



Technical Manual

Screening

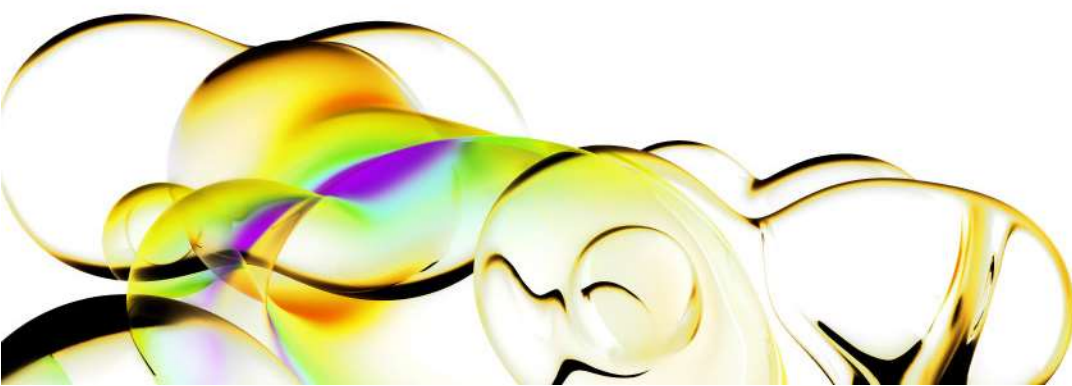
Dharmacon™ CRISPRmod™ CRISPRa All-in-One Lentiviral sgRNA Pooled Libraries

Storage : -80°C

Volume : 100 µL per vial

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1 Introduction

A. CRISPR-Cas: An adaptive immunity defense mechanism in bacteria and archaea

The CRISPR (clustered regularly interspaced palindromic repeats)-Cas (CRISPR-associated protein) system is an adaptive bacterial and archaeal defense mechanism that serves to recognize and silence incoming foreign nucleic acids. Upon infection by bacteriophage or other foreign DNA elements, a host organism can incorporate short sequences from the invading genetic material, called protospacers, into a specific region of its genome (the CRISPR locus) between short palindromic DNA repeats of variable lengths. Multiple spacer-repeat units are clustered at the CRISPR locus to form the CRISPR array. The entire locus including the CRISPR array is transcribed by RNA polymerase into a primary transcript, the pre-CRISPR RNA (pre-crRNA), which is then processed into small, mature CRISPR RNAs (crRNAs) such that they include sequences complementary to the foreign, invading DNA. crRNAs guide a multifunctional protein or protein complex (the CRISPR-associated or Cas proteins) to cleave complementary target DNA that is adjacent to short sequences known as protospacer-adjacent motifs (PAMs). Thus, the organism acquires a way to protect itself from subsequent infection¹.

B. CRISPR-Cas9 platform for transcriptional activation in mammalian cells

In addition to genome engineering applications in mammalian cells², the *Streptococcus pyogenes* CRISPR-Cas9 system has been adapted to technologies for transcriptional regulation^{3,4,5}. The nuclease activity of the *S. pyogenes* Cas9 was abolished by point mutations introduced into two catalytic residues (D10A and H840A) yielding a deactivated Cas9 (dCas9) that maintains the ability to bind to target DNA when guided by sequence-specific guide RNAs. When the dCas9 is fused to transcriptional regulators and guided to gene promoter regions, it induces RNA-directed transcriptional regulation. CRISPR-Cas9 based technologies for transcriptional regulation include CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa).

CRISPRa utilizes dCas9 fused to different transcriptional activation domains^{5,6,7,8}, which can be directed to promoter regions by specifically designed guide RNA (depicted in Figure 1). The VPR activation system utilizes a fusion of three transcriptional activators (VP64, p65 and Rta) to the C-terminal end of dCas9 and demonstrates robust gene activation in mammalian systems⁸. Unlike other CRISPRa systems that require expression of the transcriptional activators from separate vectors, the VPR system requires delivery of just two components to the cells of interest: dCas9-VPR and a guide RNA, making it easier to utilize across different biological applications.

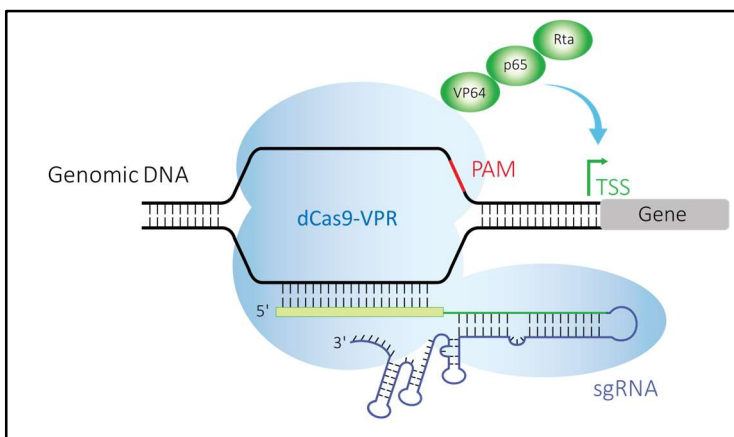


Figure 1. Illustration of dCas9-VPR with sgRNA targeting a gene's promoter region

2 CRISPRmod CRISPRa All-in-one lentiviral pooled screening platform

CRISPRmod CRISPRa All-in-one lentiviral pooled screening libraries are pools of lentiviral constructs expressing sgRNAs targeting coding genes for transcriptional activation. Gene overexpression pooled screening can be performed to identify the function of a coding gene in the regulation of cellular responses and signaling pathways without the need for costly automation and equipment required for arrayed screening. The CRISPRmod CRISPRa All-in-One Lentiviral vectors include the two critical components based on the natural *S. pyogenes* system, the VPR-fused dCas9 effector and the gene-specific sgRNA. CRISPRmod CRISPRa Lentiviral sgRNA Pooled Screening Libraries are comprised of four or eight pre-designed gene-

specific sgRNAs per gene and 50 or 100 non-targeting controls for hit normalization. All libraries are delivered with a data file containing complete library information including gene annotations, sgRNA target sequences, complete list of controls, and counts per millions of mapped reads obtained from high-throughput sequencing of library prior to lentiviral packaging.

A. CRISPRmod CRISPRa All-in-one Lentiviral vector

The CRISPRmod CRISPRa All-in-one Lentiviral vectors contain a human codon-optimized version of the *S. pyogenes* dCas9 gene fused to the VPR activation system and the puromycin resistance marker (Puro^R). Both are expressed as a bicistronic transcript with a 2A peptide sequence linker under the control of a single promoter (Figure 2). The gene-specific sgRNA is expressed under the control of a human U6 promoter. A brief description of the lentiviral vector elements is listed in Table 1. Two promoter options are available (Figure 2) for selection of a vector with the most active promoter for specific cells of interest.

Each CRISPRmod CRISPRa All-in-one Lentiviral sgRNA is specific to the gene of choice. CRISPRmod All-in-one Lentiviral sgRNAs are designed based on a published CRISPRa algorithm⁹ and target genomic DNA in the proximity of transcriptional start site (TSS). When more than one TSS exists for a gene, a second set of sgRNA reagents is available (labeled P2). The crRNA region of the sgRNA is comprised of 19-20 nucleotides identical to the genomic DNA target site, followed by the non-variable sgRNA scaffold containing the tracrRNA sequence from *S. pyogenes*. The optimized sgRNA scaffold used in the CRISPRmod CRISPRa All-in-one Lentiviral sgRNA can further improve gene activation efficiency.

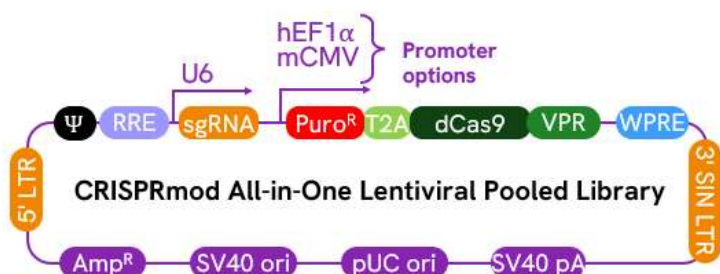


Figure 2. Schematic map of the CRISPRmod CRISPRa All-in-one Lentiviral vector.

Table 1. Elements of the CRISPRmod CRISPRa All-in-one Lentiviral vectors.

Vector element	Utility
dCas9-VPR	<i>S. pyogenes</i> dCas9-VPR for gene activation of targeted DNA when programmed with a guide RNA
T2A	Self-cleaving peptide allows for simultaneous expression of puromycin resistance and dCas9-VPR from a single transcript
Puro ^R	Puromycin resistance marker permits antibiotic selection of transduced mammalian cells
mCMV	Mouse cytomegalovirus immediate early promoter
hEF1α	Human elongation factor 1 alpha short promoter
U6	Human RNA polymerase III promoter U6
sgRNA	Optimized single guide RNA, a fusion of gene-specific crRNA with the tracrRNA scaffold
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
SV40 pA	Simian virus 40 polyadenylation signal
pUC ori	pUC origin of replication
SV40 ori	Simian virus 40 origin of replication
Amp ^R	Ampicillin resistance gene

B. Overview of CRISPRmod CRISPRa All-in-one Lentiviral Pooled Library screening workflow

Once basic transduction and assay conditions have been optimized, cells are transduced at a low multiplicity of infection (MOI) with a lentiviral pooled library (see Figure 3 for a screening workflow diagram). Following transduction and puromycin enrichment for sgRNA-expressing cells, 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 sgRNA construct are either enriched or depleted in the cell population.

To identify “hits,” gDNA is isolated from the initial transduced cell population (reference sample) and from the transduced cell population that remained following the application of selective pressure and/or phenotypic selection (experimental sample). sgRNA constructs within the isolated gDNA are amplified using vector-specific, Illumina-compatible NGS Hit Identification Primers that have been designed and optimized to minimize amplification bias and introduce diversity into the high-throughput sequencing run. Amplicons can then be indexed and prepared for high-throughput sequencing on an Illumina platform using commercially available Illumina TruSeq® prep kits. The differences in sgRNA construct abundance between reference and experimental cell populations can then be determined to identify hits from the primary screen. Each step of the CRISPRmod CRISPRa All-in-one Lentiviral Pooled Library workflow, from transduction to hit identification, has been empirically tested.

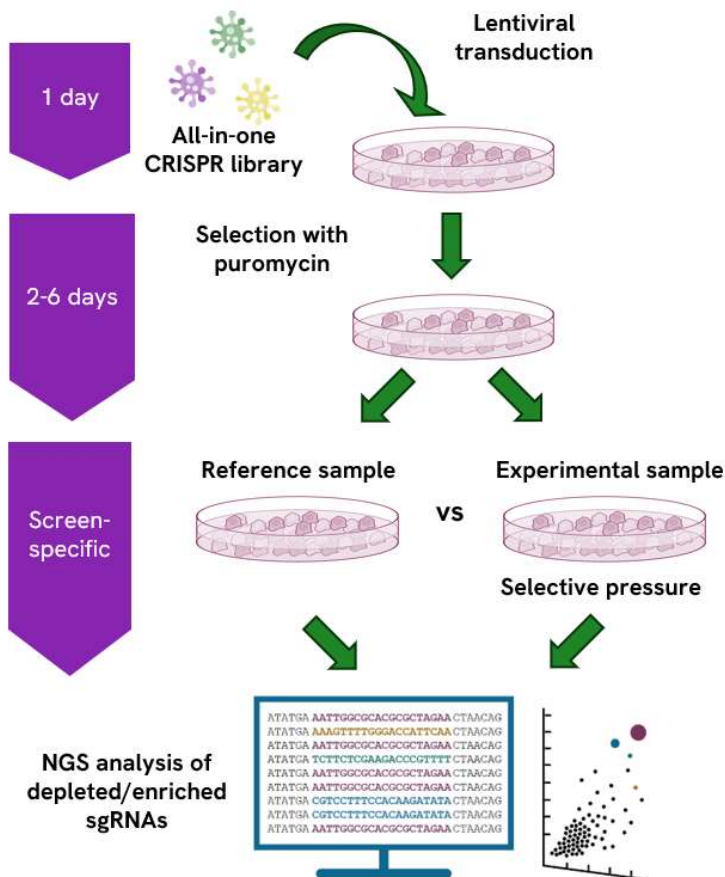


Figure 3. Gene overexpression screening workflow using the CRISPRmod CRISPRa All-in-one Lentiviral Pooled Library platform.

3 Laboratory protocols and calculation tracking worksheet

The [Pooled-sgRNA-screening-protocol-tracking-worksheet](#) is a tool for the recording of key calculations and experimental parameters. Please review all the detailed protocols provided in this manual prior to using the worksheet. An electronic version of the worksheet can be downloaded from the web page. The downloadable Excel worksheet allows users to incorporate specific input related to their screening and performs key calculations to simplify protocol planning.

4 Required materials for All-in-one lentiviral pooled library screening

A. Materials required

- CRISPRmod CRISPRa All-in-one Lentiviral Pooled Library
- NGS Hit Identification Primers
- **Optional:** CRISPRmod CRISPRa All-in-one GFP Delivery Controls

B. Additional materials required

The following additional materials are required but not supplied:

- Transduction enhancer such as LentiBOOST® (<https://sirion-biotech.com/lentiboost>)
- 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))
- Live/Dead indicator such as Zombie Dyes (BioLegend®, <https://www.biolegend.com/en-us/live-dead>)
- HyClone™ Puromycin 2 HCl (Cytiva, Cat #SV30075.01 or similar)
- Phusion Hot Start II High-Fidelity DNA Polymerase and 5x Phusion HF Buffer (Thermo Scientific, Cat #F-549L; NEB, Cat #M0535L)
- Genomic DNA purification kit (Blood and Cell Culture DNA Purification Kit, Qiagen, Cat #13362 or similar)
- PCR purification kit (QIAquick™ PCR Purification Kit, Qiagen, Cat #28104 or similar)
- Nucleotide mix dNTP set, 100 mM each (NEB, Cat # N0446S or similar)

5 Assay development and optimization

Please use the [Pooled-sgRNA-screening-protocol-tracking-worksheet](#) as a guide.

A. Selection of the optimal promoter for dCas9-VPR expression

Expression levels of dCas9-VPR can have a significant effect on gene activation efficiency. Therefore, select the CRISPRmod CRISPRa All-in-one lentiviral vector with the most active promoter in your cell line based on known promoter activity or use the CRISPRmod CRISPRa All-in-one GFP Delivery Controls to empirically determine the optimal promoter.

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 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.

CRISPRmod CRISPRa All-in-one Lentiviral Pooled Library vectors do not contain a fluorescent reporter. Therefore, CRISPRmod CRISPRa All-in-one GFP Delivery Control lentiviral particles (see Section 4.A. Materials required) co-expressing EGFP can be substituted for rapid optimization of transduction conditions. Use conditions that produce the lowest or no loss of cell viability. 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) or transduction enhancers such as LentiBOOST® may be added to enhance lentiviral particle binding to the cell surface. We recommend testing a range of concentrations for identification of optimal transduction efficiency with minimal or no cell toxicity.

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

C. Determination of functional titer

The functional titers are found in the data file provided with all Dharmacon™ lentiviral pooled screening libraries. However, lentiviral transduction efficiency varies widely from one cell type to another. After determining optimal lentiviral transduction conditions, it is required to determine the functional titer in the experimental cell line of choice and calculate an MOI low enough such that the majority of cells will have single integrations.

Promoter-matched CRISPRmod CRISPRa All-in-one GFP Delivery Control lentiviral particles can be used to determine functional titer, see **Appendix A** for detailed protocols.

Once the functional titer has been determined, use the formula below to calculate the relative transduction efficiency of your cell line of interest.

$$\begin{array}{ccc} \text{Functional titer of CRISPRmod CRISPRa GFP Delivery Control in your cell line (TU/mL)} & \div & \text{Titer of CRISPRmod CRISPRa GFP Delivery Control reported on the C of A (TU/mL)} \\ & & = \\ & & \text{Relative transduction efficiency of your cell line} \end{array}$$

Use the calculated relative transduction efficiency of your cell line to then calculate the relative functional titer for the CRISPRmod CRISPRa All-in-one Lentiviral Pooled Library using the following formula:

$$\begin{array}{ccc} \text{Relative transduction efficiency of your cell line} & \times & \text{Titer of the pooled library reported in data file (TU/mL)} \\ & & = \\ & & \text{Relative functional titer of the pooled library in your cell line (TU/mL)} \end{array}$$

Calculation example

If the functional titer for the CRISPRmod CRISPRa All-in-one GFP Delivery Control particles in your cells of interest is determined to be 2.1×10^7 TU/mL and the titer on the C of A is listed as 5.0×10^7 TU/mL, then the relative transduction efficiency of your cell type is calculated as follows:

$$2.1 \times 10^7 \text{ TU/mL (functional titer in your cell line)} \div 5.0 \times 10^7 \text{ TU/mL (titer reported on the C of A)} = 0.42 \text{ relative transduction efficiency}$$

If the relative transduction efficiency of your cell line is 0.42 and the titer of a CRISPRmod CRISPRa Lentiviral sgRNA Pooled Library, as indicated on the C of A, is 5.6×10^7 TU/mL, the relative functional titer of the pool in your cell line would be determined as follows:

$$0.42 \text{ (relative transduction efficiency)} \times 5.6 \times 10^7 \text{ TU/mL (titer reported on the C of A)} = 2.35 \times 10^7 \text{ TU/mL relative functional titer in your cell line}$$

D. Optimization of puromycin selection

The CRISPRmod CRISPRa All-in-one Lentiviral Pooled Library vectors contain the puromycin resistance marker for selection of cells after transduction. Prior to treating cells with puromycin, determine the optimal concentration of antibiotic required to kill non-transduced cells. This concentration can be identified by generating an [antibiotic kill curve](#). The puromycin concentration range for many mammalian cells is 0.5-10 $\mu\text{g/mL}$.

Day 1:

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

Day 2:

2. Change the medium to Selection Medium (Growth Medium supplemented with the antibiotic being tested) at a range of concentrations.

Days 4-7:

3. Approximately every 2-3 days replace medium with freshly prepared Selection Medium.

4. Monitor the cells daily and visually observe the percentage of surviving cells. Optimum effectiveness should be reached in 2-6 days under puromycin selection.

5. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 2-6 days from the start of antibiotic selection.

E. Determination of assay-specific screening conditions

The pooled 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), and method of phenotypic selection (for example, measuring viability, proliferation, surface marker expression or migration). We recommend that you optimize all assay conditions prior to beginning a lentiviral sgRNA pooled library screen using, if possible, a lentiviral sgRNA construct targeting a gene 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 sgRNA or the number of biological replicates of each sgRNA integration event and subsequent phenotypic selection. High sgRNA fold representation results in improved reproducibility between biological replicates and ensures that there is a sufficient window for detection of changes in sgRNA abundance¹⁰.

Recommendations:

- Between 250 and 1000 independent integrations per sgRNA (*i.e.*, 250- to 1000-fold representation)
- At least two biological replicates

We have observed that 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 sgRNA 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.

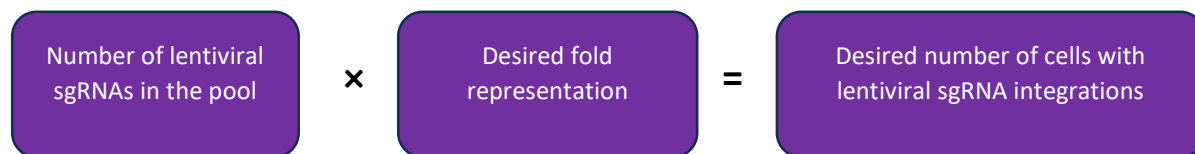
Multiplicity of infection (MOI) is defined as the ratio of transducing units of lentiviral particles to target cells. At high MOIs, each cell will likely be transduced by more than one lentiviral particle. Conversely, at low MOIs, the probability that any cell is transduced with more than one lentiviral particle is lower (Table 2). For lentiviral pooled screens, each cell will ideally express a single sgRNA. This ensures that the overexpression of a single gene in a particular cell is responsible for the resulting phenotype. Therefore, we recommend performing lentiviral transductions at an MOI ≤ 0.3 .

Table 2. Poisson distribution showing the distribution of cells with the indicated number of lentiviral integrations at various MOIs.

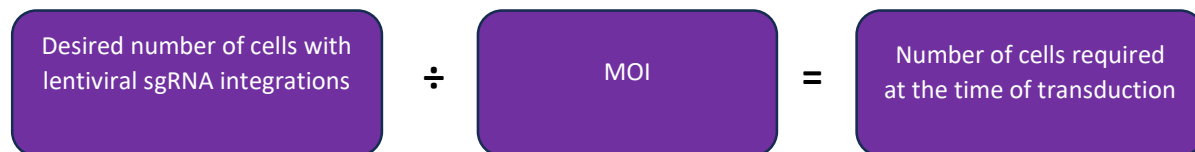
MOI	Number of lentiviral integrants per cell				
	0	1	2	3	4
0.1	0.90	0.09	0.00	0.00	0.00
0.2	0.82	0.16	0.02	0.00	0.00
0.3	0.74	0.22	0.03	0.00	0.00
0.4	0.67	0.27	0.05	0.01	0.00
0.5	0.61	0.30	0.08	0.01	0.00
0.6	0.55	0.33	0.10	0.02	0.00
0.7	0.50	0.35	0.12	0.03	0.00
0.8	0.45	0.36	0.14	0.04	0.01
0.9	0.41	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 sgRNA fold representation, use the following formula:



Use the following formula to calculate the number of cells you will need per sample at the time of transduction:



Please note: An MOI of 0.3 or lower is recommended for this library. In the equation above, MOI is used as a simplification for the proportion of cells with lentiviral integrants (shown in Table 3).

Calculation example

If you have a pool of 82000 sgRNAs and you wish to achieve a 200-fold average representation, the desired number of lentiviral integration events would be calculated as follows:

$$82000 \text{ (number of sgRNA)} \times 200 \text{ (average representation)} = 1.64 \times 10^7 \text{ lentiviral integration events}$$

Then calculate the number of cells needed at the time of transduction as follows:

$$1.64 \times 10^7 \text{ (lentiviral integration events required)} \div 0.3 \text{ (maximum recommended MOI)} = 5.5 \times 10^7 \text{ cells needed at the time of transduction}$$

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 that 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, use the following formula:

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

Table 3 provides examples of cell and plate numbers and plate sizes for various targeted sgRNA fold representations, assuming a pool size of 82000 sgRNAs and a cell line with optimal transduction density of 250 cells/mm².

Table 3. Number of cells required at the time of transduction and cell plating recommendations for indicated average fold representation assuming a whole genome pool of 82000 sgRNAs, MOI of 0.3, optimal transduction density of 250 cells/mm² and an effective surface area on 100 mm plates of 7800 mm².

Fold representation	Number of lentiviral integrations required	Number of cells required at transduction	Recommended cell plating
200	1.64×10^7	5.5×10^7	28 plates \times 100 mm
400	3.3×10^7	10.9×10^8	52 plates \times 100 mm

H. Calculation of volume of lentiviral particles needed for transduction

Before proceeding with the lentiviral sgRNA 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 your screen. 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 the screen, use the following formula:

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

Calculation example

For example, at an MOI of 0.3 and a 200-fold average sgRNA representation at transduction, you will have prepared 5.5×10^7 cells at the time of transduction. Based on the above formula, calculate the number of lentiviral particles needed:

$$0.3 \text{ (MOI)} \times 5.5 \times 10^7 \text{ cells} = 1.64 \times 10^7 \text{ TU of lentiviral particles are required for transduction}$$

If you have determined that you need 1.64×10^7 TU and you have a functional titer of 2.35×10^7 TU/mL in your cell line, you can calculate the volume of lentiviral particles needed as follows:

$$1.64 \times 10^7 \text{ TU} \div 2.35 \times 10^7 \text{ TU/mL} = 0.7 \text{ mL of lentiviral particles}$$

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

$$0.7 \text{ mL of lentiviral particles} \times 2 \text{ biological replicates} = 1.4 \text{ mL of lentiviral particles}$$

6 Primary screen

The following sections describe the primary screening workflow (Figure 3). Please use the [Pooled-sgRNA-screening-protocol-tracking-worksheet](#) as a guide.

A. Cell transductions and selection screen

The experimental conditions described here 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 sgRNA fold representation should be determined specifically for each cell line of interest and each intended screening experiment, as outlined in **Section 5**. Similarly, conditions should be clearly defined prior to starting the screen for application of selective pressure and/or sorting of cells exhibiting the phenotype of interest.

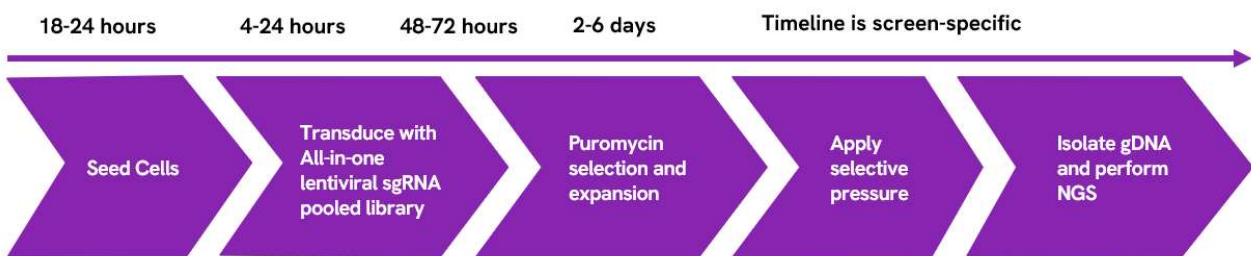


Figure 4. Timeline of primary screen.

Day 1:

1. Seed cells in Growth Medium in the number of plates determined in **Section 5.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 **Section 5.B**.) with the appropriate volume of lentiviral particles (**Section 5.H**.) so that the cells are just covered. If the lentiviral pool will be added to multiple plates, as determined in Section 5, divide the volume of lentiviral particles evenly among plates.

2. After the appropriate transduction time (see **Section 5.B.**), add additional Growth Medium to your cells such that the cells can be incubated for 48-72 hours.

Days 4-18:

1. At 48-72 hours post-transduction, begin puromycin selection to remove non-transduced cells.
2. Every 48-72 hours, replace with fresh Selection 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 5.D.).

Once a pure population of puromycin-resistant cells has been obtained (2-6 days), begin screening. Split cells into at least two populations: one as a reference and another (experimental) for application of selective pressure and/or sorting of cells exhibiting the phenotype of interest. To maintain your desired sgRNA fold representation 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 a **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 sgRNA 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.

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 230 nm ($A_{260/230}$). 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 step described here has been designed to amplify sgRNA constructs from gDNA without bias, such that differences in sgRNA 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 desired fold representation at transduction is maintained. This ensures assay reproducibility and facilitates hit identification¹⁰. Note that the NGS Hit Identification Primers have been designed to add partial TruSeq® adapters designed for use with Illumina® sequencing platforms. They are not compatible with other high-throughput sequencing systems.

i. Number of PCR reactions

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

Number of cells with
lentiviral integrations

×

6.6×10^{-3} ng/ genome (see
note below)

=

Mass of gDNA required to
maintain representation of
each sgRNA (ng)

The calculation above assumes a diploid human genome. For cells that are not diploid, calculations should be adjusted accordingly.

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 PCRs required for each sample using the following formula:

Mass of gDNA required to maintain representation of each sgRNA (ng)

÷

825 ng per reaction

=

Number of PCR reactions required to maintain representation of each sgRNA (ng)*

* Round up the value obtained to the next whole number

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

Number of PCR reactions per sample

×

Number of samples

=

Total number of PCR reactions

Calculation example

If you anticipate 1.64×10^7 cells with lentiviral integrations, the mass of gDNA required would be calculated as follows:

$$1.64 \times 10^7 \text{ lentiviral integrations} \times 6.6 \times 10^{-3} \text{ ng/genome} = 1.8 \times 10^5 \text{ ng}$$

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

$$1.8 \times 10^5 \text{ ng gDNA} \div 825 \text{ ng/PCR} = 131 \text{ PCR reactions}$$

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

$$131 \text{ PCR reactions} \times 4 \text{ samples} = 524 \text{ PCR reactions}$$

ii. PCR from genomic DNA

Once you have determined the number of PCR reactions you need per sample, you can proceed with the PCR amplification of the gDNA samples. Sequence amplification from gDNA has been optimized using Phusion Hot Start II DNA Polymerase. The amplification conditions recommended below have been determined to provide maximum yields of PCR product while staying within the linear phase of log amplification.

1. Perform PCR in 50 μ L reactions using 96-well PCR plates. PCR conditions have been optimized to amplify 825 ng of gDNA per 50 μ 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 4 provides the components and volumes needed for one 50 μ L PCR amplification reaction.

Table 4. PCR components for amplification of genomic DNA.

Component	Volume per reaction (μL)	Final concentration
5x Phusion HF Buffer	10	1x
10 mM dNTPs	1	200 μM
NGS Hit Identification Primers* (50 μM)	1	1 μM
gDNA (825 ng) + PCR grade H ₂ O	36	16.5 ng/μL
Phusion Hot Start II DNA Polymerase (2 U/μL)	2	0.08 U/μL
TOTAL	50 μL	

* The NGS Hit Identification reverse primers are vector-specific as they bind to a region in the promoter driving Cas9 expression. **Please ensure that you are using primers matched to the promoter driving Cas9 expression in your vector.**

2. To PCR amplify the CRISPRmod CRISPRa Lentiviral sgRNA constructs, use the cycling conditions outlined in Table 5. 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 5. PCR cycle parameters

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

3. Confirm that amplicons of the appropriate size are obtained from each sample by running 10 μL of PCR product on a 2% agarose gel. The amplicons from the mCMV promoter vector will be 328-337 bp, the amplicons from the hEF1α promoter vector will be 282-291 bp.

Due to the low number of PCR cycles, the product will be a faint band on a gel before purification. Note too that the staggered diversity primers will generate ten roughly equimolar amplicons varying by 1 bp in length.

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 fluorometric methods. Therefore, it is recommended to evaluate the concentration of each amplicon using quantitative PCR or chromatographic methods such as analysis on a Revvity LabChip Nucleic Acid Analyzer. Alternatively, the amplicons can be purified and quantified by a fluorometric DNA quantification method, such as QuBit (Thermo Fisher).

7 Library prep and Illumina platform sequencing

Illumina P5 and P7 adapters and TruSeq indexes can be added to the amplified library generated in **Section 6.C.ii** through a second low amplification PCR step prior to sequencing. Please follow the manufacturer's instructions for Illumina platform sequencing.

For accurate hit identification, we recommend obtaining a minimum of 1000 1×150 bp reads per sgRNA. 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.

8 Hit identification and follow up

Alignment of sequence files and hit identification can be performed using open-source programs that require some bioinformatics knowledge. We recommend that you consult with a bioinformatician with proficiency in using command line tools for data analysis and hit identification. A detailed protocol utilizing freely available software can be downloaded from the CRISPRmod CRISPRa All-in-one Lentiviral Pooled Library webpage under the Resources tab. Samples should be de-multiplexed on the sequencer to bin each index tag with the appropriate sample. Sequences should be trimmed so that the first 19 bases of each read correspond to the sgRNA target sequence. The remaining bases in the read include the sgRNA vector backbone and can be ignored in the subsequent analysis. Using an alignment tool, such as Bowtie 2¹¹, align the first 19 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 19 bases of the sgRNA 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 DESeq2¹², to determine primary hits. Hits can be confirmed and studied further using individual CRISPRmod CRISPRa All-in-one Lentiviral sgRNAs.

9 Appendix

A. Determination of functional titer

Functional titer can be determined either by counting EGFP-positive colonies using fluorescence microscopy or flow cytometry. The following protocol describes how to estimate functional titer via flow cytometry using CRISPRmod CRISPRa All-in-one GFP Delivery Control lentiviral particles. A protocol describing how to determine functional titer by fluorescence microscopy can be found in the appendix of the [SMARTvector Lentiviral shRNA Technical Manual](#).

1. The day before transduction, seed a 96-well cell culture plate (Destination Plate) with your cells at the density determined during transduction optimization. Grow cells overnight.
2. Make dilution medium using serum and transduction enhancer conditions determined during transduction optimization. Make dilutions of Delivery control lentiviral particles in a round-bottom 96-well plate (Dilution Plate). As shown in **Table 6**, use one row of the plate for each replicate of the dilution series of the lentiviral stock. We recommend performing two replicates. The procedure for dilution of the lentiviral stock is described below and results in a series of five-fold dilutions to reach a final dilution of 390,625-fold.
 - a. Add 40 μ L of dilution medium to wells A1 and B1. Add 80 μ L of dilution medium to each well A2-A8 and B2-B8.
 - b. Thaw Delivery control lentiviral particles on ice and then add 10 μ L each to wells A1 and B1. Mix contents of each well by pipetting up and down 10-15 times. Discard pipette tip before proceeding to the next dilutions.
 - c. Transfer 20 μ L 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 before proceeding to the next dilutions.
 - d. Repeat transfer of 20 μ L for columns 2 through 8, mixing 10-15 times for each dilution.
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).

Table 6. Example setup for lentiviral particle dilution series.

Well	Lentiviral particle serial dilution volume	Volume of dilution media	Dilution factor	Volume of diluted lentiviral particles used in transduction
A1, B1	10 μL (control particles)	40 μL	5	25 μL
A2, B2	20 μL (from A1 or B1)	80 μL	25	25 μL
A3, B3	20 μL (from A2 or B2)	80 μL	125	25 μL
A4, B4	20 μL (from A3 or B3)	80 μL	625	25 μL
A5, B5	20 μL (from A4 or B4)	80 μL	3125	25 μL
A6, B6	20 μL (from A5 or B5)	80 μL	15625	25 μL
A7, B7	20 μL (from A6 or B6)	80 μL	78125	25 μL
A8, B8	20 μL (from A7 or B7)	80 μL	390625	25 μL

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

7. Culture cells for 48-72 hours.

8. Prepare cells for flow cytometry following your laboratory's protocols. A Live/ Dead indicator such as the BioLegend® Zombie Dyes is recommended.

9. Determine the percentage of EGFP-expressing cells in each dilution by flow cytometry using populations of untransduced cells as negative controls.

To ensure that multiple integration events don't result in an underestimation of functional titer, cell populations with 5 to 25% EGFP-expressing cells should be used for analysis. Multiply the percentage of EGFP-expressing cells by the number of cells per well at the time of transduction to obtain the number of transducing units delivered to that well. Average the replicates for the selected dilution and then use the below formulas to calculate a functional titer and relative transduction efficiency.

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

$$\text{TU at selected dilution} \times \text{Dilution factor (Table 6)} \div 0.025 \text{ mL (Volume of diluted lentiviral particles used)} = \text{Functional titer of Delivery control lentiviral particles in your cell line (TU/mL)}$$

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

$$\text{Functional titer of Delivery control lentiviral particles in your cell line (TU/mL)} \div \text{Titer of Delivery control lentiviral particles in HEK293T (TU/mL)} = \text{Relative transduction efficiency of your cell line}$$

Use the calculated relative transduction efficiency of your cell line to calculate the anticipated functional titer using the following formula:

Relative transduction efficiency of your cell line

×

Titer of the lentivirus as calculated by in HEK293T cells (TU/mL)

=

Anticipated functional titer in your cell line (TU/mL)

Calculation example

If 12.3% of your cells were EGFP-positive at the 1:125 dilution (A3, B3) and you had 2×10^4 cells per well at the time of transduction, those wells were each transduced with 2460 TU of CRISPRmod CRISPRa All-in-one GFP Delivery Control particles. The functional titer for the CRISPRmod CRISPRa All-in-one GFP Delivery Control particles in your cells of interest is calculated as follows:

$$2460 \text{ TU} \times 125 \text{ (dilution factor)} \div 0.025 \text{ mL (volume of diluted particles)} = 1.23 \times 10^7 \text{ TU/mL relative functional titer}$$

If the titer on the C of A is listed as 5.0×10^7 TU/mL, then the relative transduction efficiency of your cell type is calculated as follows:

$$1.2 \times 10^7 \text{ TU/mL (functional titer in your cell line)} \div 5.0 \times 10^7 \text{ TU/mL (titer reported on the C of A)} = 0.25 \text{ relative transduction efficiency}$$

If the relative transduction efficiency of your cell line is 0.25 and the titer of an CRISPRmod CRISPRa All-in-one Lentiviral sgRNA Pooled Library, as indicated on the C of A, is 6.6×10^7 TU/mL, the relative functional titer of the pool in your cell line would be determined as follows:

$$0.25 \text{ (relative transduction efficiency)} \times 6.6 \times 10^7 \text{ TU/mL (titer reported on the C of A)} = 1.7 \times 10^7 \text{ TU/mL relative functional titer in your cell line}$$

B. Stability and storage

CRISPRmod CRISPRa All-in-one Lentiviral Pooled Libraries are shipped on dry ice and must be stored at -80°C . Under these conditions, lentiviral particles are stable for at least 3 years. Repeated freeze-thaw cycles should be avoided, as this is expected to negatively affect titer. When setting up a pooled lentiviral screening experiment, lentiviral particles should be thawed on ice, divided into smaller aliquots (if necessary) and immediately returned to -80°C .

C. Quality assurance and control

CRISPRmod CRISPRa All-in-one Lentiviral Pooled Libraries are subject to stringent quality control at multiple steps during the manufacturing process, including:

1. Evaluation of sgRNA representation by high-throughput sequencing on Illumina sequencing platform:

Lentiviral pooled libraries are sequenced using 2×250 base reads, filtered by PHRED score > 30 , and > 1000 -fold read coverage. Pools have greater than 95% of the constituent constructs recovered. 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 gene annotation, sgRNA target sequences information and counts per million mapped reads is provided with each screening library.

2. Lentiviral titering using quantitative PCR (qPCR) after transduction in HEK293T cells for functional titers.
3. Examination of each batch to ensure preparations are free from mold and bacterial contamination.
4. Specified lentiviral titers are included in data file with each shipment.

10 Frequently asked questions

How many lentiviral particles do I need for my screen?

The number of lentiviral particles (reported as transducing units (TU)) required for a screen will depend on:

- 1) The relative transduction efficiency of your specific cell line/cell type
- 2) The fold representation you choose to maintain throughout the screen
- 3) The number of biological replicates you intend to include

Once these parameters have been determined, the [Pooled-sgRNA-screening-protocol-tracking-worksheet](#) can be used to calculate the number of TU needed for your screen.

How can I determine the relative transduction efficiency of CRISPRmod CRISPRa All-in-one Lentiviral Pooled Library particles in my cells?

Relative transduction efficiency will depend on your cell type of choice and transduction conditions. The CRISPRmod CRISPRa All-in-one GFP delivery controls are matched lentiviral particles that co-express EGFP and dCas9-VPR along with a pool of non-targeting guide RNAs and thus can be used to determine the relative transduction efficiency for your cell type via a fluorescent readout. If you do not know the best conditions for transduction of your cell type of interest, these controls can also be used to optimize plating density, transduction medium, transduction medium, and the concentration of any transduction enhancers.

How do I choose between the two CRISPRmod CRISPRa All-in-one Lentiviral Pooled Library promoter options?

Choose the promoter option that has been demonstrated by your own experimental observations or shown to actively express a transgene in your cells of choice through references in the published literature. For optimal experimental confidence (or if such information is unavailable), consider testing both promoter options using the CRISPRmod CRISPRa All-in-one GFP delivery controls. Because EGFP is co-expressed with dCas9-VPR from a single bicistronic transcript, EGFP fluorescence will be directly proportional to Cas9 expression.

I am using human cell lines in my research; should I always choose a human promoter for optimal dCas9-VPR 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.

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 worldwide without incident. Handling of lentiviral products requires extensive experience with cell culture techniques. It is vital that the protocols provided by Revvity and the safety guidelines described for appropriate handling and storage are fully understood and followed precisely (see Section 12, Lentiviral particle product safety level information).

Can Dharmacon™ lentiviral particles be further propagated in the lab?

No. Dharmacon™ lentiviral products are engineered for biosafety and are provided as replication-incompetent lentiviral particles.

Can I order the CRISPRmod CRISPRa All-in-one Lentiviral Pooled libraries as plasmid DNA or glycerol stocks?

No. Our CRISPRmod CRISPRa All-in-one Lentiviral Pooled Libraries are sold exclusively as lentiviral particles.

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 of future freeze-thaws. However, we recommend avoiding multiple freeze-thaw cycles 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.

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.

Where can I find titer information for my pooled library?

The titer of your lentiviral pools will be indicated in the data file sent with your library shipment and can also be requested from [Scientific Support](#).

Where can I find the targeting sequences for sgRNAs included in my pooled library?

The sgRNA target sequences are in the data files sent with your library shipment. Please contact [Scientific Support](#) with your Sales Order number (SO-XXXXXXG) if you did not receive the data files in your shipment.

How specific are the CRISPRmod CRISPRa sgRNAs in targeting the gene of interest?

Multiple publications have shown CRISPRa to be highly specific by RNA-seq expression analysis. Remember that for CRISPRa off-targeting, the guide RNA needs to bind to the promoter region of another gene to have an off-target effect, dramatically decreasing the potential off-target space. Furthermore, the guide RNAs are designed based on published algorithms that incorporate chromatin, nucleosome position, and sequence features to accurately predict highly effective guide RNAs and apply a filter for off-target binding. However, there might be examples of genes where the promoter region for one gene is in close proximity to another gene's promoter region. Investigation of the genomic locations of the hits of your screen might be important for proper interpretation of the results.

What if a gene has more than one transcriptional start site?

The published CRISPRa algorithm⁹ used FANTOM and Ensembl databases to predict the transcriptional start site (TSS) more accurately. Some genes (6.8%) were identified as having alternative distal TSSs that could not be efficiently activated from a single locus. For the CRISPRmod CRISPRa pooled libraries, we include the top four or eight guide RNAs for the primary TSS, and, when applicable, four or eight guide RNAs for the secondary TSS. These are labeled as P1 and P2, respectively. Because which TSS is active and to what level is cell line dependent, it is possible for P1 guide RNAs but not P2 guide RNAs against a gene to be identified as hits in your screen, or vice versa.

Do the CRISPRmod CRISPRa All-in-one Lentiviral Pooled Libraries target lncRNAs or regulatory regions?

No. The libraries only contain guide RNAs targeting protein-coding genes.

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 7) below.

Table 7. Constants needed for calculating gDNA input.

Constants	Value
Mass of a base pair	660 g/mol/bp
Base pairs per diploid human genome	6×10^9 bp
Avogadro constant	6.02×10^{23} mol ⁻¹

Example:

Calculate the mass of human genome:

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

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

The calculation above assumes a diploid human genome. For cells that are not diploid, calculations should be adjusted accordingly.

How can I index and sequence the gDNA from my screen?

gDNA can be amplified, indexed, and prepared for NGS sequencing with a two-step PCR protocol. We provide vector-specific NGS hit identification primers for the first round of amplification of your gDNA. The forward and reverse primers contain the following Illumina® compatible TruSeq® partial adapters:

Forward primer extension: 5' AACTCTTCCCTACACGACGCTCTCCGATCT 3'

Reverse primer extension: 5' GTGACTGGAGTTCAGACGTGTGCTCTCCGATCT 3'

Illumina P5 and P7 adapters and TruSeq indexes can then be added to the amplified library through a second low amplification PCR step prior to sequencing. Note that the forward primers contain a stagger site between the 5' Illumina extension and the priming site to introduce nucleotide diversity during sequencing, which will need to be adjusted for when the sequences are trimmed. For more information, please see our Bioinformatic Analysis of Dharmacon™ Lentiviral sgRNA Pooled Library Screens Protocol.

11 References

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12 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 all Dharmacon™ lentiviral particle products.

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 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 Scientific Support team.

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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 (https://osp.od.nih.gov/wp-content/uploads/Lenti_Containment_Guidance.pdf);
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) (https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.pdf)

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).

13 Limited use licenses

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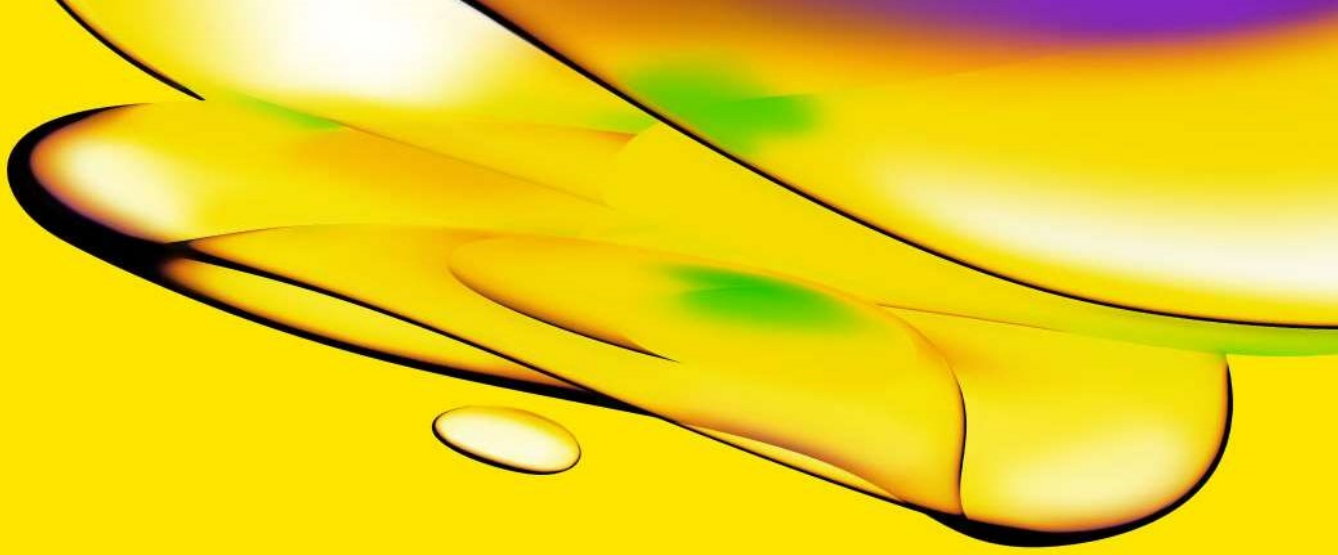
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