hot Start II High-Fidelity DNA Polymerase and 5x Phusion HF Buffer (Thermo Scientific, Cat #F549X; NEB, Cat #M0535X).



Please see Section 4 to determine the amount of Phusion DNA polymerase required for a screening workflow.

### B. Additional recommended materials

- For constitutive promoters, use the SMARTchoice Promoter Selection Plate (Dharmacon, Cat #SP-001000-01) for optimal promoter selection
- For inducible vectors, use the SMARTchoice Inducible Non-targeting Control 4-Pack (Dharmacon, Cat #VSC6847) for optimal promoter selection
- Doxycycline hyclate (required for inducible promoter options only; Thermo Scientific, Cat #ICN19895510)
- Polybrene (American Bioanalytical, Cat #AB01643)
- Betaine Solution 5 M (Sigma-Aldrich, Cat #B0300)
- PCR purification kit (QIAquick™ PCR Purification Kit, Qiagen, Cat #28104 or similar)
- Low range DNA Ladder (GeneRuler™ Low Range DNA Ladder, ready-to-use, 25-700 bp, Thermo Scientific, Cat #SM1193 or similar)
- Genomic DNA purification kit (Blood and Cell Culture DNA Purification Kit, Qiagen, Cat #13362 or similar)

- Nucleotide mix dNTP set, 10 mM each (GE Life Sciences, Cat #28-4065-64 or similar)
- HyClone™ Puromycin 2 HCl (GE Life Sciences, Cat #SV30075.01 or similar)
- Base Medium: antibiotic-free cell culture medium (without supplements or serum)
- Growth Medium: antibiotic-free cell culture medium (with serum and/or supplements) recommended for maintenance of the cells
- Transduction Medium: the base cell culture medium containing lentiviral particles (with transduction additives and serum, if necessary)
- Selection Medium: Growth Medium supplemented with the appropriate concentration of puromycin
- Resazurin cell viability reagent or similar

## 4 Assay development and optimization

Please use the [Laboratory protocols and calculation tracking worksheet](#) (see [Appendix A](#)) as a guide.

### A. Selection of the optimal promoter for your lentiviral pooled library

#### SMARTchoice Promoter Selection Plate for constitutive expression

The SMARTchoice Promoter Selection Plate (purchased separately) is designed to allow data-driven promoter selection prior to ordering a SMARTvector Lentiviral shRNA Pooled Library or a shMIMIC Lentiviral microRNA Pooled Library. This unique tool permits the simultaneous evaluation of seven different constitutive promoters (hCMV, mCMV, PGK, hEF1 $\alpha$ , mEF1 $\alpha$ , CAG, and UBC) in order to select one that is most effective in your cell line or cell type of interest. Detailed protocols for adherent or suspension cells are described in the SMARTvector Lentiviral shRNA Technical Manual ([dharmacon.horizon-discovery.com/uploadedFiles/Resources/smartvector-constitutive-manual.pdf](http://dharmacon.horizon-discovery.com/uploadedFiles/Resources/smartvector-constitutive-manual.pdf)).

#### SMARTchoice Inducible Non-targeting Control 4-Pack for inducible expression

SMARTvector and shMIMIC Inducible lentiviral vector configuration options include four RNA pol II promoter options: mouse CMV, PGK, human EF1 $\alpha$  and mouse EF1 $\alpha$ ; all of which control the expression of puromycin resistance and the Tet-On 3G transactivator. Use the SMARTchoice Inducible Non-targeting Control 4-Pack to evaluate which of these promoters are most active in the cells of interest. Consult the SMARTvector Inducible Lentiviral shRNA Technical Manual ([dharmacon.horizon-discovery.com/uploadedFiles/Resources/smartvector-inducible-lentiviral-shRNA-manual.pdf](http://dharmacon.horizon-discovery.com/uploadedFiles/Resources/smartvector-inducible-lentiviral-shRNA-manual.pdf)) for detailed protocols.

### B. Optimization of lentiviral transduction

While 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 selected during these optimization steps must be compatible with primary screening protocols and conditions. SMARTvector Non-targeting Control lentiviral particles can be used for optimization of transduction conditions. Detailed protocols for transduction optimization are described in the SMARTvector Lentiviral shRNA Technical Manual ([dharmacon.horizon-discovery.com/uploadedFiles/Resources/smartvector-constitutive-manual.pdf](http://dharmacon.horizon-discovery.com/uploadedFiles/Resources/smartvector-constitutive-manual.pdf)). For inducible lentiviral vector options consult the SMARTvector Inducible Lentiviral shRNA Technical Manual ([dharmacon.horizon-discovery.com/uploadedFiles/Resources/smartvector-inducible-lentiviral-shRNA-manual.pdf](http://dharmacon.horizon-discovery.com/uploadedFiles/Resources/smartvector-inducible-lentiviral-shRNA-manual.pdf)). 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 the cells of interest.

**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. If Polybrene is toxic to the cells of interest, DEAE-Dextran (1-10 µg/mL) or Protamine Sulfate (1-50 µg/mL) may be substituted.

**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.

### C. Determination of functional titer

Functional titer can be determined either by counting GFP or RFP-positive colonies using fluorescence microscopy or by fluorescence activated cell sorting (FACS) analysis of GFP/RFP-positive cells. For no reporter lentiviral vector options we suggest using a functional titration protocol such as limiting dilution with cell viability assay by crystal violet staining ([dharmacon.horizondiscovery.com/uploadedfiles/resources/titer-crystal-violet-protocol.pdf](http://dharmacon.horizondiscovery.com/uploadedfiles/resources/titer-crystal-violet-protocol.pdf)) or genomic qPCR assay.<sup>1</sup>

Lentiviral transduction efficiency can vary by target cell type. The titer of SMARTvector Lentiviral shRNA particles reported on the Certificate of Analysis (C of A) was measured in HEK293T cells, so the relative transduction efficiency of your cell type will likely be different. Therefore, it is recommended that a relative functional titer is determined in your cells using SMARTvector Non-targeting Control Particles and then used to convert the reported titer of each lentiviral pool for the primary screen. SMARTvector Non-targeting Control Particles are purchased separately and should be promoter-matched to the pooled library.

#### Day 1:

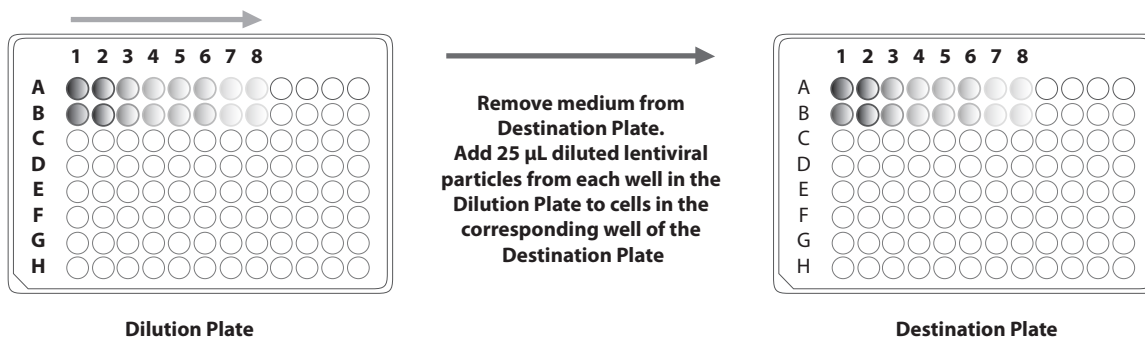
1. The day before transduction, seed a 96-well cell culture plate (Destination Plate) with your cells at the density determined during transduction optimization. Grow cells overnight.
2. Make dilution medium using serum, Polybrene conditions and cell medium determined during transduction optimization. Make dilutions of SMARTvector Non-targeting Control Particles in a round-bottom 96-well plate (Dilution Plate). As shown in Figure 3 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 of 390 625-fold dilution.
  - 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 SMARTvector Non-targeting Control 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 3 through 8, mixing 10-15 times for each dilution.
  - e. Allow lentiviral particle: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  $\mu\text{L}$  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  $\mu\text{L}$  of normal growth medium to cells.

**Days 4-5:**

1. Culture cells for 48-72 hours (as determined during transduction optimization).
2. Choose one well in the Destination Plate for counting GFP/RFP-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 4). Calculate the average number of GFP/RFP-positive colonies from the same destination well of each replicate.

Make five-fold dilutions into Dilution Plate according to protocol and Table 2.



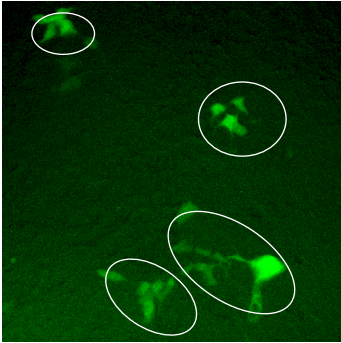
**Figure 3.** Diagram for dilution series of lentiviral particles (Dilution Plate) and addition to cells (Destination Plate).

