Dharmacon™
SMARTvector™ Lentiviral shRNA Pooled Libraries
SMARTvector™ Inducible Lentiviral shRNA Pooled Libraries
shMIMIC Lentiviral microRNA Pooled Libraries
shMIMIC Inducible Lentiviral microRNA Pooled Libraries
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>
<thead>
<tr>
<th>SMARTvector Lentiviral shRNA Pooled Library</th>
<th>Human</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of targeted genes</td>
<td>Number of pools × number of constructs per pool</td>
<td>Number of targeted genes</td>
</tr>
<tr>
<td>Whole Genome</td>
<td>19241</td>
<td>16 pools × 9870 constructs</td>
</tr>
<tr>
<td>Druggable Genome</td>
<td>7341</td>
<td>6 pools × 10080 constructs</td>
</tr>
<tr>
<td>GPCR</td>
<td>377</td>
<td>1 pool × 3384 constructs</td>
</tr>
<tr>
<td>Ion Channel</td>
<td>340</td>
<td>1 pool × 3081 constructs</td>
</tr>
<tr>
<td>Protein Kinase</td>
<td>702</td>
<td>1 pool × 5942 constructs</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>245</td>
<td>1 pool × 2312 constructs</td>
</tr>
<tr>
<td>Protease</td>
<td>466</td>
<td>1 pool × 4074 constructs</td>
</tr>
<tr>
<td>Ubiquitin Conjugate</td>
<td>557</td>
<td>1 pool × 4795 constructs</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>shMIMIC Lentiviral microRNA Pooled Library</th>
<th>Number of mature microRNAs</th>
<th>Number of unique designs</th>
<th>Number of pools × number of constructs per pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>2580</td>
<td>2555</td>
<td>1 pool × 2665 constructs</td>
</tr>
<tr>
<td>Mouse</td>
<td>1913</td>
<td>1896</td>
<td>1 pool × 2066 constructs</td>
</tr>
<tr>
<td>Human-Mouse Conserved</td>
<td>355 human + 341 mouse</td>
<td>386</td>
<td>1 pool × 496 constructs</td>
</tr>
</tbody>
</table>

1Libraries 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).
2Constitutive and inducible lentiviral pooled libraries are provided at ≥ 5 × 10⁸ TU/mL ± 20% and ≥ 5 × 10⁷ ± 20% TU/mL, respectively.
3Additional gene families are available on the website and by request [ts.dharmacon@ge.com].
4The number of genes and constructs per pool are subject to change at any time without notice.
5Number of constructs per pool includes gene-specific and negative control constructs.
6Custom libraries targeting rat gene families or researcher-defined human, mouse or rat gene lists are available by request [ts.dharmacon@ge.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), 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).
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 Dharmaco web page (dharmaco.gelifesciences.com/uploadedFiles/Resources/pooled-screening-protocol-tracking-worksheet.xls). 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: dharmaco.gelifesciences.com/shrna/smartvector-lentiviral-controls/
  - Catalog numbers for gene-specific and negative controls with an inducible promoter may be found here: dharmaco.gelifesciences.com/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 #M0535K).

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, hEF1α, mEF1α, 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 ([dharmarcon.gelifesciences.com/uploadedFiles/Resources/smartvector-constitutive-manual.pdf](dharmarcon.gelifesciences.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 EF1α and mouse EF1α; 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 ([dharmarcon.gelifesciences.com/uploadedFiles/Resources/smartvector-inducible-lentiviral-shRNA-manual.pdf](dharmarcon.gelifesciences.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 ([dharmarcon.gelifesciences.com/uploadedFiles/Resources/smartvector-constitutive-manual.pdf](dharmarcon.gelifesciences.com/uploadedFiles/Resources/smartvector-constitutive-manual.pdf)). For inducible lentiviral vector options consult the SMARTvector Inducible Lentiviral shRNA Technical Manual ([dharmarcon.gelifesciences.com/uploadedFiles/Resources/smartvector-inducible-lentiviral-shRNA-manual.pdf](dharmarcon.gelifesciences.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 ([dharmaco.gelifesciences.com/uploadedfiles/resources/titer-crystal-violet-protocol.pdf](http://dharmaco.gelifesciences.com/uploadedfiles/resources/titer-crystal-violet-protocol.pdf)) or genomic qPCR assay.1

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 μL of each dilution of lentiviral particles from the Dilution Plate to the corresponding wells in the Destination Plate, being careful to not create bubbles.

