Technical Manual **Screening**

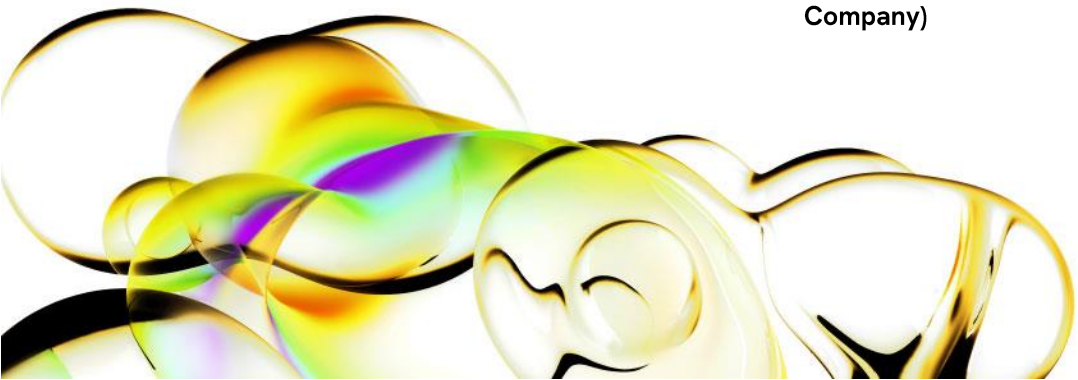
Dharmacon™ CRISPRmod CRISPRi Lentiviral sgRNA Pooled Libraries

Storage : -80°C

Version: 0001 **Revision date:** 052026

For research only. Not for use in diagnostic procedures.

Horizon Discovery Biosciences Ltd. (A Revvity Company)



Contents

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 infection[1].

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

In addition to genome engineering applications in mammalian cells[2], 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).

CRISPRi systems initially utilized the Krüppel associated box (KRAB) domain from zinc finger protein 10 (KOX1) as a transcriptional repressor[4,6] but recent publications have demonstrated improved transcriptional repression with a variety of different effectors[7,8]. Revvity's CRISPRi system utilizes a novel fusion protein comprised of domains from two human transcriptional repressors, Sal-like protein 1 (SALL1) and Sin3 histone deacetylase corepressor complex component SDS3 (SDS3), fused to the C-terminal end of dCas9[9]. Revvity's CRISPRi system was developed to provide robust and consistent gene repression across the genome.

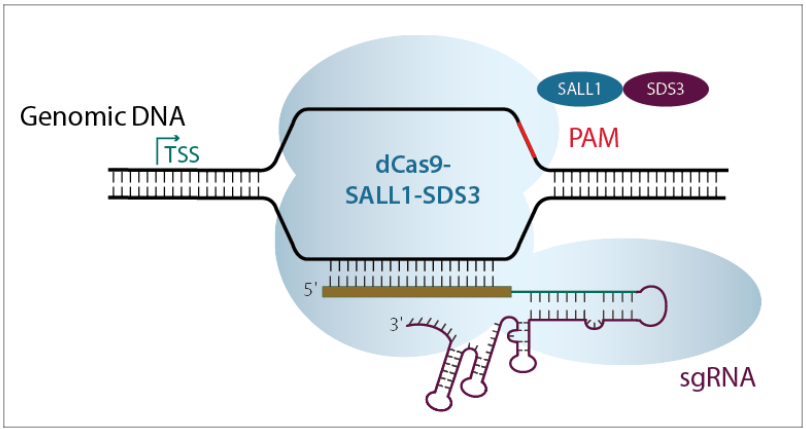


Figure 1. Illustration of dCas9-SALL1-SDS3 with sgRNA targeting the region immediately downstream of a gene's transcriptional start site (TSS).