**Table 2.** Example setup for lentiviral particle dilution series.

Well	Dilution Plate		Dilution factor	Volume of diluted lentiviral particles used in transduction (Destination Plate)
	Lentiviral particle serial dilution volume	Volume of dilution medium		
A1	10 $\mu\text{L}$ (*control)	40 $\mu\text{L}$	5	25 $\mu\text{L}$
A2	20 $\mu\text{L}$ (from A1)	80 $\mu\text{L}$	25	25 $\mu\text{L}$
A3	20 $\mu\text{L}$ (from A2)	80 $\mu\text{L}$	125	25 $\mu\text{L}$
A4	20 $\mu\text{L}$ (from A3)	80 $\mu\text{L}$	625	25 $\mu\text{L}$
A5	20 $\mu\text{L}$ (from A4)	80 $\mu\text{L}$	3125	25 $\mu\text{L}$
A6	20 $\mu\text{L}$ (from A5)	80 $\mu\text{L}$	15 625	25 $\mu\text{L}$
A7	20 $\mu\text{L}$ (from A6)	80 $\mu\text{L}$	78 125	25 $\mu\text{L}$
A8	20 $\mu\text{L}$ (from A7)	80 $\mu\text{L}$	390 625	25 $\mu\text{L}$

\* SMARTvector Non-targeting Control Particles. Repeat identical dilution series in wells B1 to B8.





**Figure 4.** Example of individual colonies in HEK293T cells 72 hours post-transduction. Four colonies are circled. Imaged at 40× magnification.

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

$$\begin{array}{c} \text{Number of} \\ \text{TurboGFP-} \\ \text{positive} \\ \text{colonies} \end{array} \times \begin{array}{c} \text{Dilution} \\ \text{factor} \\ \text{(Table 2)} \end{array} \div \begin{array}{c} 0.025 \\ \text{(volume of} \\ \text{diluted lentiviral} \\ \text{particles in mL)} \end{array} = \begin{array}{c} \text{Functional titer of} \\ \text{SMARTvector Non-targeting} \\ \text{Control Particles stock in} \\ \text{your cell line (TU/mL)} \end{array}$$

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

$$\begin{array}{c} \text{Functional titer of} \\ \text{SMARTvector Non-targeting} \\ \text{Control Particles} \\ \text{your cell line (TU/mL)} \end{array} \div \begin{array}{c} \text{Titer of SMARTvector} \\ \text{Non-targeting Control Particles stock} \\ \text{reported on the C of A (TU/mL)} \end{array} = \begin{array}{c} \text{Relative} \\ \text{transduction} \\ \text{efficiency of} \\ \text{your cell line} \end{array}$$

**Use the calculated relative transduction efficiency of your cell line to calculate the relative functional titer for each lentiviral pooled library using the following formula:**

$$\begin{array}{c} \text{Relative} \\ \text{transduction} \\ \text{efficiency of} \\ \text{your cell line} \end{array} \times \begin{array}{c} \text{Titer of the lentiviral} \\ \text{pooled library} \\ \text{reported on the} \\ \text{C of A (TU/mL)} \end{array} = \begin{array}{c} \text{Relative} \\ \text{functional titer of the} \\ \text{lentiviral pooled library} \\ \text{in your cell line (TU/mL)} \end{array}$$

#### Calculation examples

If you counted 58 GFP/RFP-positive colonies in well A7 of the destination plate, the titer of the SMARTvector Non-targeting Control Particles in your cell line would be calculated as follows:

$$58 \text{ (GFP/RFP positive colonies)} \times 78 \text{ 125 (dilution factor)} \div 0.025 \text{ (volume of diluted lentiviral particles in mL)} = 1.8 \times 10^8 \text{ TU/mL functional titer of SMARTvector Non-targeting Control Particles in your cell line}$$

If the titer for the SMARTvector Non-targeting Control Particles on the product insert was listed as  $9.0 \times 10^8$  TU/mL, the relative transduction efficiency of your cell type would be determined as follows:

$$1.8 \times 10^8 \text{ TU/mL (functional titer in your cell line)} \div 9.0 \times 10^8 \text{ TU/mL (titer as indicated on product insert)} = 0.2 \text{ relative transduction efficiency}$$

If the relative transduction efficiency of your cell line is 0.2 and the titer of a lentiviral pooled library, as indicated on the C of A, is  $5.0 \times 10^8$  TU/mL, the relative functional titer of the pool in your cell line would be determined as follows:

$$0.2 \text{ (relative transduction efficiency)} \times 5.0 \times 10^8 \text{ TU/mL (titer as indicated on product insert)} = 1.0 \times 10^8 \text{ TU/mL relative functional titer in your cell line}$$

## D. Optimization of puromycin selection

The SMARTvector lentiviral vector backbone contains the puromycin selectable marker which allows for selection of cells that have integrated a SMARTvector shRNA or shMIMIC microRNA construct. Depending on your choice, the SMARTvector lentiviral vector may also contain either the TurboGFP or TurboRFP fluorescent reporter protein to allow selection by FACS to isolate cells expressing TurboGFP or TurboRFP. Prior to 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.

**Day 1:**

1. Plate cells at a density appropriate for your cell type. Incubate overnight.

**Day 2:**

1. Change to fresh medium supplemented with puromycin at a range of concentrations (0-10 µg/mL). Incubate for 3-6 days.

**Day 4-8:**

1. Approximately every 2-3 days replace with freshly prepared puromycin medium.
2. Monitor the cells daily and visually observe the percentage of surviving cells. Optimum effectiveness should be reached in 3-6 days under puromycin selection.
3. The optimal antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 3-6 days from the start of antibiotic selection.

## E. Determination of assay-specific screening conditions

The lentiviral pooled library screening workflow described here facilitates identification of genetic regulators of a range of biological processes. Variables to consider when planning screening conditions include, but are not limited to, assay duration, conditions of selective pressure (for example, concentration or duration), method of phenotypic selection (for example, measuring viability, proliferation, surface marker expression or migration), and doxycycline application parameters (if using inducible promoter options). We recommend that you optimize all assay conditions prior to beginning a lentiviral pooled library screen using, if possible, either an shRNA or a shMIMIC microRNA construct known to be involved in the phenotype(s) of interest.

## F. Selection of average fold representation and number of biological replicates

A critical consideration of lentiviral pooled screening is the extent to which any given 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 construct or the number of biological replicates of each construct integration event and subsequent phenotypic selection. High construct representation results in high reproducibility between biological replicates and ensures that there is a sufficient window for detection of changes in construct representation after phenotypic selection.<sup>2</sup>

**Recommendations:**

- Between 500 and 1000 independent integrations per construct
- At least two biological replicates



We have observed that construct fold representation has a greater impact on the ability to identify hits than the number of biological replicates. Therefore, we recommend performing at least two biological replicates, while maintaining an average construct fold representation as high as is practical or technically feasible for your screen.

## G. Calculation of number of cells needed for transduction

Once transduction optimization experiments have been performed and the assay-specific screening parameters determined, cells need to be prepared for transduction. The calculations below outline how to determine the number of cells required at the time of transduction.

MOI is defined as the ratio of transducing units of lentiviral particles to target cells. At high MOIs, each cell will likely be transduced by more than one lentiviral particle. Conversely, at low MOIs, the probability that any cell is transduced with more than one lentiviral particle is low (Table 3). For lentiviral pooled screen, each cell will ideally express a single construct. This ensures that the knockdown of a single gene (in the case of shRNA constructs) or over-expression of a single microRNA (in the case of shMIMIC microRNA constructs) in a particular cell is responsible for the resulting phenotype. Therefore, we recommend performing lentiviral transductions at an MOI  $\leq 0.3$ .

**Table 3.** Poisson distribution showing the distribution of cells with the indicated number of lentiviral integrations at various MOIs.

MOI	Number of lentiviral integrants per cell				
	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

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 one lentiviral integrant.

**To calculate the number of lentiviral integration events required to achieve a given construct representation, use the following formula:**

$$\text{Number of constructs in the lentiviral pool} \times \text{Desired fold representation} = \text{Desired number of cells with lentiviral integrations}$$

**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 3), and then use the following formula:**

$$\text{Desired number of cells with lentiviral integrations} \div \text{Proportion of cells with lentiviral integrations} = \text{Number of cells required at the time of transduction}$$

### Calculation examples

If you have a pool of 1000 constructs and you wish to achieve a 500-fold average representation, the desired number of lentiviral integration events would be calculated as follows:

$$1000 \text{ (number of constructs)} \times 500 \text{ (average representation)} = 5.0 \times 10^5 \text{ lentiviral integration events}$$

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

$$5.0 \times 10^5 \text{ (lentiviral integration events required)} \div 0.25 \text{ (proportion of cells that undergo at least one integration)} = 2.3 \times 10^6 \text{ cells needed at the time of transduction}$$

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 you wish to transduce. The calculations below outline how to determine cell plating requirements.

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

$$\text{Number of cells required at the time of transduction} \div \text{Cell density at transduction} \div \text{Size of plate (mm}^2\text{)} = \text{Number of plates required per sample}$$

**To determine the total number of plates to seed per pooled RNAi library, use the following formula:**

$$\text{Number of plates required per sample} \times \text{Number of biological replicates} = \text{Number of plates required per pool}$$



Table 4 provides examples of cell and plate numbers and plate sizes for various targeted construct fold representations, assuming a pool size of 1000 constructs and a cell line with optimal transduction density of 250 cells/mm<sup>2</sup>.

**Table 4.** Number of cells required at the time of transduction and cell plating recommendations for indicated average construct fold representation assuming pools of 1000 constructs, MOI of 0.3, optimal transduction density of 250 cells/mm<sup>2</sup> and an effective surface area on 100 mm plates of 7800 mm<sup>2</sup>.

Construct fold representation	Number of lentiviral integrations required	Number of cells required at transduction	Recommended cell plating
500	$5 \times 10^5$	$2.3 \times 10^6$	1 plate $\times$ 100 mm
1000	$1 \times 10^6$	$4.5 \times 10^6$	2 plates $\times$ 100 mm

## H. Calculation of volume of lentiviral particles needed for transduction

Before proceeding with the lentiviral pooled library screen, confirm that you have sufficient lentiviral particles to perform the 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.

Follow the steps below to calculate the volume of lentiviral particles required for each pool. All pooled libraries are supplied as multiple aliquots of 25  $\mu$ L of lentiviral particles, at titers of  $\geq 5 \times 10^8$  TU/mL for constitutive vectors and  $\geq 5 \times 10^7$  TU/mL for inducible vectors.

Table 5 provides examples of volume of lentiviral particles needed for various fold representations and relative transduction efficiencies. Additional lentiviral particles can be ordered separately.

**First, identify the number of lentiviral particles (or transducing units, TU) necessary for each biological replicate based on the required number of cells at the time of transduction by applying the following formula:**

$$\text{MOI} \times \text{Number of cells required at the time of transduction} = \text{Required number of lentiviral particles (TU)}$$

**Next, determine the volume of lentiviral particles required based on the following formula:**

$$\text{Required number of lentiviral particles (TU)} \div \text{Functional titer in your cell line (TU/mL)} = \text{Volume of lentiviral particles per biological replicate (mL)}$$

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

$$\text{Volume of lentiviral particles per biological replicate (mL)} \times \text{Number of biological replicates} = \text{Volume of lentiviral particles per pool (mL)}$$

### Calculation examples

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

$$0.3 \text{ (MOI)} \times 2.0 \times 10^6 \text{ cells} = 6.0 \times 10^5 \text{ TU of lentiviral particles are required for transduction}$$

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

$$6.0 \times 10^5 \text{ TU} \div 1.0 \times 10^8 \text{ TU/mL} = 0.006 \text{ mL of lentiviral particles}$$

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

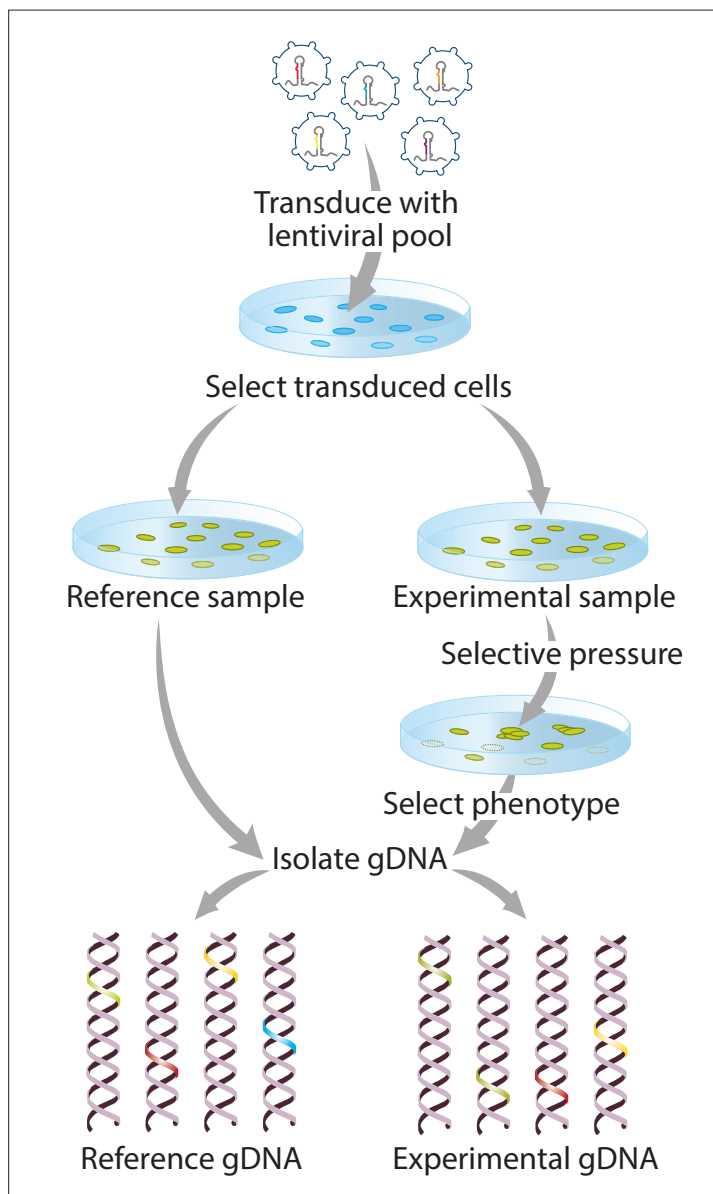
$$0.006 \text{ mL of lentiviral particles} \times 2 \text{ biological replicates} = 0.012 \text{ mL of lentiviral particles}$$

**Table 5.** Volume of lentiviral particles at  $5 \times 10^8$  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 1000 constructs with one biological replicate at an MOI of 0.3.

Construct fold representation	Relative transduction efficiency			
	1	0.5	0.2	0.1
500	1.2 $\mu$ L	2.4 $\mu$ L	6 $\mu$ L	12 $\mu$ L
1000	2.4 $\mu$ L	4.8 $\mu$ L	12 $\mu$ L	24 $\mu$ L

## 5 Primary screen

The following sections describe the primary screen workflow (Figure 5). Please use the **Laboratory protocols and calculation tracking worksheet** (see Appendix A) as a guide.

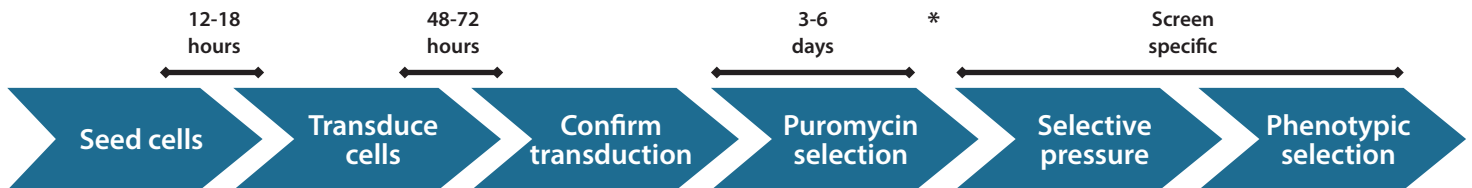


**Figure 5.** Primary screen workflow.

## A. Cell transduction and selection

The experimental conditions described here, and in Figure 6, 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 construct fold representation should be determined specifically for each cell line of interest and each intended screening experiment, as outlined in Section 4.

Similarly, conditions should be clearly defined prior to starting the screen for application of selective pressure and selection of phenotype of interest.



**Figure 6.** Timeline of primary screening. \*Timing of doxycycline induction varies by assay when using inducible promoter options.

### Day 1:

1. Seed cells in normal Growth Medium in the number of plates determined in Section 4.G 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.

### Day 2:

1. The next day, remove the medium and add optimized Transduction Medium (see Appendix) with the appropriate amount of lentiviral particles (Section 4.H) so that the cells are just covered. If a single lentiviral pool will be added to multiple plates, as determined in Section 4.G, divide the volume of lentiviral particles evenly among plates.
2. After the appropriate transduction time (see Section 4.B), add additional Growth Medium to your cells such that the cells can be incubated for 48-72 hours.

### Days 4-8:

1. At 48-72 hours post-transduction examine the cells microscopically for the presence of fluorescent reporter expression (if a reporter was chosen for the lentiviral vector); this will be the first indication as to the efficiency of transduction. If using an inducible promoter option, induction by doxycycline for a minimum of 24 hours will be necessary to visualize the fluorescent reporter (See note below).



**When using an MOI = 0.3 you should expect approximately 25% of the cells to express TurboGFP, or a 25% cell survival rate following puromycin selection for no-reporter or inducible products prior to induction.**

2. Begin puromycin selection to remove non-transduced cells. Monitor the cells daily and observe the percentage of fluorescent-positive cells (if a reporter was chosen for the lentiviral vector). Every 48-72 hours, replace with fresh medium containing puromycin and passage cells as needed.



**Use the appropriate concentration of puromycin and the minimum number of days required to kill non-transduced cells as determined by a puromycin kill curve (see Section 4.D)**

Once a pure population of transduced cells has been obtained (3-6 days), begin screening. Split cells into at least two populations: one as a reference and another (experimental) for application of selective pressure and phenotypic selection. To maintain your desired construct 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.



Isolation of gDNA from cells transduced with lentiviral pooled libraries has been optimized in the protocol below using Qiagen Blood and Cell Culture DNA Maxi Kit; however, kits from other manufacturers may also be suitable.

1. Collect cells by trypsinization for counting and gDNA isolation. 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.

To ensure high 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.



It is important that you elute gDNA samples in EDTA-free buffer to prevent inhibition of subsequent PCR reactions.

2. 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  $A_{230}$  ( $A_{260/230}$ ). High-quality gDNA samples should have an  $A_{260/280}$  ratio of 1.8 to 2.0, indicating the absence of contaminating proteins, and an  $A_{260/230}$  ratio of  $> 2.0$ , indicating the absence of other organic contaminants.

## C. PCR amplification of constructs from genomic DNA

The PCR amplification and high-throughput sequencing workflow described here (Figure 7) has been designed to amplify shRNA and shMIMIC microRNA constructs from gDNA without bias, such that differences in construct representation after sequencing are due to enrichment or depletion that occurs during the primary screen. It is important to use adequate template copies per construct in the PCR amplification step such that the average fold representation at transduction is maintained. This ensures assay reproducibility and facilitates hit identification.<sup>2</sup>



The SMARTvector Indexing 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 construct fold representation, assuming a single lentiviral integration event per cell (genome), using the following formula:

$$\begin{array}{|c|} \hline \text{Number of} \\ \text{cells with lentiviral} \\ \text{integrants} \\ \hline \end{array} \times \begin{array}{|c|} \hline 6.6 \times 10^{-3} \\ \text{ng/genome} \\ \text{(see note below)} \\ \hline \end{array} = \begin{array}{|c|} \hline \text{Mass of gDNA} \\ \text{required to maintain} \\ \text{representation of} \\ \text{each construct} \\ \hline \end{array}$$



The calculation above assumes a diploid human genome. Many cell lines are not diploid and calculations must be adjusted accordingly. For diploid mouse cell lines use  $6.1 \times 10^{-3}$  ng/genome.



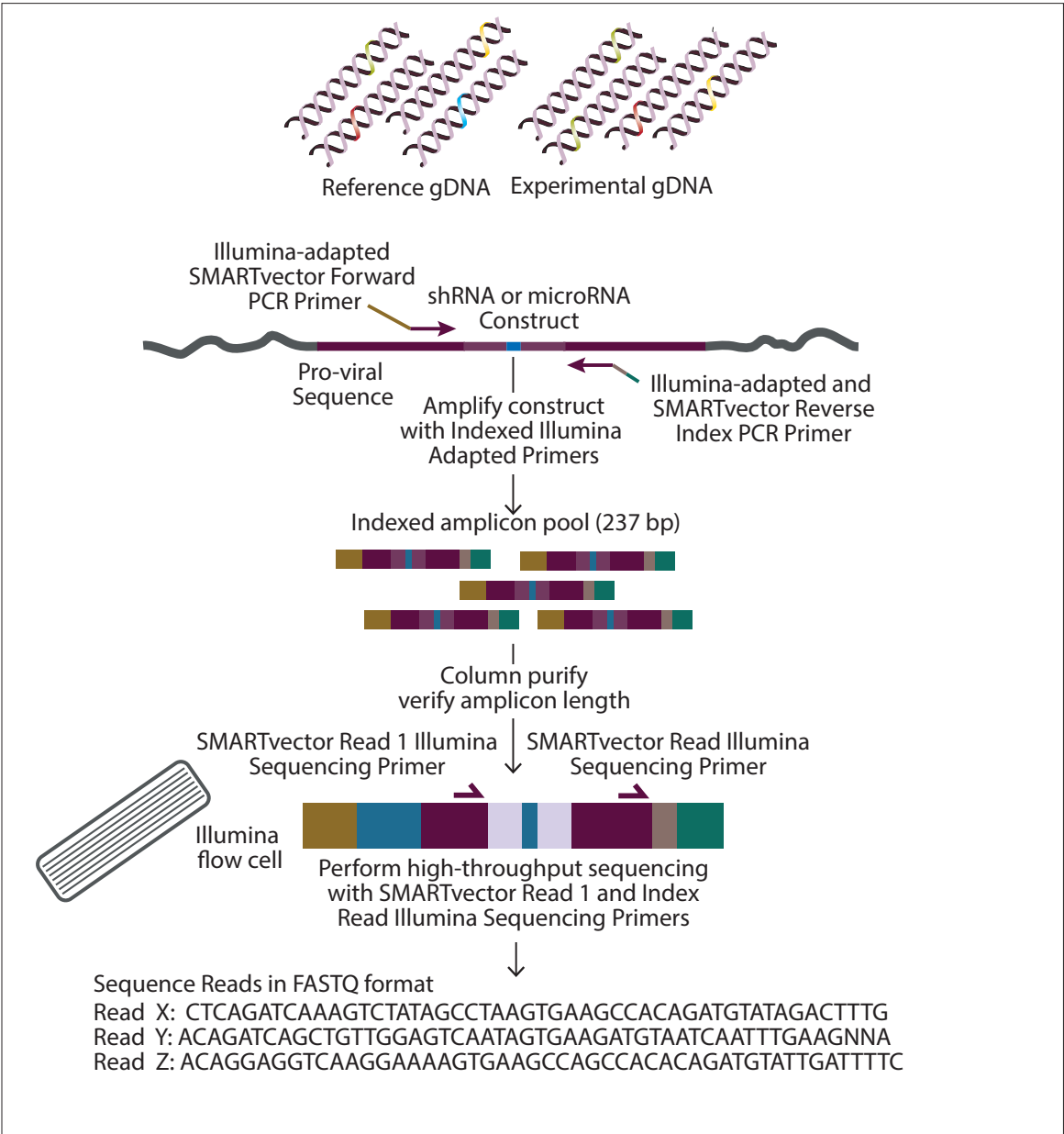


Figure 7. PCR amplification and Illumina high-throughput sequencing workflow.

We have optimized the PCR conditions to remain in the linear phase of log amplification using Phusion Hot Start II DNA Polymerase. We recommend a maximum of 825 ng of gDNA per PCR; using more gDNA per PCR will inhibit the efficiency of the reaction and will result in either failure of the PCR or biased amplification.

**Calculate the number of PCR reactions required for each sample using the following formula:**

$$\text{Mass of gDNA required to maintain representation of each construct (ng)} \div 825 \text{ ng per reaction} = \text{Number of PCR reactions required to maintain representation of each construct*}$$

\* Round up the value obtained to the next whole number)

Table 6 gives an example of the input gDNA and number of PCR reactions required for various fold representations with a pool of 1000 constructs.

**Table 6.** Input gDNA for PCR amplification and number of PCR reactions at indicated fold representation for a pool of 1000 constructs.

Construct fold representation	Input gDNA ( $\mu\text{g}$ )	Number of PCR reactions
500	3.3	4
1000	6.6	8

**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:**

$$\text{Number of PCR reactions per sample} \times \text{Number of samples per pool} = \text{Number of PCR reactions per pool}$$

**To determine the amount of Phusion DNA Polymerase required for each lentiviral pooled library use the following formula:**

$$\text{Number of PCR reactions per sample} \times 4 \text{ units of polymerase per PCR} = \text{Units of polymerase per pool}$$

### Calculation examples

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

$$5 \times 10^5 \text{ lentiviral integrants} \times 6.6 \times 10^{-3} \text{ ng/genome} = 3.3 \times 10^3 \text{ ng}$$

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

$$3.3 \times 10^3 \text{ ng gDNA} \div 825 \text{ ng/PCR} = 4 \text{ PCR reactions}$$

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

$$4 \text{ PCR reactions} \times 4 \text{ samples} = 16 \text{ PCR reactions}$$

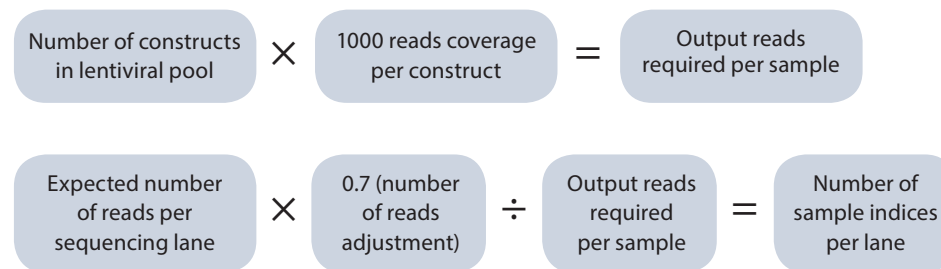
To perform 16 PCR reactions you need the following amount of Phusion Hot Start II DNA Polymerase:

$$16 \text{ PCR reactions} \times 4 \text{ units of Phusion polymerase/PCR} = 64 \text{ units of Phusion polymerase required per pool}$$

## ii. Multiplexing of high-throughput sequencing samples

Most high-throughput sequencing platforms provide a sufficiently high number of sequence reads to allow multiplexing of samples in sequencing runs (running multiple samples in one lane). For more accurate hit identification, we recommend obtaining a minimum of 1000 reads per construct. If the output of your sequencing platform is higher than the number of reads required per sample, it may be possible to multiplex samples in the same run. To calculate the maximum number of samples that can be loaded per lane, it is advisable to assume a lower number of sequence reads than the maximum obtained on an optimum sequencing run. We suggest estimating the number of reads by adjusting the manufacturer's specifications by a factor of 0.7.

**From this information the number of samples you can run per lane can be determined using the following calculations:**



### Calculation examples

If your pool contains 1000 constructs, then the number of output reads required per sample can be calculated as follows:

$$1000 \text{ constructs} \times 1000 \text{ reads/construct} = 1 \text{ million output reads required per sample}$$

If you are using a sequencing platform with a projected output of 15 million reads per lane then the number of samples per lane can be calculated as:

$$1.5 \times 10^7 \text{ reads per lane} \times 0.7 \text{ (number of reads adjustment)} \div 1 \times 10^6 \text{ output reads required per sample} = 10 \text{ samples per lane}$$

Illumina-adapted SMARTvector Indexing PCR primers are provided as 50  $\mu$ M forward and reverse indexed primers. The index tags are shown in Table 7.

**Table 7.** The index tags on SMARTvector Indexing PCR primers are compatible with Illumina TruSeq LT indices.

Item	Index	Index sequence
Kit A		
SMARTvector Reverse Index PCR Primer 2	2	CGATGT
SMARTvector Reverse Index PCR Primer 4	4	TGACCA
SMARTvector Reverse Index PCR Primer 5	5	ACAGTG
SMARTvector Reverse Index PCR Primer 6	6	GCCAAT
SMARTvector Reverse Index PCR Primer 7	7	CAGATC
SMARTvector Reverse Index PCR Primer 12	12	CTTGTA
SMARTvector Reverse Index PCR Primer 13	13	AGTCAA
SMARTvector Reverse Index PCR Primer 14	14	AGTTCC
SMARTvector Reverse Index PCR Primer 15	15	ATGTCA

Item	Index	Index sequence
SMARTvector Reverse Index PCR Primer 16	16	CCGTCC
SMARTvector Reverse Index PCR Primer 18	18	GTCCGC
SMARTvector Reverse Index PCR Primer 19	19	GTGAAA
Kit B (Optional)		
SMARTvector Reverse Index PCR Primer 1	1	ATCACG
SMARTvector Reverse Index PCR Primer 3	3	TTAGGC
SMARTvector Reverse Index PCR Primer 8	8	ACTTGA
SMARTvector Reverse Index PCR Primer 9	9	GATCAG
SMARTvector Reverse Index PCR Primer 10	10	TAGCTT
SMARTvector Reverse Index PCR Primer 11	11	GGCTAC
SMARTvector Reverse Index PCR Primer 20	20	GTGGCC
SMARTvector Reverse Index PCR Primer 21	21	GTTTCG
SMARTvector Reverse Index PCR Primer 22	22	CGTACG
SMARTvector Reverse Index PCR Primer 23	23	GAGTGG
SMARTvector Reverse Index PCR Primer 25	25	ACTGAT
SMARTvector Reverse Index PCR Primer 27	27	ATTCCT

### 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. Sequence amplification from gDNA has been optimized using Phusion Hot Start II DNA Polymerase. The amplification conditions recommended have been determined to provide maximum yields of PCR product while staying within the linear phase of log amplification.

1. Perform PCR in 50  $\mu$ L reactions using 96-well PCR plates. PCR conditions have been optimized to amplify 825 ng of gDNA per 50  $\mu$ L reaction. As described above, calculate the number of reactions needed for the amplification of total gDNA necessary to maintain the fold representation used at transduction. Table 8 provides the components and volumes needed for one 50  $\mu$ L PCR amplification reaction.

**Table 8.** PCR components for amplification of genomic DNA.

Component	Volume per reaction	Final concentration
5x Phusion HF Buffer	10 $\mu$ L	1x
10 mM dNTPs	1 $\mu$ L	200 $\mu$ M each
SMARTvector Forward PCR Primer (50 $\mu$ M)	0.5 $\mu$ L	0.5 $\mu$ M
SMARTvector Reverse Index PCR Primer (50 $\mu$ M)	0.5 $\mu$ L	0.5 $\mu$ M
5 M betaine	5 $\mu$ L	0.5 M
gDNA + PCR grade H <sub>2</sub> O	31 $\mu$ L	~ 825 ng/reaction
Phusion Hot Start II DNA Polymerase (2 U/ $\mu$ L)	2 $\mu$ L	0.08 U/ $\mu$ L
<b>Total</b>	<b>50 <math>\mu</math>L</b>	



- To PCR amplify the SMARTvector or shMIMIC constructs, use the cycling conditions outlined in Table 9. 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.

**Table 9.** PCR cycle parameters

Temperature	Time	23 cycles
98 °C	3 minutes	
98 °C	10 seconds	
60 °C	15 seconds	
72 °C	15 seconds	
72 °C	5 minutes	

- Confirm that a 237 base pair amplicon is obtained from each sample by running 10 µL of PCR product on a 2% agarose gel.



**Due to the low number of PCR cycles, the product will be a faint band on a gel before purification.**

- Combine reactions amplifying the same gDNA sample into a single 1.5 mL tube. Purify the combined PCR amplicons for each gDNA sample using a PCR purification kit.



**Please be aware that gDNA often co-purifies with the PCR products. Although contaminating gDNA does not interfere with the next-generation sequencing, it does affect the quantification of the amount of PCR amplicons by spectrophotometric or fluorimetric methods. Therefore, it is recommended to evaluate the concentration of each amplicon using quantitative PCR or chromatographic methods such as analysis on an Agilent Bioanalyzer System. Alternatively, the amplicons can be purified by an agarose gel electrophoresis protocol and quantified by a fluorimetric DNA quantification method, such as QuBit™ (Life Technologies).**

## 6 Illumina platform sequencing

Please use the [Laboratory protocols and calculation tracking worksheet](#) (see Appendix A) as a guide.

Manufacturer's instructions should be followed for Illumina platform sequencing including (a) quantifying the library using recommended quality standards and (b) dilution and denaturation of the library.

For the Illumina MiSeq Desktop Sequencer, we recommend spiking the library with 10% PhiX Control to serve as an internal control and loading the library onto the flow cell at 7 to 10 pM using standard loading volumes. After loading your sample into the Illumina MiSeq Reagent Cartridge, add 5 µL of the 100 µM SMARTvector Read 1 Sequencing Primer stock to the "Read 1 Primer mix" reservoir and 5 µL of the 100 µM SMARTvector 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**).



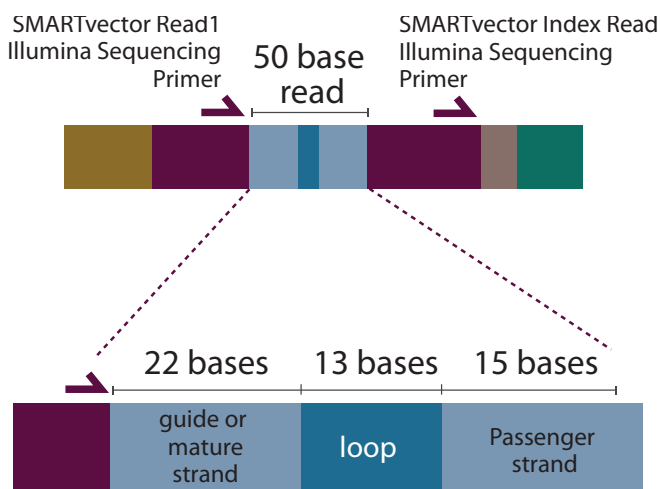
**PhiX Control contains a balanced representation of A, T, G and C nucleotides helping with sequencing unbalanced or low diversity libraries that may result during a screen.**

For the Illumina HiSeq 2500 system, we recommend spiking the library with 10% PhiX Control to serve as an internal control and loading the library onto the flow cell at 6 pM for Rapid Run with onboard clustering or 7.5 pM for High Output Run with c-bot clustering using standard loading volumes. Dilute the SMARTvector Read 1 Sequencing and SMARTvector Index Read Sequencing primers into the respective Illumina primer reagents to a final concentration of 0.5  $\mu$ M.

At least 22 base single-end reads are required to sequence the guide strand of the SMARTvector shRNA or shMIMIC microRNA, as outlined in Figure 8.



The SMARTvector Reverse Index PCR Primers for Illumina platforms use the same index sequences as the Illumina TruSeq LT Index sequences, A001 to A027 (see Table 7).



**Figure 8.** Position of SMARTvector Sequencing primers and contents of 50 base sequencing reading including the shRNA guide, loop and part of the passenger strand or the shMIMIC microRNA expression construct mature strand, loop and part of passenger strand.

## 7 Hit identification and follow up

Please use the [Laboratory protocols and calculation tracking worksheet](#) (see Appendix A) as a guide.

Alignment of sequence files and hit identification can be performed efficiently using open source programs that require some bioinformatics knowledge. 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 SMARTvector Lentiviral shRNA Pooled Library or the shMIMIC Lentiviral microRNA Pooled Library under Resources ([dharmacon.horizondiscovery.com/uploadedFiles/Resources/lentiviral-pooled-library-bioinformatic-analysis-protocol.pdf](http://dharmacon.horizondiscovery.com/uploadedFiles/Resources/lentiviral-pooled-library-bioinformatic-analysis-protocol.pdf)). The first 21 to 22 bases of each read correspond to the sequence of the guide strand of shRNA constructs or the mature strand of shMIMIC microRNA constructs and are sufficient for differentiating among constructs. The remaining bases in the read include the loop and a partial passenger strand sequence and can be ignored in the subsequent analysis (Figure 8). Using an alignment tool, such as Bowtie 2,<sup>3</sup> align the first 22 bases of each read to the reference file provided with your lentiviral pooled library. Bowtie 2 has an option to ignore bases on the 3' end of the read; use this option to align only the first 22 bases of the guide strand. Using the output of the aligner, count the number of alignments for each construct. Next, use a differential expression tool built for discrete count data, such as DESeq,<sup>4</sup> to determine primary hits. Hits can be confirmed and studied further using individual constructs that can be ordered from the [SMARTvector Lentiviral shRNA](#) or [shMIMIC Lentiviral microRNA](#) collections.

## 8 Appendix

### A. Laboratory protocols and calculation tracking worksheet

An electronic version of this worksheet is available for download from the Dharmacon web page under the Resources section ([Pooled Screening Protocol Tracking Worksheet](#)).

#### Dharmacon™ SMARTvector™ Lentiviral shRNA Pooled Library and shMIMIC™ Lentiviral microRNA Pooled Library Laboratory protocols and calculation tracking worksheet

This laboratory protocols and calculation tracking worksheet is provided as a benchtop guide for using this product. We strongly recommend that you thoroughly read through the technical manual before using this guide. In this guide we refer to the term **construct** to denote shRNA or shMIMIC microRNA constructs. This guide is numbered to correspond to Technical Manual sections.

INPUT your values into light blue boxes

OUTPUT values are displayed in light grey boxes

#### 3 Required materials for lentiviral pooled screening

Determine materials required

#### 4 Assay development and optimization

##### A. Selection of the optimal promoter for your lentiviral pooled library

Select promoter and reporter option for your library and controls

##### B. 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<sup>2</sup>

##### C. Determination of functional titer

SMARTvector or shMIMIC Lentiviral Pooled Library titer as provided in Certificate of Analysis (C of A):  TU/mL

SMARTvector Non-targeting Control titer as provided in Certificate of Analysis (C of A):  TU/mL

- The day before transduction seed a 96-well cell culture plate (Destination Plate) with your cells at  cells/mm<sup>2</sup>
- Make dilution medium using  % FBS and  µg/mL Polybrene.  
 Make dilutions of SMARTvector Non-targeting 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.
  - 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.
  - Thaw SMARTvector Non-targeting 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.
  - 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.
  - Repeat transfer of 20 µL for columns 2 through 8, mixing 10-15 times for each dilution.
  - Allow lentiviral particle-Polybrene complexes to form for 3-5 minutes at room temperature.
- Remove culture medium from the cells in the 96-well plate.
- 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.
- Incubate the cells for  hours.
- Add 75 µL of normal growth medium to cells.
- Culture cells for 48-72 hours.
- Choose one well in the destination 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-targeting control in your cell line:

$$\text{TurboGFP-positive colonies} \times \text{Dilution factor} \div \text{Volume of lentiviral particles} = \text{Functional titer}$$

$$\text{TurboGFP-positive colonies} \times \text{dilution factor} \div 0.025 \text{ mL} = \text{TU/mL functional titer}$$

**Relative transduction efficiency of your cell line:**

$$\frac{\text{Functional titer of non-targeting control in your cell line}}{\text{Titer of non-targeting control in C of A}} = \text{Relative transduction efficiency of your cell line}$$

\_\_\_\_\_ TU/mL functional ÷ \_\_\_\_\_ TU/mL = \_\_\_\_\_ relative transduction efficiency

**Functional titer in your cell line (calculate for each lentiviral pooled library):**

$$\text{Relative transduction efficiency of your cell line} \times \text{Titer of the lentiviral pool in C of A} = \text{Relative functional titer in your cell line}$$

\_\_\_\_\_ relative transduction efficiency × \_\_\_\_\_ TU/mL = \_\_\_\_\_ TU/mL relative functional titer

**D. 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 (0-10 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  
**Days of antibiotic selection:** \_\_\_\_\_ days

**E. Determination of 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.

**F. Selection of average fold representation 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 (> 1 recommended)  
**Number of constructs per pooled library:** \_\_\_\_\_ constructs

**G. Calculation of number of cells needed for transduction**

**Desired number of cells with lentiviral integrants (calculate for each lentiviral pooled library):**

$$\text{Number of constructs in the lentiviral pool} \times \text{Desired fold representation} = \text{Desired number of cells with lentiviral integrations}$$

\_\_\_\_\_ constructs × \_\_\_\_\_ fold representation = \_\_\_\_\_ cells with lentiviral integrations

**Desired MOI:** \_\_\_\_\_

**Number of cells required at the time of transduction (calculate for each lentiviral pooled library):**

$$\frac{\text{Desired number of cells with lentiviral integrations}}{\text{Proportion of cells with lentiviral integrations}} = \text{Number of cells required at the time of transduction}$$

\_\_\_\_\_ cells with lentiviral integrations ÷ \_\_\_\_\_ proportion of cells with integrations = \_\_\_\_\_ cells required at transduction

**Number of plates required per sample (calculate for each lentiviral pooled library):**

$$\frac{\text{Cells required at transduction}}{\text{Cell density at transduction}} \div \frac{\text{Size of plate (mm}^2\text{)}}{\text{mm}^2\text{ per plate}} = \text{Number of plates required per sample}$$

\_\_\_\_\_ cells required at transduction ÷ \_\_\_\_\_ cells/mm<sup>2</sup> ÷ \_\_\_\_\_ mm<sup>2</sup> per plate = \_\_\_\_\_ plates per sample

**Number of plates required per pooled library (calculate for each lentiviral pool):**

$$\text{Number of plates required per sample} \times \text{Number of biological replicates} = \text{Number of plates required per pool}$$

\_\_\_\_\_ plates required per sample × \_\_\_\_\_ biological replicates = \_\_\_\_\_ plates per pool



## H. Calculation of volume of lentiviral particles needed for transduction

**Transducing units of lentiviral particles (calculate for each lentiviral pooled library):**

$$\begin{array}{|c|} \hline \text{Desired MOI} \\ \hline \end{array} \times \begin{array}{|c|} \hline \text{Number of cells required at the time of transduction} \\ \hline \end{array} = \begin{array}{|c|} \hline \text{Required number of lentiviral particles (TU)} \\ \hline \end{array}$$

$$\text{MOI} \times \text{cells required at transduction} = \text{TU}$$

**Volume of lentiviral particles per sample (calculate for each lentiviral pooled library):**

$$\begin{array}{|c|} \hline \text{Required number of lentiviral particles (TU)} \\ \hline \end{array} \div \begin{array}{|c|} \hline \text{Functional titer in your cell line (TU/mL)} \\ \hline \end{array} = \begin{array}{|c|} \hline \text{Volume of lentiviral particles per biological replicate (mL)} \\ \hline \end{array}$$

$$\text{TU} \div \text{TU/mL functional titer} = \text{mL lentiviral particles}$$

**Volume of lentiviral particles per pool (calculate for each lentiviral pooled library):**

$$\begin{array}{|c|} \hline \text{Volume of lentiviral particles per biological replicate (mL)} \\ \hline \end{array} \times \begin{array}{|c|} \hline \text{Number of biological replicates} \\ \hline \end{array} = \begin{array}{|c|} \hline \text{Volume of lentiviral particles per pool (mL)} \\ \hline \end{array}$$

$$\text{mL lentiviral particles} \times \text{biological replicates} = \text{mL lentiviral particles per pool}$$

## 5. Primary screen

### A. Cell transduction and selection

#### Day 1

1. Seed cells in normal growth medium. Incubate overnight.

#### Day 2

2. 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 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.

#### Day 4-8

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.

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 construct 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. Combine gDNA isolations after elution, as needed.
2. Assess DNA purity and quantify the isolated gDNA by spectrophotometry.

### C. PCR amplification of constructs from genomic DNA

#### i. Number of PCR reactions

**Amount of gDNA required to maintain fold representation (calculate for each lentiviral pooled library):**

$$\begin{array}{|c|} \hline \text{Number of cells with lentiviral integrants} \\ \hline \end{array} \times \begin{array}{|c|} \hline \text{Nanograms per genome} \\ \hline \end{array} = \begin{array}{|c|} \hline \text{Mass of gDNA required to maintain representation of each construct} \\ \hline \end{array}$$

$$\text{cells with lentiviral integrants} \times 6.6 \times 10^{-3} \text{ ng/genome}^* = \text{ng gDNA} \quad * \text{ assumes human diploid cell}$$

**Number of PCR reactions per sample (calculate for each lentiviral pooled library):**

$$\begin{array}{|c|} \hline \text{Mass of gDNA required to maintain representation of each construct} \\ \hline \end{array} \div \begin{array}{|c|} \hline \text{ng per reaction} \\ \hline \end{array} = \begin{array}{|c|} \hline \text{Number of PCR reactions required to maintain representation of each construct} \\ \hline \end{array}$$

$$\text{ng gDNA} \div 825 \text{ ng/reaction} = \text{PCR reactions per sample}$$

**Number of PCR reactions per pool (calculate for each lentiviral pooled library):**

$$\begin{array}{|c|} \hline \text{Number of PCR reactions per sample} \\ \hline \end{array} \times \begin{array}{|c|} \hline \text{Number of samples per pool} \\ \hline \end{array} = \begin{array}{|c|} \hline \text{Number of PCR reactions per pool} \\ \hline \end{array}$$

$$\text{PCR reactions per sample} \times \text{samples per pool} = \text{PCR reactions per pool}$$

Units of Phusion HotStart II DNA Polymerase required per pool (calculate for each lentiviral pooled library):

$$\boxed{\text{Number of PCR reactions per pool}} \times \boxed{4 \text{ units of polymerase per PCR}} = \boxed{\text{Units of polymerase per pool}}$$

$$\boxed{\text{PCR reactions per pool}} \times 4 \text{ units/PCR} = \boxed{\text{units Phusion HSII}}$$

ii. Multiplexing of high-throughput sequencing samples

Sequencing reads per sample

$$\boxed{\text{Number of constructs in lentiviral pool}} \times \boxed{\text{Read coverage per construct}} = \boxed{\text{Output reads required per sample}}$$

$$\boxed{\text{constructs}} \times 1000 \text{ reads/construct} = \boxed{\text{output reads required per sample}}$$

Sample indices per sequencing lane

$$\boxed{\text{Expected deep sequencing read output}} \times \boxed{0.7 \text{ (number of reads adjustment)}} \div \boxed{\text{Output reads required per sample}} = \boxed{\text{Sample indices per lane}}$$

$$\boxed{\text{reads}} \times 0.7 \div \boxed{\text{output reads per sample}} = \boxed{\text{sample indices per lane}}$$

iii. PCR from genomic DNA

1. PCR components for construct amplification (calculate Master Mix for each sample)

Component	Volume per reaction (μL)	Final Concentration	Master Mix (μL)	# of reactions per sample (rounded up to nearest whole number)
5x Phusion HF Buffer	10	1x		
10 mM dNTPs	1	200 μM each		
SMARTvector Forward PCR Primer (50 μM)	0.5	0.5 μM		
SMARTvector Reverse Index PCR Primer (50 μM)	0.5	0.5 μM		
5 M Betaine	5	0.5 M		
gDNA (825 ng) + PCR grade H <sub>2</sub> O	31	16.5 ng/μL		
Phusion Hot Start II DNA polymerase (2 U/μL)	2	0.08 U/μL		
<b>Total</b>	<b>50 μL</b>			

2. PCR cycling conditions

Cycles	Temperature	Time
1	98 °C	3 minutes
23	98 °C	10 seconds
	60 °C	15 seconds
	72 °C	15 seconds
1	72 °C	5 minutes

3. Combine reactions amplifying the same gDNA sample into a single 1.5 mL tube.  
Confirm that a 237-base pair amplicon is achieved from each sample by running 10 μL of PCR product on a 2% agarose gel.
4. Purify PCR products.
5. Evaluate purified amplicons using the quality standards recommended by your Illumina platform.

## 6. Illumina platform sequencing

Follow the manufacturer's instructions for Illumina platform sequencing.  
Load Illumina flow cell with amplicon samples (we recommend 7-10 pM using standard loading volumes).  
Obtain at least 22 base single-end reads with the provided **SMARTvector Read 1 Sequencing Primer**.  
Perform index read with **SMARTvector Index Read Sequencing Primer**.

## 7. Hit identification and follow up

Bin each index tag.  
Trim sequences to 22 base pairs.  
Align sequence reads with FASTA files provided with your lentiviral pooled library.  
Count the number of alignments for each construct that correspond to a gene-targeting shRNA construct or a shMIMIC microRNA.  
Perform differential expression analysis to determine primary hits.

## B. Key terms and definitions

**Antibiotic Drug Selection** SMARTvector Lentiviral shRNA and shMIMIC Lentiviral miRNA constructs contain the puromycin resistance gene that allows selection of transduced cells. Following transduction, cells can be selected and clonal populations can be derived.

**Puromycin Resistance Gene (Puro<sup>R</sup>)** The puromycin resistance gene is a mammalian selectable marker gene that blocks the ability of the antibiotic puromycin to inhibit protein synthesis through ribosomal binding.

**Multiplicity of Infection (MOI)** Multiplicity of infection is the ratio of lentiviral transducing particles to cells. An MOI of 10 indicates that there are 10 transducing units (TU) for every cell in the well. It is important to note that different cell types require different MOIs for successful transduction.

**Polybrene** Hexadimethrine bromide, also referred to as Polybrene, has been shown to enhance transduction of mammalian cells two- to ten-fold by binding to the cell surface and neutralizing surface charge.

**Transduction Efficiency** This term refers to the fraction of cells that successfully integrate one or more lentiviral genomes during the transduction process.

**Transducing Unit** Unit of measure that refers to the number of lentiviral particles that can transduce a population of cells and integrate into the host genome. Titers are expressed as transducing units per milliliter (TU/mL).

**Lentiviral Titer** This is the number of transducing units (TU) present in a given volume (1 mL). Titer can be assessed by a number of techniques including antibody- and PCR-based methodologies, flow cytometry and observation of transgene expression following transduction of cells with serial dilutions of lentiviral particles.

**Long Terminal Repeat (LTR)** The long terminal repeat is the control center for retroviral gene expression. All of the requisite signals for gene expression are found in the LTRs: enhancer, promoter, transcription initiation (capping), transcription terminator and polyadenylation signal. Expression directed by the viral LTR signals is carried out entirely by host cell enzymes (RNA polymerase II, poly A synthetase, guanylyl transferase).

**Self-Inactivating (SIN) Vector** Self-inactivating lentiviral vectors are constructed by deleting the transcriptional enhancers and/or the enhancers and promoter in the U3 region of the 3' LTR. After one round of vector replication, these changes are copied into both the 5' and the 3' LTRs, producing inactive lentiviral particles that are replication-incompetent.

**Vesicular Stomatitis Virus Glycoprotein (VSVg)** VSVg is an envelope protein derived from vesicular stomatitis lentiviral particles (VSV), which has previously been shown to provide broad tropism.

**Precursor microRNA (pre-miRNA)** A stem-loop RNA resulting from microprocessor complex processing of the primary microRNA transcript; precursors are shuttled out of the nucleus into the cytoplasm for further enzymatic processing by Dicer to yield mature microRNAs.

**Primary microRNA (pri-miRNA)** The capped and polyadenylated RNA product of transcription of a microRNA gene

in the nucleus, which contains local hairpin structures with imperfect base-pairing through the stem and, through enzymatic processing by the microprocessor complex, yields the precursor microRNA.

### C. Stability and storage

Lentiviral pooled libraries are shipped on dry ice as 25  $\mu$ L aliquots and must be stored at  $-80^{\circ}\text{C}$ . Under these conditions, lentiviral particles are stable for at least 12 months. Repeated freeze-thaw cycles should be avoided, as this is expected to negatively affect titer. When setting up a screening experiment using lentiviral pooled library reagents, lentiviral particles should be thawed on ice, divided into smaller aliquots (if necessary) and immediately returned to  $-80^{\circ}\text{C}$ .

### D. Quality assurance and control

SMARTvector Lentiviral shRNA and shMIMIC Lentiviral microRNA Pooled Libraries are subject to stringent quality control at multiple steps during the manufacturing process, including:

1. Evaluation of construct representation by high-throughput sequencing on Illumina sequencing platform:  
Lentiviral pool master library glycerol stocks are sequenced using  $2 \times 100$  base reads, filtered by PHRED score  $> 30$ , and  $> 1000$ -fold read coverage. Pools have greater than 95% of the constituent constructs recovered. Greater than 80% of the guide strand sequences are perfect. The library has high standards for uniform construct distribution with 70% and 90% of the population within 10-fold and 25-fold of each other, respectively. A spreadsheet data file with construct annotation, sequence information and counts per million of mapped reads is provided with each screening library.
2. Lentiviral titering using quantitative PCR (qPCR) after transduction in HEK293T cells for functional titers.
3. Confirmation of fluorescent reporter expression (TurboGFP or TurboRFP) when present by fluorescence microscopy following transduction in HEK293T cells.
4. Examination of each batch to ensure preparations are free from mold and bacterial contamination.
5. A Certificate of Analysis (C of A) with specified lentiviral titers is included with each shipment.

## 9 Frequently asked questions

### How many screenings can I perform with my pools?

The number of screenings which can be performed per lentiviral pool will depend on:

- 1) The transducibility of your specific cell line/cell type;
- 2) The fold representation you choose to maintain throughout the screening; and
- 3) The number of biological replicates you intend to include.

### Is target gene down-regulation by the SMARTvector Lentiviral shRNA or shMIMIC Lentiviral microRNA transient or permanent?

Lentiviral constructs, by way of lentiviral particle delivery of the engineered vector, integrate their payload into the host cell genome. When cells are maintained under puromycin selection and as they divide, each recombinant genome is replicated and transferred to the daughter cell progeny and can permanently express the RNAi construct (shRNA or shMIMIC microRNA). Thus, expression of the RNAi construct is stable and permanent in these cells. However, RNAi biology is complex and long-term down-regulation of putative gene targets depends on whether or not there is redundancy or involvement in feedback loops. Also, cell-dependent epigenetic regulatory mechanisms may affect the long term expression.

### How do I know if my target cell type can be transduced with a SMARTvector Lentiviral shRNA or shMIMIC Lentiviral microRNA construct?

SMARTvector and shMIMIC lentiviral constructs are pseudotyped with the VSVg envelope protein to allow broad tropism across many cells. Therefore, most cell types are transduced, albeit, with varying degrees of efficiency. However, the efficiency of target gene regulation is not only a factor of successful transduction, but is also dependent upon the efficiency of transgene expression; this can be assessed by utilizing the SMARTchoice Promoter Selection Plate (Cat #SP-001000-01) with the cells of interest. This will allow the simultaneous evaluation of seven SMARTchoice promoters.

### Can SMARTvector Lentiviral shRNA or shMIMIC Lentiviral microRNA particles be further propagated in the lab?

No. SMARTvector and shMIMIC lentiviral constructs are engineered for biosafety and are provided as replication-incompetent lentiviral particles.

### Where can I find the sequences of the mature microRNAs?

We provide mature microRNA sequences in the library data file, provided on a USB drive which was shipped with your pooled library. You can also download mature microRNA sequences used to design the shMIMIC Lentiviral microRNAs from the miRBase database website: <ftp://mirbase.org/pub/mirbase>

### Can I use the SMARTchoice Promoter Selection Plate to optimize all of the transduction conditions for my cells of interest?

No. The SMARTchoice Promoter Selection Plate is not meant to be used to establish fundamental lentiviral transduction conditions, such as optimal cell density, in your cells of interest. The purpose of the Promoter Selection Plate is to visually assess relative promoter activity. Basic experimental conditions (such as cell density, whether or not to include serum, Polybrene concentration) should first be established prior to using the Promoter Selection Plate. The Promoter Selection Plate contains duplicate wells of serially diluted lentiviral particles representing each promoter so that two separate



conditions can be tested simultaneously. After identification of the most active promoter for your cells of interest, additional transduction optimization should be performed using control lentiviral particles.

**I think I know the best promoter for my cells of interest. Do I still need to evaluate promoter activity with the SMARTchoice Promoter Selection Plate or SMARTchoice Inducible Non-targeting Control 4-pack?**

Promoter activity varies in different cell lines and cell types. This may affect the expression of the shRNA or shMIMIC microRNA construct and, subsequently, the level of target gene regulation that can be achieved. You may choose the promoter option that has been demonstrated, either by your own experimental observations or through references in the published literature, to actively express a transgene in your cells of choice. However, for optimal experimental confidence we recommend selecting the best promoter empirically using the SMARTchoice Promoter Selection Plate or Inducible Non-targeting Control 4-pack.

**I have used the human CMV promoter previously for my mouse cell line with low performance. Will a different promoter increase shRNA or shMIMIC microRNA function?**

Poor performance may be due to low transduction efficiency or suboptimal promoter activity. Optimization of transduction conditions and using promoters with optimal activity in your cells are essential to successful gene regulation. Promoter activity varies in different cell lines and cell types, and we highly recommend using the SMARTchoice Promoter Selection Plate or SMARTchoice Inducible Non-targeting Control 4-pack to determine the most active promoter in your cells of interest. For example, the mouse CMV promoter is very active in NIH/3T3 cells and results in greater gene silencing compared to the same construct expressed from the human CMV promoter in this cell line.

**I am using human cell lines in my research; should I always choose a human promoter for best activity?**

In some instances promoter activity correlates with the species from which it is derived. However, promoter activity does not always follow a species-specific expression pattern. For example, we have observed mouse promoters to be the most active in some human cell lines, whereas both human and mouse promoters were most active in some rat cells. Choosing the most effective promoter in a particular cell line is not always predictable, and therefore should be determined empirically.

**What quality control is performed on SMARTvector Lentiviral shRNA and shMIMIC Lentiviral microRNA Pooled Libraries?**

SMARTvector and shMIMIC lentiviral pools undergo multiple levels of analysis throughout production to ensure the quality of the final product. Pooled library glycerol stocks are subjected to high-throughput sequencing using  $2 \times 100$  base reads, filtered by PHRED score  $> 30$ , and  $> 1000$ -fold read coverage. Pools have greater than 95% of the constructs recovered. Greater than 80% of the guide strand sequences are perfect. The library has high standards for uniform construct distribution with 70% and 90% of the population within 10-fold and 25-fold of each other, respectively. A data file with NGS QC metrics is provided with each screening library. All components of the manufacturing process are carefully monitored for bacterial and/or fungal contamination. Following packaging, lentiviral particles are titered by qPCR.

**Are Dharmacon lentiviral particle products safe to use in the laboratory? What precautions should be taken when handling lentiviral particles?**

Lentiviral delivery systems have been employed in many research laboratories around the world without incident.



Handling of lentiviral products requires extensive experience with cell culture techniques. It is vital that the protocols provided by Dharmacon and the safety guidelines described for appropriate handling and storage are fully understood and followed precisely (see Section 11, Lentiviral particle product safety level information).

**How are Dharmacon lentiviral particle products manufactured?**

High-titer lentiviral particle products are manufactured using the Trans-Lentiviral Packaging System to achieve high titers as well as maximal biosafety. The transfer vector plasmid encoding the small RNA and the Trans-Lentiviral Packaging plasmids are co-transfected into the HEK293T cell line. Subsequently, lentiviral particles are harvested, concentrated and purified. All Dharmacon lentiviral particles are manufactured under stringent quality control guidelines (see Appendix).

**How are lentiviral particle products shipped?**

Lentiviral particle products are shipped on dry ice for overnight domestic delivery or priority international for delivery outside of the U.S.

**How should lentiviral particle products be stored?**

All lentiviral particle products must be stored at -80 °C. If necessary, the particles can be aliquoted upon the first thaw to convenient volumes and the aliquots stored at -80 °C to minimize the number future freeze-thaws. However, we recommend avoiding multiple freeze-thaw cycles as much as possible.

**What is the shelf-life of Dharmacon lentiviral particle products?**

Lentiviral particles can be stored at -80 °C for at least 12 months without loss of titer. Our internal stability testing has shown that if stored properly undisturbed, lentiviral particles can be used years after being produced without significant loss of titer. Each thaw can reduce titer by an order of magnitude, so multiple freeze-thaws should be avoided as much as possible.

**Can lentiviral particle products be kept at 4 °C?**

No. Once thawed, lentiviral particle preparations begin to decrease in titer. We advise against storing any lentiviral particles at 4 °C. All lentiviral particle products must be kept frozen and stored at -80 °C until transductions are performed.

**Where can I find titer information for my pools?**

The titer of your lentiviral pools will be indicated on the Certificate of Analysis (C of A). C of As for purchased SMARTvector Lentiviral shRNA and shMIMIC Lentiviral microRNA Pooled Libraries can also be requested from Technical Support.

**Where can I find the sequence of an individual constructs included in my pool(s)?**

The insert sequences are in the material sent with your shipment. Please contact Technical Support with your Purchase Order number if you did not receive this material in your shipment.

**How is gDNA input calculated to maintain desired construct fold representation?**

The quantity of gDNA required for maintaining a desired fold representation can be calculated using the formulas and values (Table 10) below.



This calculation is for a diploid genome. Many cell lines are not diploid and calculations should be adjusted accordingly.

**Table 10.** Constants needed for calculating gDNA input.

Constants	Value
Mass of a base pair	660 g/mol/bp
Base pairs per diploid human genome	$6 \times 10^9$ bp
Base pairs per diploid mouse genome	$5.6 \times 10^9$ bp
Avogadro constant	$6.02 \times 10^{23}$ mol <sup>-1</sup>

**Example:**

Calculate the mass of human genome:

$$(6 \times 10^9 \text{ bp/genome}) \times (660 \text{ g/mol/bp}) = 4 \times 10^{12} \text{ g/mol/genome}$$

$$(4 \times 10^{12} \text{ g/mol/genome}) \div (6.02 \times 10^{23} \text{ mol}^{-1}) = 6.6 \times 10^{-12} \text{ g/genome}$$

## 10 References

1. R.H. Kutner, X.-Y. Zhang, and J. Reiser. Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. *Nat. Protoc.* **4**, 495-505 (2009).
2. Ž. Strezoska, *et al.* Optimized PCR conditions and increased shRNA fold representation improve reproducibility of pooled shRNA screens. *PLoS One.* **7**, e42341 (2012).
3. B. Langmead and S. Salzberg. Fast gapped-read alignment with Bowtie 2. *Nat. Methods.* **9**, 357-359 (2012).
4. S. Anders and W. Huber. Differential expression analysis for sequence count data. *Genome Biol.* **11**, R106 (2010).

## 11 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. It is applicable to the following lentiviral particle products:

SMARTvector™ Lentiviral shRNA

SMARTvector™ Inducible Lentiviral shRNA

shMIMIC Lentiviral microRNA

shMIMIC Inducible Lentiviral microRNA

SMARTchoice™ Promoter Selection Plate

SMARTvector™ Non-targeting Control

SMARTvector™ Inducible Non-targeting Control

SMARTvector™ Lentiviral shRNA Pooled Library

SMARTvector™ Inducible Lentiviral shRNA Pooled Library

shMIMIC Lentiviral microRNA Pooled Library

shMIMIC Inducible Lentiviral microRNA Pooled Library



**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 use and the acceptance of replication-incompetent SIN lentiviral vectors and replication-defective lentiviral particles into their local jurisdiction and institution.**

The Products 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).

For questions concerning the design or production of the products, please contact our technical support team.

Dharmacon™, A Horizon Discovery Group Company

2650 Crescent Drive

Lafayette, CO 80026 USA

Tel: +1 800 235 9880; +1 303 604 9499

Fax: +1 800 292 6088; +1 303 604 9680

Email: [ts.dharmacon@horizondiscovery.com](mailto:ts.dharmacon@horizondiscovery.com) (North America) or [ts.dharmacon@horizondiscovery.com](mailto:ts.dharmacon@horizondiscovery.com) (Europe)

#### **In the US:**

For US guidance on containment for lentiviral vectors, please refer to:

1. The Recombinant DNA Advisory Committee (RAC) guidelines for research with lentiviral vectors ([osp.od.nih.gov/office-biotechnology-activities/biosafety/biosafety-guidance](http://osp.od.nih.gov/office-biotechnology-activities/biosafety/biosafety-guidance)).
2. 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);
3. The NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines), ([osp.od.nih.gov/office-biotechnology-activities/biosafety/nih-guidelines](http://osp.od.nih.gov/office-biotechnology-activities/biosafety/nih-guidelines)).

#### **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
2. Council Directive 2001/18/EC of the European Parliament and of the Council of 12 March on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC.

**In Germany:**

Required Containment Measures: 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 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).

## 12 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 Licensing Statements](#). 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.

**If you have any questions contact**

**t** +44 (0) 1223 976 000 (UK) **or** +1 800 235 9880 (USA); +1 303 604 9499 (USA)

**f** +44 (0)1223 655 581

**w** [horizondiscovery.com/contact-us](http://horizondiscovery.com/contact-us) **or** [dharmacon.horizondiscovery.com/service-and-support](http://dharmacon.horizondiscovery.com/service-and-support)

**Horizon Discovery**, 8100 Cambridge Research Park, Waterbeach, Cambridge, CB25 9TL, United Kingdom

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