5. Incubate the cells for 4-24 hours (as determined during transduction optimization).

6. Add 75 μL of normal growth medium to cells.

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.

Table 2. Example setup for lentiviral particle dilution series.

<table>
<thead>
<tr>
<th>Well</th>
<th>Lentiviral particle serial dilution volume</th>
<th>Volume of dilution medium</th>
<th>Dilution factor</th>
<th>Volume of diluted lentiviral particles used in transduction (Destination Plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>10 μL (*control)</td>
<td>40 μL</td>
<td>5</td>
<td>25 μL</td>
</tr>
<tr>
<td>A2</td>
<td>20 μL (from A1)</td>
<td>80 μL</td>
<td>25</td>
<td>25 μL</td>
</tr>
<tr>
<td>A3</td>
<td>20 μL (from A2)</td>
<td>80 μL</td>
<td>125</td>
<td>25 μL</td>
</tr>
<tr>
<td>A4</td>
<td>20 μL (from A3)</td>
<td>80 μL</td>
<td>625</td>
<td>25 μL</td>
</tr>
<tr>
<td>A5</td>
<td>20 μL (from A4)</td>
<td>80 μL</td>
<td>3125</td>
<td>25 μL</td>
</tr>
<tr>
<td>A6</td>
<td>20 μL (from A5)</td>
<td>80 μL</td>
<td>15 625</td>
<td>25 μL</td>
</tr>
<tr>
<td>A7</td>
<td>20 μL (from A6)</td>
<td>80 μL</td>
<td>78 125</td>
<td>25 μL</td>
</tr>
<tr>
<td>A8</td>
<td>20 μL (from A7)</td>
<td>80 μL</td>
<td>390 625</td>
<td>25 μL</td>
</tr>
</tbody>
</table>

* SMARTvector Non-targeting Control Particles. Repeat identical dilution series in wells B1 to B8.
Functional titer in transducing units per mL (TU/mL) can be determined using the following formula:

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

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

\[
\frac{\text{Functional titer of SMARTvector Non-targeting Control Particles stock in your cell line (TU/mL)}}{\text{Titer of SMARTvector Non-targeting Control Particles stock reported on the C of A (TU/mL)}} = \text{Relative transduction efficiency of your cell line}
\]

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:

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

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:

\[
58 \text{ (GFP/RFP positive colonies)} \times 78125 \text{ (dilution factor)} \div 0.025 \text{ (volume of diluted lentiviral particles in mL)} = 1.8 \times 10^8 \text{ TU/mL functional titer of SMARTvector Non-targeting Control Particles in your cell line}
\]

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.

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 ≤ 0.3.

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.

### 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 22% 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:

\[
5.0 \times 10^5 \text{ (lentiviral integration events required)} \div 0.22 \text{ (proportion of cells that undergo at least one integration)} = 2.3 \times 10^6 \text{ cells needed at the time of transduction}
\]

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

<table>
<thead>
<tr>
<th>MOI</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.90</td>
<td>0.09</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.2</td>
<td>0.82</td>
<td>0.16</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.3</td>
<td>0.74</td>
<td>0.22</td>
<td>0.03</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.4</td>
<td>0.67</td>
<td>0.27</td>
<td>0.05</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>0.5</td>
<td>0.61</td>
<td>0.30</td>
<td>0.08</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>0.6</td>
<td>0.55</td>
<td>0.33</td>
<td>0.10</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>0.7</td>
<td>0.50</td>
<td>0.35</td>
<td>0.12</td>
<td>0.03</td>
<td>0.00</td>
</tr>
<tr>
<td>0.8</td>
<td>0.45</td>
<td>0.36</td>
<td>0.14</td>
<td>0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>0.9</td>
<td>0.41</td>
<td>0.37</td>
<td>0.16</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>1.0</td>
<td>0.37</td>
<td>0.37</td>
<td>0.18</td>
<td>0.06</td>
<td>0.02</td>
</tr>
</tbody>
</table>

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:

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

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:

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

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/mm².