2 CRISPRmod CRISPRi Lentiviral sgRNA Pooled Screening Platform

CRISPRmod CRISPRi Lentiviral sgRNA Pooled Libraries are pools of lentiviral constructs expressing sgRNAs targeting coding genes for repression. Loss-of-function 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. CRISPRi Lentiviral sgRNA Pooled Libraries are comprised of gene-specific sgRNAs, as well as a panel of core essential genes that serve as technical controls, and 50 or 100 non-targeting controls per pool for hit normalization. Table 1 lists the pre-defined pooled lentiviral libraries that are available. 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.

Table 1. Approximate number of targeted genes in respective human CRISPRmod CRISPRi Lentiviral sgRNA pooled libraries. Please reach out to Scientific Support for more information regarding gene composition of each library.

Lentiviral sgRNA Pooled Library	Approximate number of targeted genes	Lentiviral sgRNA Pooled Library	Approximate number of targeted genes
Whole Genome	18900	Cytokine Receptors	140
Druggable Genome	8350	Membrane Trafficking	140
GPCR	480	Deubiquitinating Enzymes	110
Ion Channel	430	Cell Cycle Regulation	170
Protein Kinase	760	Tyrosine Kinases	90
Phosphatase	310	Nuclear Receptors	50
Protease	700	Apoptosis	550
Ubiquitin Conjugate	660	DNA Damage Response	240
Epigenetics	860		

A. CRISPRmod CRISPRi dCas9-SALL1-SDS3 and sgRNA vectors

The CRISPRmod CRISPRi Lentiviral dCas9-SALL1-SDS3 vectors contain a human codon-optimized version of the *S. pyogenes* dCas9 gene fused to the SALL1-SDS3 repressor and the blasticidin resistance marker (Blast^R). Both are expressed as a bicistronic transcript with a 2A peptide sequence linker under the control of a single promoter (Figure 2). Multiple promoter options are available (Figure 2A) so that the most active promoter driving dCas9-SALL1-SDS3 expression can be chosen for specific cells of interest. In the CRISPRmod CRISPRi Lentiviral sgRNA vector backbone (Figure 2B), the gene-specific sgRNA is expressed under the control of a human U6 promoter, while expression of the puromycin resistance marker (PuroR) is driven from the mouse CMV promoter and allows for rapid selection of cells with integrated sgRNA. The functional elements for both lentiviral vectors are listed and described in Table 2.

Each CRISPRmod CRISPRi Lentiviral sgRNA is specific to the gene of choice and are designed based on a published CRISPRi algorithm[10] to 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 CRISPRi Lentiviral sgRNA can further improve gene repression efficiency.

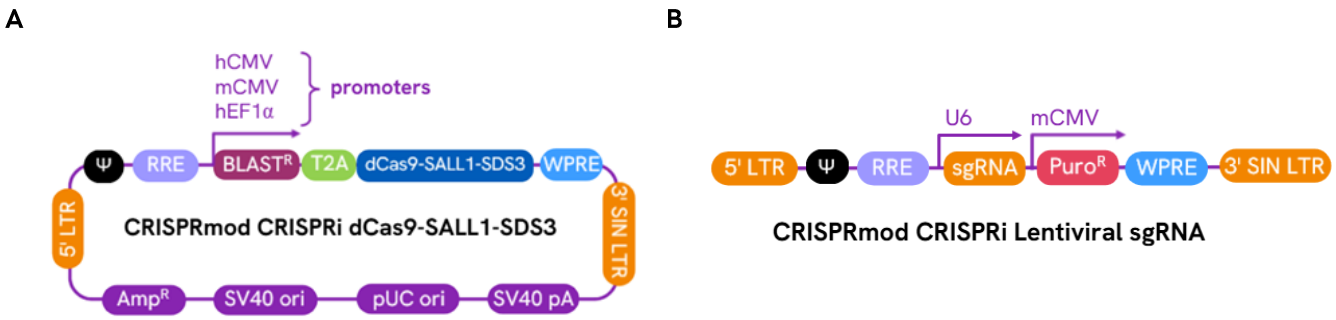


Figure 2. Schematic maps of the CRISPRmod CRISPRi dCas9-SALL1-SDS3 and Lentiviral sgRNA vectors.