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/mm² and an effective surface area on 100 mm plates of 7800 mm².

<table>
<thead>
<tr>
<th>Construct fold representation</th>
<th>Number of lentiviral integrations required</th>
<th>Number of cells required at transduction</th>
<th>Recommended cell plating</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>$5 \times 10^5$</td>
<td>$2.3 \times 10^4$</td>
<td>1 plate × 100 mm</td>
</tr>
<tr>
<td>1000</td>
<td>$1 \times 10^5$</td>
<td>$4.5 \times 10^4$</td>
<td>2 plates × 100 mm</td>
</tr>
</tbody>
</table>

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 μL 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:

$$\text{MOI} \times \frac{\text{Number of cells required at the time of transduction}}{\text{Volume of lentiviral particles per biological replicate (mL)}} = \frac{\text{Required number of lentiviral particles (TU)}}{\text{Number of biological replicates}}$$

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

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

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

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

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:

$$0.3 \times 2.0 \times 10^6 \text{ cells} = 6.0 \times 10^4 \text{ TU of lentiviral particles are required for transduction}$$

If you have determined that you need $6.0 \times 10^4$ TU and you have a functional titer of $1.0 \times 10^9$ TU/mL in your cell line, you can calculate the volume of lentiviral particles needed as follows:

$$6.0 \times 10^4 \text{ TU} \div 1.0 \times 10^9 \text{ TU/mL} = 0.006 \text{ mL of lentiviral particles}$$

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

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

**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.

<table>
<thead>
<tr>
<th>Construct fold representation</th>
<th>Relative transduction efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>500</td>
<td>1.2 µL</td>
</tr>
<tr>
<td>1000</td>
<td>2.4 µL</td>
</tr>
</tbody>
</table>
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.

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).
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.

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.

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.

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.

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 $A_{260/230}$. 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.²

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:

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

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.
Experimental gDNA

Reference gDNA

Illumina-adopted SMARTvector Forward PCR Primer

shRNA or microRNA Construct

Pro-viral Sequence

Amplify construct with Indexed Illumina Adapted Primers

Indexed amplicon pool (237 bp)

Column purify verify amplicon length

SMARTvector Read 1 Illumina Sequencing Primer

SMARTvector Read 1 and Index Read Illumina Sequencing Primers

Perform high-throughput sequencing with SMARTvector Read 1 and Index Read Illumina Sequencing Primers

Illumina flow cell

Sequence Reads in FASTQ format

Read X: CTCAGATCAAAGTCTATAGCCTAAGTGAAGCCACAGATGTATAGACTTTG
Read Y: ACAGATCAAGCTTGAGTGAAGATGTAATCAATTTGAAGNNA
Read Z: ACAGGAGGTCAAGGAAAAGTGAAGCCACACACAGATGTATTGATTTTC

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:**

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

* 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.

<table>
<thead>
<tr>
<th>Construct fold representation</th>
<th>Input gDNA (μg)</th>
<th>Number of PCR reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>3.3</td>
<td>4</td>
</tr>
<tr>
<td>1000</td>
<td>6.6</td>
<td>8</td>
</tr>
</tbody>
</table>

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:

\(5 \times 10^5\) lentiviral integrants \(\times 6.6 \times 10^{-3}\) ng/genome \(= 3.3 \times 10^2\) ng

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

\(3.3 \times 10^2\) ng gDNA \(\div 825\) ng/PCR \(= 4\) PCR reactions

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

\(4\) PCR reactions \(\times 4\) samples \(= 16\) PCR reactions

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

\(16\) PCR reactions \(\times 4\) units of Phusion polymerase/PCR \(= 64\) 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:

\[
\text{Number of constructs in lentiviral pool} \times \frac{1000 \text{ reads coverage per construct}}{0.7 (\text{number of reads adjustment})} \times \frac{\text{Output reads required per sample}}{\text{Output reads required per sample}} = \text{Number of sample indices per lane}
\]

**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 μM 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.

<table>
<thead>
<tr>
<th>Item</th>
<th>Index</th>
<th>Index Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kit A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMARTvector Reverse Index PCR Primer 2</td>
<td>2</td>
<td>CGATGT</td>
</tr>
<tr>
<td>SMARTvector Reverse Index PCR Primer 4</td>
<td>4</td>
<td>TGACCA</td>
</tr>
<tr>
<td>SMARTvector Reverse Index PCR Primer 5</td>
<td>5</td>
<td>ACAGTG</td>
</tr>
<tr>
<td>SMARTvector Reverse Index PCR Primer 6</td>
<td>6</td>
<td>GCCAAT</td>
</tr>
<tr>
<td>SMARTvector Reverse Index PCR Primer 7</td>
<td>7</td>
<td>CAGATC</td>
</tr>
<tr>
<td>SMARTvector Reverse Index PCR Primer 12</td>
<td>12</td>
<td>CTTGTA</td>
</tr>
<tr>
<td>SMARTvector Reverse Index PCR Primer 13</td>
<td>13</td>
<td>AGTCAA</td>
</tr>
<tr>
<td>SMARTvector Reverse Index PCR Primer 14</td>
<td>14</td>
<td>AGTCCC</td>
</tr>
<tr>
<td>SMARTvector Reverse Index PCR Primer 15</td>
<td>15</td>
<td>ATGTCA</td>
</tr>
</tbody>
</table>
### 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 μL reactions using 96-well PCR plates. PCR conditions have been optimized to amplify 825 ng of gDNA per 50 μL reaction. As described above, calculate the number of reactions needed for the amplification of total gDNA necessary to maintain the fold representation used at transduction. Table 8 provides the components and volumes needed for one 50 μL PCR amplification reaction.

Table 8. PCR components for amplification of genomic DNA.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Phusion HF Buffer</td>
<td>10 μL</td>
<td>1x</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1 μL</td>
<td>200 μM each</td>
</tr>
<tr>
<td>SMARTvector Forward PCR Primer (50 μM)</td>
<td>0.5 μL</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>SMARTvector Reverse Index PCR Primer (50 μM)</td>
<td>0.5 μL</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>5 M betaine</td>
<td>5 μL</td>
<td>0.5 M</td>
</tr>
<tr>
<td>gDNA + PCR grade H2O</td>
<td>31 μL</td>
<td>~ 825 ng/reaction</td>
</tr>
<tr>
<td>Phusion Hot Start II DNA Polymerase (2 U/μL)</td>
<td>2 μL</td>
<td>0.08 U/μL</td>
</tr>
<tr>
<td>Total</td>
<td>50 μL</td>
<td>**</td>
</tr>
</tbody>
</table>
2. 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

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>98 °C</td>
<td>3 minutes</td>
<td></td>
</tr>
<tr>
<td>98 °C</td>
<td>10 seconds</td>
<td></td>
</tr>
<tr>
<td>60 °C</td>
<td>15 seconds</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>15 seconds</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>5 minutes</td>
<td></td>
</tr>
</tbody>
</table>

3. 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.

4. 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 µM.

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).

SMARTvector Read1 Illumina Sequencing Primer
SMARTvector Index Read Illumina Sequencing Primer

50 base read

22 bases 13 bases 15 bases
guide or mature strand loop Passenger strand

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.gelifesciences.com/uploadedFiles/Resources/lentiviral-pooled-library-bioinfomatic-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, 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, 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].
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. Optimality 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: __________

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):

\[ \text{Number of constructs in the lentiviral pool} \times \frac{1}{\text{Desired fold representation}} = \text{Desired number of cells with lentiviral integrations} \]

Desired MOI: __________

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

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

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

\[ \frac{\text{Cells required at transduction} \times \text{Cell density at transduction}}{\text{Size of plate (mm²)}} = \text{Number of plates required per sample} \]

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

\[ \text{Number of plates required per sample} \times \text{Number of biological replicates} = \text{Number of plates required per pool} \]
H. Calculation of volume of lentiviral particles needed for transduction

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

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

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

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

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

\[
\text{TU/mL functional titer} = \text{mL lentiviral particles}
\]

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

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

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

5. Primary screen

A. Cell transduction and selection

Day 1


Day 2

2. Remove the growth medium and add medium with \% FBS, \(\text{ug/mL Polybrene}\) 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. \text{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

\[
\text{Amount of gDNA required to maintain fold representation (calculate for each lentiviral pooled library):}
\]

\[
\text{Number of cells with lentiviral integrants} \times \text{Nanograms per genome} = \text{Mass of gDNA required to maintain representation of each construct}
\]

\[
\text{cells with lentiviral integrants} \times 6.6 \times 10^7 \text{ ng/genome} = \text{ng gDNA}
\]