Table 2. Elements of the CRISPRmod CRISPRi Lentiviral dCas9-SALL1-SDS3 and sgRNA vectors.

Vector element	Utility
dCas9-SALL1-SDS3	*S. pyogenes* dCas9-SALL1-SDS3 for transcriptional repression of targeted DNA when programmed with a guide RNA
T2A	Self-cleaving peptide allows for simultaneous expression of blasticidin resistance and dCas9-SALL1-SDS3 from a single transcript
Puro^R	Puromycin resistance marker permits antibiotic selection of transduced mammalian cells
Blast^R	Blasticidin resistance marker permits antibiotic selection of transduced mammalian cells
hCMV	Human cytomegalovirus immediate early promoter
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 the CRISPRi Lentiviral sgRNA Pooled Library screening workflow

Once basic transduction and assay conditions have been optimized, dCas9-SALL1-SDS3 expressing cell lines can easily be generated with the CRISPRmod CRISPRi dCas9-SALL1-SDS3 lentiviral particles. Cells are transduced at a low multiplicity of infection (MOI) with a lentiviral pooled library (see Figure 3 for a screening workflow diagram). Individual cells in the resulting transduced population will contain a single construct integrated into their genomes. 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 and prepared for high-throughput sequencing using Dharmacon™ NGS Library Prep Kits. These vector-specific kits have 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. These libraries can then be multiplexed and sequenced on an Illumina® platform using standard Illumina sequencing primers. 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 CRISPRi Lentiviral sgRNA Pooled Library workflow, from transduction to hit identification, has been empirically tested.

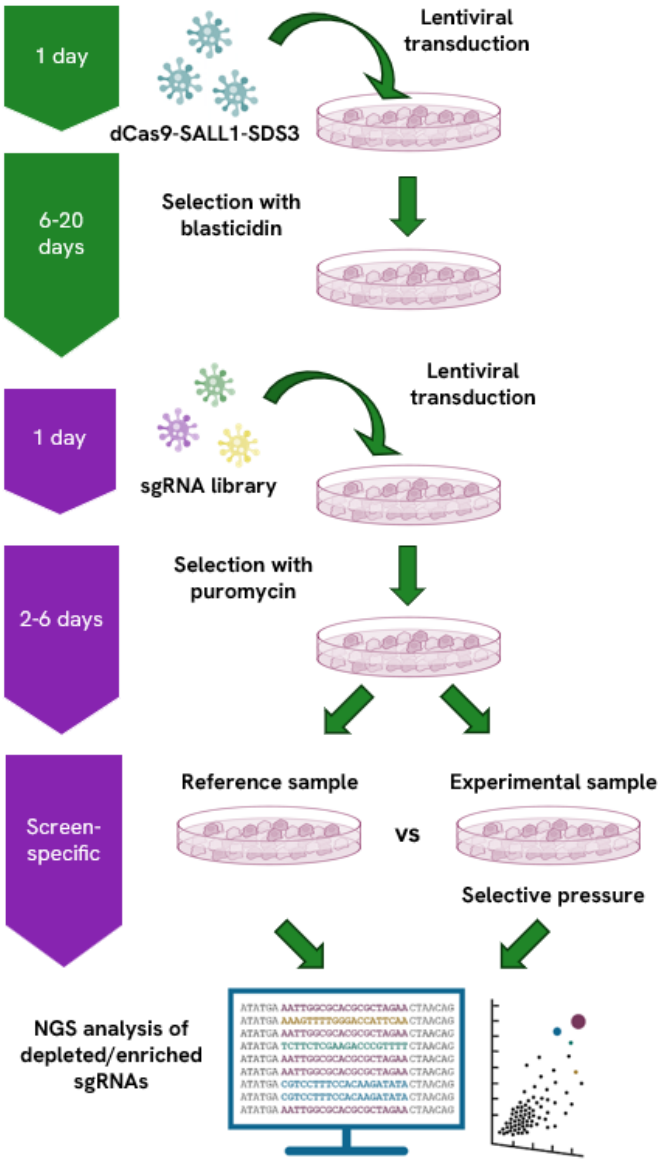


Figure 3. Loss of function screening workflow using the CRISPRmod CRISPRi Lentiviral sgRNA Pooled Library platform.

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 also 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 lentiviral sgRNA pooled library screening

A. Materials required

- CRISPRmod CRISPRi Lentiviral sgRNA Pooled Library
- Dharmacon™ NGS Library Prep Kit- mCMV

B. Additional materials required

The following additional materials are required but not supplied:

- CRISPRmod CRISPRi dCas9-SALL1-SDS3 lentiviral particles
- CRISPRmod CRISPRi lentiviral positive and non-targeting sgRNA controls for gene repression validation and experimental optimization
- SMARTvector Non-targeting Control Particles with optimal promoter and fluorescent reporter (TurboGFP or TurboRFP) for determining relative transduction efficiency and optimizing transduction conditions in desired cells of interest.
- Transduction enhancer such as LentiBOOST® or polybrene
- 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), Resazurin, or similar metabolic assay
- Blasticidin S (Fisher Scientific, Cat #BP2647-25; InvivoGen, Cat #ant-bl-1)
- HyClone™ Puromycin 2 HCl (Cytiva, Cat #SV30075.01 or similar)
- Genomic DNA purification kit (Blood and Cell Culture DNA Purification Kit, Qiagen, Cat #13362) 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-SALL1-SDS3 expression

Expression levels of dCas9-SALL1-SDS3 can have a significant effect on transcriptional repression efficiency. Therefore, select the lentiviral dCas9-SALL1-SDS3 expression vector with the most active promoter in your cell line based on empirical testing or known promoter activity.

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 CRISPRi dCas9-SALL1-SDS3 and sgRNA vectors do not contain a fluorescent reporter. Therefore, SMARTvector shRNA Non-targeting Control lentiviral particles (see Section 4.B. Additional materials required) containing either TurboGFP or TurboRFP reporter genes can be substituted for optimization of transduction conditions.

Detailed protocols for transduction optimization are described in the SMARTvector Lentiviral shRNA Technical Manual (SMARTvector Lentiviral shRNA Technical Manual).

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 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.

To determine the relative titer in the experimental cell line, perform a functional titer protocol such as limiting dilution with cell viability assay by crystal violet staining. Alternatively, SMARTvector Non-targeting Control lentiviral particles (see Section 4.B. Additional materials required) containing the optimal promoter and either TurboGFP or TurboRFP reporter gene can be substituted for determining relative transduction efficiency in your desired cells. Detailed protocols for determining functional titer in your cells are described in the SMARTvector Lentiviral shRNA Technical Manual.

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

Functional titer of shRNA Non-targeting Control reported on the C of in your	÷	Titer of shRNA Non-targeting Control reported on the C of A (TU/mL)	=	Relative transduction efficiency of your cell line

Use the calculated relative transduction efficiency of your cell line to then calculate the relative functional titer for the CRISPRmod CRISPRi Lentiviral sgRNA pooled library using the following formula:

Relative transduction efficiency of your cell line	x	Titer of the pooled library reported in data file (TU/mL)	=	Relative functional titer of the pooled library in your cell line (TU/mL)

Calculation example

If the functional titer for the Lentiviral shRNA Non-targeting 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 × 10^7 TU/mL (functional titer in your cell line) ÷ 5.0 × 10^7 TU/mL (titer reported on the C of A) = 0.42 relative transduction efficiency

If the relative transduction efficiency of your cell line is 0.42 and the titer of an CRISPRmod CRISPRi Lentiviral sgRNA Pooled Library, as indicated on the C of A, is 5.6 × 10^8 TU/mL, the relative functional titer of the pool in your cell line would be determined as follows:

> 0.42 (relative transduction efficiency) × 5.6 × 10^8 TU/mL (titer reported on the C of A) = 2.35 × 10^8 TU/mL relative functional titer in your cell line

D. Optimization blasticidin and puromycin selection

The CRISPRmod CRISPRi dCas9-SALL1-SDS3 lentiviral vectors contain the blasticidin resistance marker while the CRISPRmod CRISPRi Lentiviral sgRNA vector contains the puromycin resistance marker for selection of cells after transduction. ***Note:*** *If using another CRISPRi effector construct (such as Strict-R inducible), ensure cells are transduced and selected as described in the corresponding manual using a selection marker that is compatible with the CRISPRi Lentiviral sgRNA construct.*

Prior to treating cells with blasticidin, puromycin or another antibiotic, determine the optimal concentration of each antibiotic required to kill non-transduced cells. This concentration can be identified by generating an antibiotic kill curve. The blasticidin concentration range for many mammalian cells is 2-20 µg/mL; for puromycin it is 0.5-10 µg/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 4-15:

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 2-7 days under puromycin selection or 7-15 days under blasticidin selection.

5. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 2-15 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 CRISPRmod CRISPRi 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 abundance[11].

Recommendations:

· At least 200 independent integrations per sgRNA (*i.e.*, at least 200-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 3). For lentiviral pooled screens, each cell will ideally express a single sgRNA. This ensures that the repression 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 3. Poisson distribution showing the distribution of cells with the indicated number of lentiviral integrations at various MOIs. At each MOI, the portion of cells having 0, 1, 2, 3, or 4 lentiviral integrants is indicated. For example, at an MOI of 0.3, only 3% of cells are predicted to contain more than one lentiviral integrant.

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

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

Number of lentiviral sgRNAs in the pool × Desired fold representation = Desired number of cells with lentiviral sgRNA integrations

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

Desired number of cells with lentiviral sgRNA integrations ÷ the proportion of cells with lentiviral integrations = Number of cells required at the time of transduction

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:

76000 (number of sgRNA) × 200 (average representation) = 1.64×10^7 lentiviral integration events

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

1.64×10^7 (lentiviral integration events required) ÷ 0.3 (maximum recommended MOI) = 5.5×10^7 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:

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

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

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

Table 4 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/mm^2.

Table 4. 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/mm^2 and an effective surface area on 100 mm plates of 7800 mm^2.

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 × 100 mm
400	3.3×10^7	1.09×10^8	56 plates × 100 mm

H. Calculation of volume of lentiviral particles needed for transduction

Before proceeding with the CRISPRmod CRISPRi 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 each pool. 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:

MOI × Number of cells required at the time of transduction = Required number of lentiviral particles (TU)

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

Required number of lentiviral particles (TU) ÷ Functional titer in your cell line (TU/mL) = Volume of lentiviral particles per biological replicate

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

Volume of lentiviral particles per biological replicate (mL) × Number of biological replicates = 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 (MOI) × 5.5 × 10^7 cells = 1.64 × 10^7 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 × 10^8TU/mL in your cell line, you can calculate the volume of lentiviral particles needed as follows:

1.64 × 10^7 TU ÷ 2.35 × 10^8 TU/mL = 0.07 mL of lentiviral particles

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

0.07 mL of lentiviral particles × 2 biological replicates = 0.14 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.

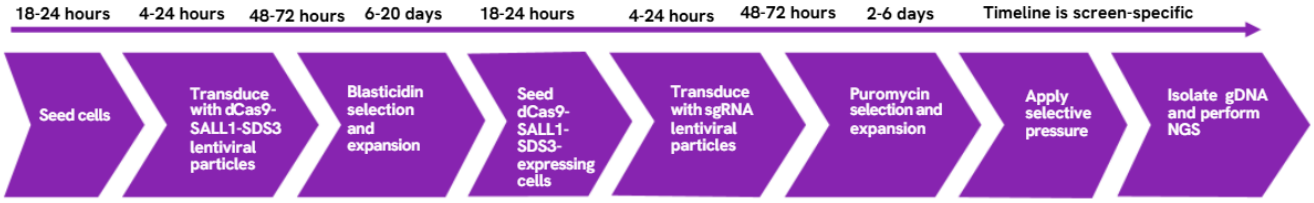


Figure 4. Timeline of primary screen.

i. Transduction of cells with CRISPRmod CRISPRi dCas9-SALL1-SDS3 lentiviral particles

Prior to performing the screen, a population of stable dCas9-SALL1-SDS3-expressing cells is first generated using CRISPRmod CRISPRi dCas9-SALL1-SDS3 lentiviral particles (with the most active cellular promoter). If a clonal dCas9-SALL1-SDS3-expressing cell line is preferred, isolate and characterize clonal cell lines using protocols appropriate for your cells of interest.

Day 1:

Seed cells in Growth Medium. The number of cells seeded should be determined by extrapolating from the number of cells desired 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 amount of CRISPRmod CRISPRi dCas9-SALL1-SDS3 lentiviral particles so that the cells are just covered.

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 4-15:

1. At 48-72 hours post-transduction begin **blasticidin** selection to remove non-transduced cells.

2. Every 48-72 hours, replace with fresh Selection Medium containing **blasticidin** 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 transduced cells has been obtained (7-15 days), expand the cell population to have enough cells for your screen with the pooled lentiviral sgRNA library as determined in Section 5.G. Calculation of number of cells needed for transduction.

We recommend evaluating the generated dCas9-SALL1-SDS3-expressing cell line by estimating transcriptional repression efficiency with sgRNA positive control lentiviral particles (see Section 4.B.) before performing the screen.

ii. Transduction of cells with CRISPRmod CRISPRi Lentiviral sgRNA Pooled Library

Day 1:

1. Seed the dCas9-SALL1-SDS3-expressing 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 a single 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 (2-7 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 correspond 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 correspond to the desired number of lentiviral integrants. The most accurate results can be obtained by counting cell numbers 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

Dharmacon™ NGS Library Prep Kits can be used to prepare gDNA samples for next-generation sequencing. First, sgRNA constructs within the isolated gDNA are amplified using vector-specific NGS Hit Identification Primers that have been designed and optimized to minimize amplification bias and introduce diversity into the high-throughput sequencing run. A second PCR amplification integrates relevant flow cell binding domains and unique 12 base pair sample indices. A limited number of cleanup steps ensures maximum recovery of amplicons for downstream sequencing. These libraries can then be multiplexed and sequenced on an Illumina® platform using standard Illumina sequencing primers.

It is important to use adequate template copies per construct in the PCR I amplification step such that the desired fold representation at transduction is maintained. This ensures assay reproducibility and facilitates hit identification[11].

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. We recommend a maximum of 5 µg of gDNA per 50 µL PCR RXN; using more gDNA per PCR could inhibit the efficiency of the reaction and may result in 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)	÷	5000 ng per reaction	=	Number of PCR reactions required to maintain representation of each sgRNA

* 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 1.52×10^7 cells with lentiviral integrations, the mass of gDNA required would be calculated as follows:

1.64×10^7 lentiviral integrations × 6.6×10^{-3} ng/genome = 1.1×10^5 ng

The maximum amount of gDNA per PCR is 5000 ng, therefore the number of PCR reactions necessary can be calculated as follows:

1×10^5 ng gDNA ÷ 5000 ng/PCR = 22 PCR reactions

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

22 PCR reactions × 4 samples = 88 PCR reactions

ii. PCR from genomic DNA

Once you have determined the number of PCR reactions needed per sample, you can proceed with the PCR amplification of the gDNA samples. A detailed protocol can be found in the Dharmacon NGS Library Prep Kit Manual.

Perform PCRs in 50 µL reactions using 96-well PCR plates. Note that while **PCR I** will likely require multiple 50 µL reactions, a single 50 µL **PCR II** reaction is sufficient to prepare a sample from a whole genome screen performed with 4 sgRNAs/ gene.

Following amplification and cleanup, it is recommended that you quantify your library by gel or fragment analysis. Amplicons will be 391-400 bp in size.

7 Illumina platform 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 CRISPRi Lentiviral sgRNA 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 2[12], 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 DESeq2[13], to determine primary hits. Hits can be confirmed and studied further using individual CRISPRmod CRISPRi Lentiviral sgRNAs.

9 Appendix

A. Stability and storage

CRISPRmod CRISPRi Lentiviral sgRNA Pooled Libraries are shipped on dry ice and must be stored at -80 °C. Under these conditions, lentiviral particles are stable for at least 12 months from date of receipt. 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.

B. Quality assurance and control

CRISPRmod CRISPRi Pooled Lentiviral sgRNA Screening 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 do I choose between the various CRISPRmod CRISPRi Lentiviral dCas9-SALL1-SDS3 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 not available, consider testing multiple lentiviral promoter-dCas9-SALL1-SDS3 constructs.

I am using human cell lines in my research; should I always choose a human promoter for optimal dCas9-SALL1-SDS3 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. Additional guidance can be found in the CRISPR promoter selection guide.

Can I use CRISPRmod CRISPRi guide RNAs with dCas9-KRAB or other CRISPRi repressors?

Yes. The CRISPRmod CRISPRi system uses canonical guide RNAs that can be used with other similar systems that use canonical Cas9 guide RNAs, like dual-inducible constructs like Strict-R dCas9-SALL1-SDS3 and KRAB-based repressors.

Do the CRISPRmod CRISPRi Lentiviral sgRNA pooled libraries target lncRNAs or regulatory regions?

No. The libraries only contain sgRNAs targeting protein-coding genes.

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. The 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. CRISPRmod CRISPRi lentiviral products are engineered for biosafety and are provided as replication-incompetent lentiviral particles.

Can I order the CRISPRmod CRISPRi Lentiviral sgRNA Pooled libraries as plasmid DNA or glycerol stocks?

No. Our CRISPRmod CRISPRi Lentiviral sgRNA 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 CRISPRmod CRISPRa Lentiviral sgRNA 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-XXXXXXXG) if you did not receive the data files 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 5**) below.

Table 5. 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:

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

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

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

11 References

1. Bhaya, *et al.* CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu. Rev. Genet.* **45**, 273-297 (2011).
2. M. Jinek, *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821 (2012).
3. L.S. Qi, *et al.*, Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell*, **152**(5): p. 1173-83 (2013).
4. L.A.Gilbert, *et al.*, CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*, **154**(2): p. 442-51 (2013).
5. A.W. Cheng, *et al.*, Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res*, **23**(10): p. 1163-71 (2013).
6. L. A. Gilbert, *et al.*, Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell.* **159**, 647-661 (2014).

7. NC. Yeo, *et al.*, An enhanced CRISPR repressor for targeted mammalian gene regulation. *Nat Methods*, **15**(8): p. 611-616 (2018).
8. N. Alerasool, *et al.*, An efficient KRAB domain for CRISPRi applications in human cells. Nat Methods. **17**(11): p. 1093-1096 (2020).
9. C. Mills, *et al.*, A novel CRISPR interference effector enabling functional gene characterization with synthetic guide RNAs. CRISPR J. (2022).
10. M. A. Horlbeck *et al.*, Compact and highly active next-generation libraries for CRISPR-mediated gene repression and activation. *eLife*. **5**, e19760 (2016).
11. Ž. Strezoska, *et al.* Optimized PCR conditions and increased shRNA fold representation improve reproducibility of pooled shRNA screens. *PLoS One* **7**, e42341 (2012).
12. Langmead and S. Salzberg. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357-359 (2012).
13. S. Anders and W. Huber. Differential expression analysis for sequence count data. *Genome Biol.* **11**, R106 (2010).

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 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 Scientific Support team.

Horizon Discovery Biosciences Ltd. (A Revvity Company)
2650 Crescent Drive
Lafayette, CO 80026 USA
Tel: +1 800 235 9880; 303 604 9499 (United States)
Fax: +1 800 292 6088; 303 604 9680 (United States)
Email: technical.horizon@revvity.com (North America and Europe)
https://horizondiscovery.com/en/contact-us

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:

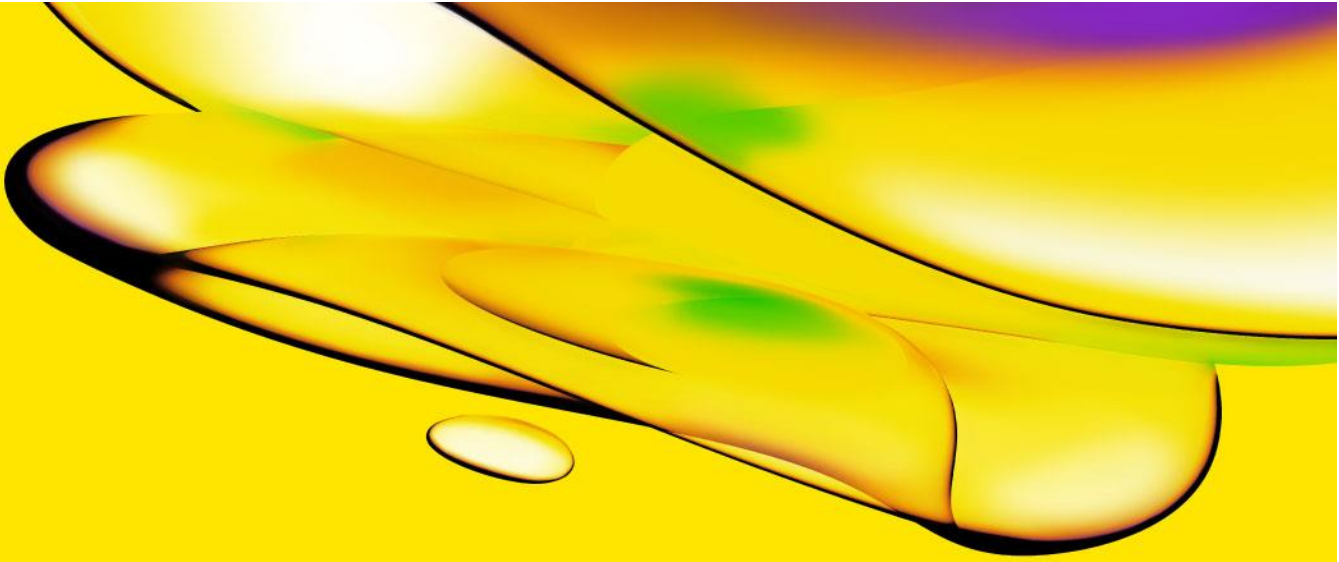
1. German Genetic Engineering Act (Gentechnikgesetz - GenTG); and
2. Genetic Engineering Safety Ordinance (Gentechnik-Sicherheitsverordnung - GenTSV).

13 Limited use licenses

The Products, use and applications, are covered by pending and issued patents. Certain Label licenses govern the use of the Products, these can be found at Dharmacon Licensing Statements. It is each Buyer's responsibility to determine which intellectual property rights held by third parties may restrict the use of Products for a particular application. Please review the Label Licenses governing all use of the Products.

For more information and questions contact
https://horizondiscovery.com/en/contact-us

Horizon Discovery Biosciences LTD is a Revvity company
Dharmacon Inc is a Revvity company



Revvity
940 Winter Street
Waltham, MA 02451 USA

www.revvity.com

For a complete listing of our global offices, visit www.revvity.com