\* assumes human diploid cell

\[
\text{Number of PCR reactions per sample (calculate for each lentiviral pooled library):}
\]

\[
\text{Mass of gDNA required to maintain representation of each construct} + \text{ng per reaction} = \text{Number of PCR reactions required to maintain representation of each construct}
\]

\[
\text{ng gDNA} + 825 \text{ ng/reaction} = \text{PCR reactions per sample}
\]

\[
\text{Number of PCR reactions per pool (calculate for each lentiviral pooled library):}
\]

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

\[
\text{PCR reactions per sample} \times \text{samples per pool} = \text{PCR reactions per pool}
\]
Units of Phusion HotStart II DNA Polymerase required per pool (calculate for each lentiviral pooled library):

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

\[
\text{PCR reactions per pool} \times 4 \text{ units/PCR} = \text{units Phusion HSII}
\]

ii. Multiplexing of high-throughput sequencing samples

\[
\text{Sequence reads per sample} = \frac{\text{Number of constructs in lentiviral pool}}{1000 \text{ reads/construct}} = \text{output reads required per sample}
\]

\[
\text{Sample indices per sequencing lane} = \text{output reads per sample} + 0.7 \times \text{number of reads adjustment} = \text{Sample indices per lane}
\]

iii. PCR from genomic DNA

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (µL)</th>
<th>Final Concentration</th>
<th>Master Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Phusion HF Buffer</td>
<td>10</td>
<td>1×</td>
<td></td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1</td>
<td>200 µM each</td>
<td></td>
</tr>
<tr>
<td>SMARTvector Forward PCR Primer (50 µM)</td>
<td>0.5</td>
<td>0.5 µM</td>
<td></td>
</tr>
<tr>
<td>SMARTvector Reverse Index PCR Primer (50 µM)</td>
<td>0.5</td>
<td>0.5 µM</td>
<td></td>
</tr>
<tr>
<td>5 M Betaine</td>
<td>5</td>
<td>0.5 M</td>
<td></td>
</tr>
<tr>
<td>gDNA 1 µg + PCR grade H2O</td>
<td>31</td>
<td>16.5 ng/µL</td>
<td></td>
</tr>
<tr>
<td>Phusion Hot Start II DNA polymerase (2 U/µL)</td>
<td>2</td>
<td>0.08 U/µL</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50 µL</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. PCR cycling conditions

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98 °C</td>
<td>3 minutes</td>
</tr>
<tr>
<td>23</td>
<td>98 °C</td>
<td>10 seconds</td>
</tr>
<tr>
<td></td>
<td>60 °C</td>
<td>15 seconds</td>
</tr>
<tr>
<td></td>
<td>72 °C</td>
<td>15 seconds</td>
</tr>
<tr>
<td>1</td>
<td>72 °C</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

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.

Purify PCR products.

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)** 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 μL aliquots and must be stored at -80 °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 °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 × 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.
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 x 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.
10 **References**


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

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

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

**Example:**

Calculate the mass of human genome:

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

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

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

GE Healthcare Dharmacon, Inc.
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@ge.com (North America) or TS.Dharmacon.eu@ge.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](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);

In the EU:
For the EU directives, please consult the following:


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](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 http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-us/about-us/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.

Illumina™, HiSeq™, MiSeq™, and TruSeq™ are trademarks or registered trademarks of Illumina, Inc. Limited permission to copy or distribute sequence information. Oligonucleotide sequences ©2007-2011 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina Instruments and products only. All other uses are strictly prohibited. Tet-On 3G is a trademark of Clontech Laboratories, Inc. TurboGFP and TurboRFP are trademarks of Evrogen Inc. Phusion is a trademark of Thermo Fisher Scientific, Inc. GE, imagination at work and GE monogram are trademarks of General Electric Company. Dharmacon is a trademark of GE Healthcare companies. All other trademarks are the property of General Electric Company or one of its subsidiaries. ©2015 General Electric Company—All rights reserved. First published June 2015. GE Healthcare UK Limited, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK.