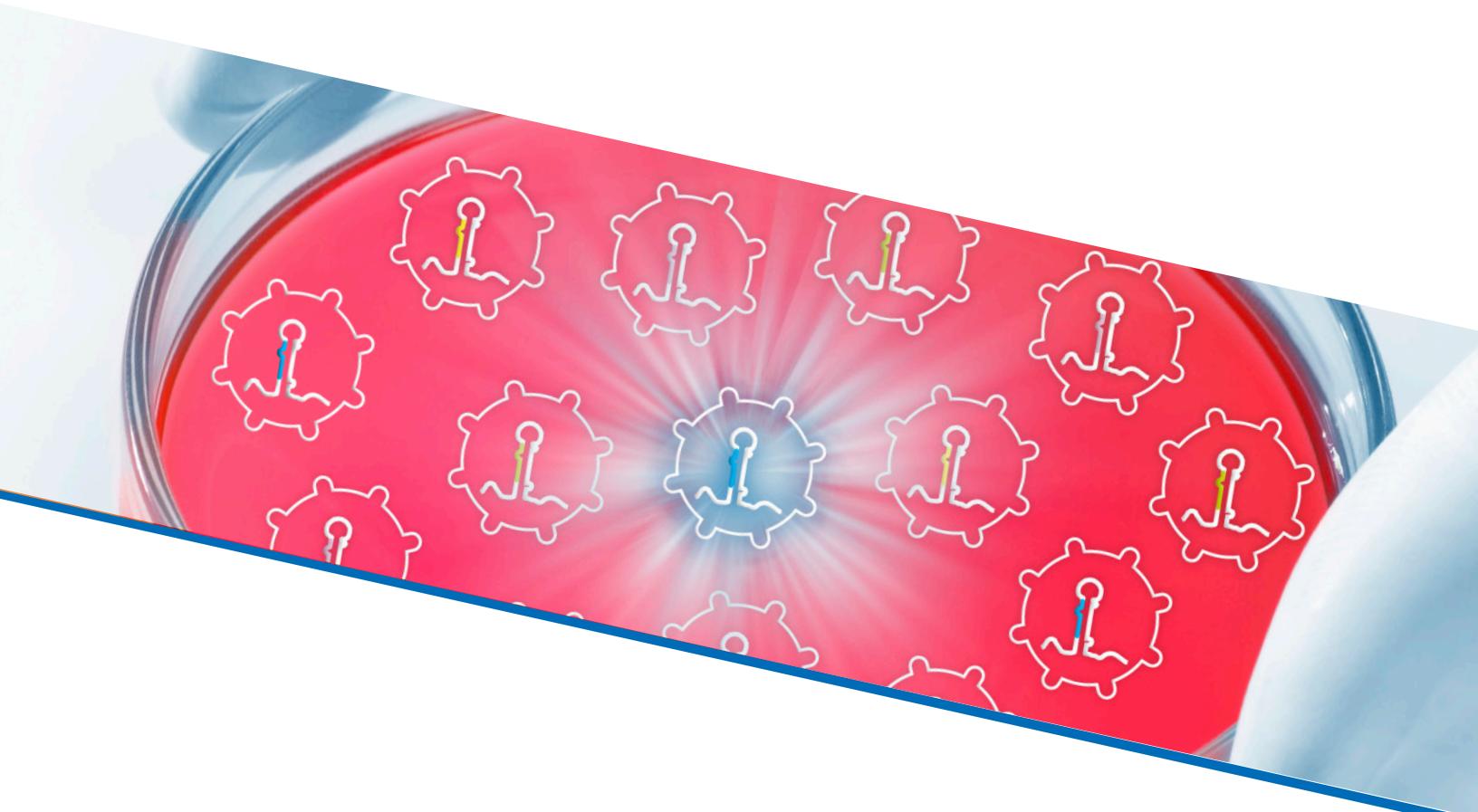


Dharmacon™ Edit-R™ Lentiviral sgRNA Pooled Screening Libraries



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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 infection.¹

B. Engineering a CRISPR-Cas9 platform for mammalian genome editing

Many bacterial and archaeal CRISPR-Cas systems have been identified with diverse sets of mechanisms, Cas proteins and multi-subunit complexes. In particular, the processes and key components of the *Streptococcus pyogenes* CRISPR-Cas9 system have been well studied and adapted for genome engineering in mammalian cells. In *S. pyogenes*, only three components are required for targeted DNA cleavage at specific target sites adjacent to a PAM:² (1) The endonuclease Cas9, programmed by (2) a mature crRNA processed from transcription of the CRISPR locus/array which complexes with (3) another CRISPR locus-encoded RNA, the trans-activating CRISPR RNA (tracrRNA, Figure 1A).³ Alternatively, the crRNA can be fused to the tracrRNA creating a chimeric structure termed a single guide RNA (sgRNA, Figure 1B).²

Upon site-specific double-strand DNA cleavage, a mammalian cell can repair such a break through either non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ is often imperfect, resulting in small insertions and deletions (indels) that can cause nonsense mutations resulting in gene disruptions to produce gene knockouts.^{4,5} This endogenous DNA break repair process, coupled with the highly tractable *S. pyogenes* CRISPR-Cas9 system, allows for a readily engineered platform to permanently disrupt gene function.

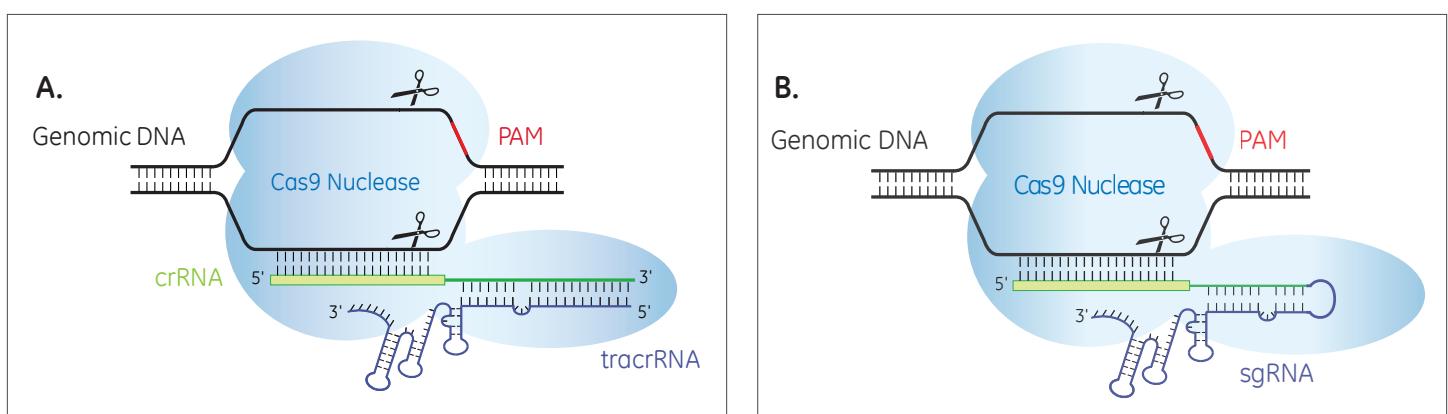


Figure 1. Illustration of CRISPR-Cas9 system. Cas9 nuclease (light blue) programmed by the crRNA (green):tracrRNA (blue) complex (A) or the sgRNA (B) cutting both strands of genomic DNA 5' of the PAM (red).

2 Edit-R Lentiviral sgRNA Pooled Screening platform

Edit-R Lentiviral sgRNA Pooled Screening Libraries are pools of lentiviral constructs expressing sgRNAs targeting coding genes for knockout. Gene knockout 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 Edit-R Lentiviral sgRNA Pooled Screening platform is a two-vector system that utilizes a lentiviral vector for Cas9 expression and a gene-specific vector for sgRNA expression designed to the target site of interest. Edit-R Lentiviral sgRNA Pooled Screening Libraries are comprised of five to ten pre-designed gene-specific sgRNAs per gene, 100 non-targeting controls for hit normalization and up to 340 gene-specific positive controls. Table 1 lists the pre-defined pooled lentiviral libraries that are available. Custom libraries can be ordered upon request.

Table 1. Available Edit-R Lentiviral sgRNA Pooled Screening Libraries.

Edit-R Lentiviral sgRNA Pooled Library ^{1,2,3}	Human		Mouse	
	Number of targeted genes ⁴	Number of pools × number of constructs per pool ^{4,5}	Number of targeted genes ⁴	Number of pools × number of constructs per pool ^{4,5}
Whole Genome	18 525	18 pools × 10 450 constructs	19 683	19 pools × 10 600 constructs
Druggable Genome	7359	7 pools × 10 730 constructs	9827	10 pools × 10 170 constructs
GPCR	378	1 pool × 4127 constructs	498	1 pool × 5311 constructs
Ion Channel	345	1 pool × 3829 constructs	340	1 pool × 3818 constructs
Protein Kinase	700	1 pool × 7262 constructs	708	1 pool × 7451 constructs
Phosphatase	247	1 pool × 2846 constructs	269	1 pool × 3089 constructs
Protease	473	1 pool × 5080 constructs	533	1 pool × 5712 constructs
Ubiquitin Conjugate	564	1 pool × 5642 constructs	517	1 pool × 5564 constructs

¹All lentiviral pooled libraries are provided as high-titer, concentrated viral particles ($\geq 5 \times 10^8$ TU/mL ± 20%).

²Additional gene families are available on the website and by request (ts.dharmacon@ge.com).

³Custom libraries targeting rat gene families or researcher-defined human, mouse or rat gene lists are available by request (ts.dharmacon@ge.com).

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

⁵Number of constructs per pool includes gene specific and negative control sgRNA constructs.

Edit-R Lentiviral sgRNA Pooled Screening Libraries include:

1. sgRNAs targeting coding genes in the NCBI Reference Sequence Database: Five to ten sgRNAs per gene designed using the proprietary Edit-R algorithm;
2. Gene-specific positive controls: sgRNA constructs targeting up to 34 protein-coding genes including *ACTB*, *GAPDH*, *LAMB1*, and *PPIB*;
3. Negative controls: 100 non-targeting sgRNA controls bioinformatically confirmed to not align with (target) any gene in the human, mouse and rat genomes.

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 master pools.

Also available for use with the pooled screening libraries are optimized PCR and sequencing primers and experimentally tested protocols for reliable identification of sgRNA hits by high-throughput sequencing on an Illumina platform (see Section 4 for details on additional recommended materials).

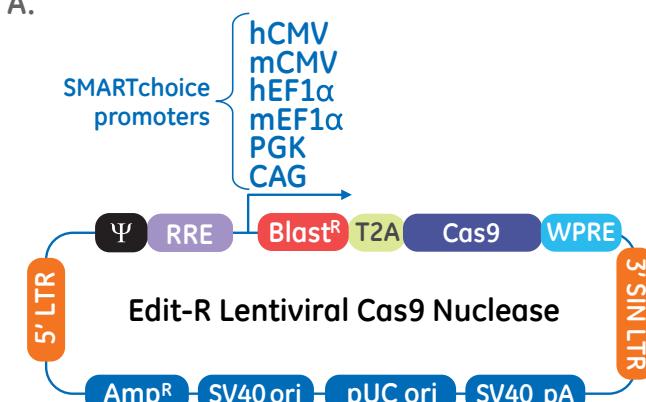
A. Edit-R Lentiviral Cas9 Nuclease and sgRNA vectors

The Edit-R Lentiviral Cas9 Nuclease expression vectors contain a human codon-optimized version of the *S. pyogenes* *cas9* (*csm1*) gene 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 2A). Multiple promoter options are available (Figure 2A) so that the most active promoter driving Cas9 nuclease expression can be chosen for specific cells of interest. In the Edit-R 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 (Puro^R) 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.

Table 2. Elements of the Edit-R Lentiviral Cas9 Nuclease Expression and sgRNA vectors.

Vector Element	Utility
Cas9	Human codon-optimized <i>S. pyogenes</i> Cas9 nuclease for cleavage of targeted DNA when programmed with a sgRNA
T2A	Self-cleaving peptide allows for simultaneous expression of blasticidin resistance and Cas9 proteins from a single transcript
Blast ^R	Blasticidin resistance marker permits antibiotic selection of transduced mammalian cells
hCMV	Human cytomegalovirus immediate early promoter
mCMV	Mouse cytomegalovirus immediate early promoter
hEF1 α	Human elongation factor 1 alpha promoter
mEF1 α	Mouse elongation factor 1 alpha promoter
PGK	Mouse phosphoglycerate kinase promoter
CAG	Human cytomegalovirus, chicken β -actin hybrid promoter
U6	Human RNA polymerase III promoter U6
Puro ^R	Puromycin resistance marker permits antibiotic selection of transduced mammalian cells
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
WPRES	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 for vector propagation in <i>E. coli</i> cultures

A.



B.

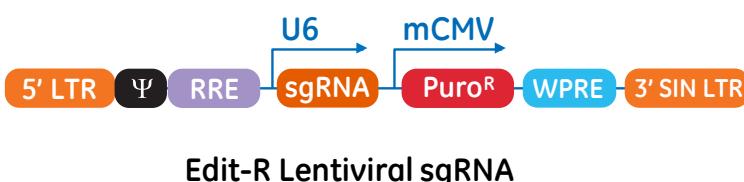


Figure 2. Schematic maps of the Edit-R Lentiviral Cas9 Nuclease (A) and sgRNA (B) vectors.

Each Edit-R Lentiviral sgRNA is specific to the gene or genomic site of choice. The crRNA region of the sgRNA is comprised of 19-20 nucleotides identical to the genomic DNA (gDNA) target site, or protospacer, followed by the non-variable sgRNA scaffold containing the tracrRNA sequence from *S. pyogenes*. The chosen genomic DNA target sequence must be immediately upstream of a PAM (protospacer-adjacent motif). The predominant *S. pyogenes* PAM nucleotide sequence is NGG.

B. Overview of Edit-R Lentiviral sgRNA Pooled Library screening workflow

Once vector configuration options, basic transduction and assay conditions have been optimized, Cas9-expressing cell lines can easily be generated with the Edit-R Lentiviral Cas9 Nuclease Expression particles. Subsequent gene knockout screening can be performed by an additional transduction with Edit-R Lentiviral sgRNA Pooled Library 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 single constructs integrated into their genomes. Following transduction, a selective pressure is applied such that those constructs involved in a specific biological response can be identified. As a result of the selective pressure, cells expressing the 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 remains following the application of selective pressure and/or phenotypic selection (experimental sample). sgRNA constructs within the isolated gDNA are amplified using Edit-R Pooled sgRNA Forward and Reverse Index PCR primers that have been designed and optimized to minimize amplification bias. Following amplification, indexed reaction products can be directly loaded onto Illumina flow cells and sequenced using Edit-R Pooled sgRNA Read 1 and Index Read 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 lentiviral pooled library screening workflow, from transduction to hit identification, has been empirically tested. The Edit-R Lentiviral sgRNA Pooled Screening Library platform includes universal Illumina-adapted PCR primers for identification of sgRNA sequences from gDNA by high-throughput sequencing on Illumina instrumentation. Edit-R Pooled sgRNA Forward and Reverse Index PCR primers have built-in adaptor and index sequences that allow the researcher to easily move from PCR amplification to high-throughput sequencing on an Illumina platform. Direct identification of sgRNA sequences facilitates data analysis and ensures accurate target gene identification.

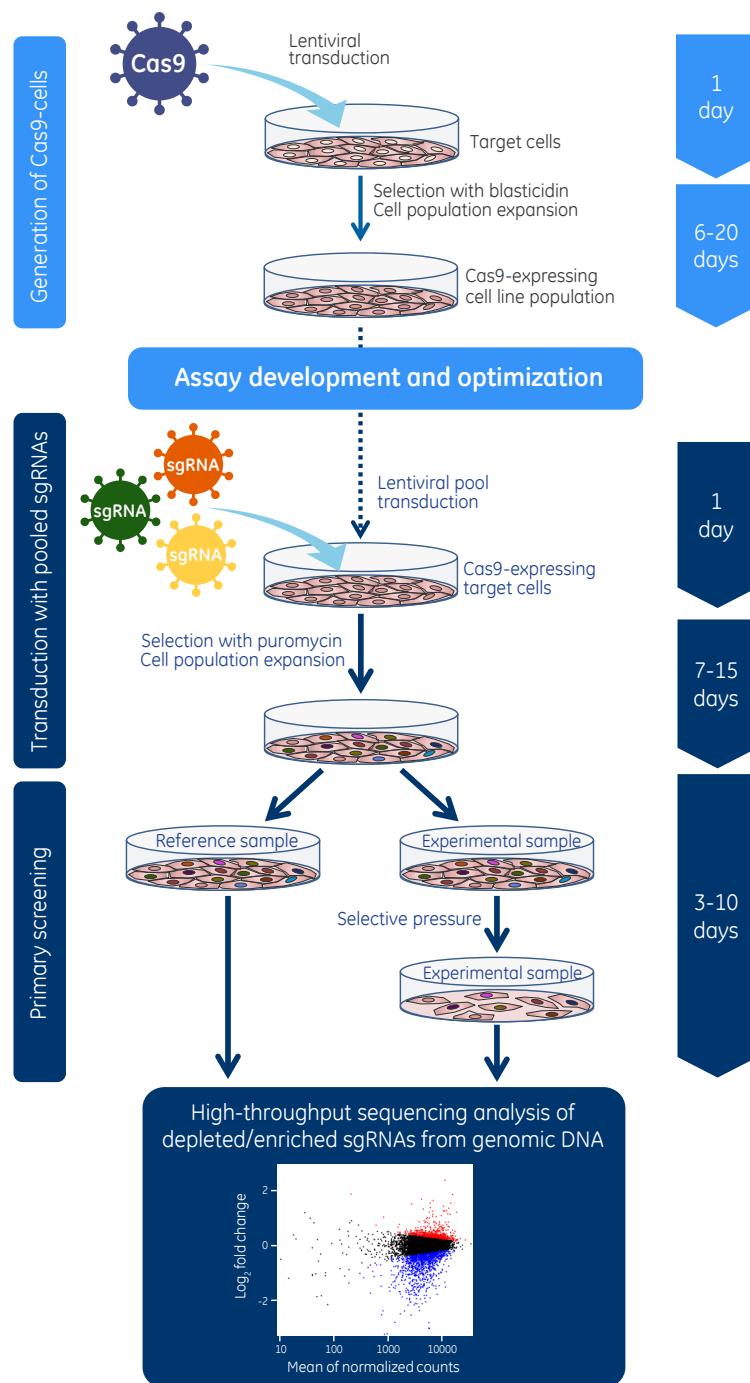


Figure 3. Gene knockout screening workflow using the Edit-R Lentiviral sgRNA Pooled Screening Library platform.

A Cas9-expressing stable cell line (mixed or clonal cell population) is first generated with Edit-R Lentiviral Cas9 Nuclease particles by selection with blasticidin. These cells are then transduced with lentiviral sgRNA pooled library and selected with puromycin. Transduced cells are split into reference and experimental populations for application of a selective pressure and/or phenotypic selection. Genomic DNA is then isolated from the reference and experimental populations of transduced cells. Edit-R Pooled sgRNA Indexing PCR primers are used to PCR amplify integrated constructs and add Illumina flow-cell binding sequences. The resulting amplicons are sequenced on Illumina platform sequencers, using the Edit-R Pooled sgRNA Read 1 and Index Read Sequencing primers. The integrated sgRNA sequences in both reference and experimental samples are identified and relative abundance compared. sgRNA constructs that are enriched or depleted are identified as hits to be confirmed and studied further using individual Edit-R Lentiviral sgRNAs in additional phenotypic and/or biochemical assays.

3 Laboratory protocols and calculation tracking worksheet

The **Laboratory protocols and calculation tracking worksheet** is a tool for the recording of key calculations and experimental parameters ([see Appendix A](#)). Please review all of the detailed protocols provided in this manual prior to using the worksheet. An electronic version of the worksheet can also be downloaded from the Dharmacon web page ([Edit-R Pooled Screening Protocols and Calculation Worksheet](#)). 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

- Edit-R Lentiviral sgRNA Pooled Screening Library(ies) (Table 1)
- Edit-R Lentiviral Cas9 Nuclease vector with chosen promoter
 - » Edit-R Lentiviral hCMV-Blast-Cas9 Nuclease Particles (Dharmacon, Cat #VCAS10124)
 - » Edit-R Lentiviral mCMV-Blast-Cas9 Nuclease Particles (Dharmacon, Cat #VCAS10125)
 - » Edit-R Lentiviral hEF1 α -Blast-Cas9 Nuclease Particles (Dharmacon, Cat #VCAS10126)
 - » Edit-R Lentiviral mEF1 α -Blast-Cas9 Nuclease Particles (Dharmacon, Cat #VCAS10127)
 - » Edit-R Lentiviral PGK-Blast-Cas9 Nuclease Particles (Dharmacon, Cat #VCAS10128)
 - » Edit-R Lentiviral CAG-Blast-Cas9 Nuclease Particles (Dharmacon, Cat #VCAS10129)
- Edit-R Pooled sgRNA Indexing PCR and Sequencing Primer Kit A (Dharmacon, Cat #PRM10184) for Illumina sequencing platform
- Edit-R Pooled sgRNA Indexing PCR and Sequencing Primer Kit B (Dharmacon, Cat #PRM10185) for additional reverse indexing PCR primers (if experimental design requires additional multiplexing capability)
- Phusion Hot Start II High-Fidelity DNA Polymerase and 5x Phusion HF Buffer (Thermo Scientific, Cat #F-549X; NEB, Cat #M0535X).



Please see Section 6 to determine the amount of Phusion DNA polymerase required for a screening workflow.

B. Additional materials required

The following additional materials are required but not supplied:

- Positive and/or negative sgRNA controls for gene editing validation and experimental optimization
 - » Edit-R PP1B Lentiviral sgRNA Positive Control [Dharmacon, Cat # VSGH10231 (Human) and Cat # VSGM10233 (Mouse)]
 - » Edit-R Lentiviral sgRNA Non-targeting Control(s) (Dharmacon, Cat # VSGC102XX)
- SMARTvector Non-targeting Control Particles with choice of promoter (hCMV, mCMV, hEF1 α , mEF1 α , PGK, CAG, UBC) and fluorescent reporter (TurboGFP or TurboRFP) for determining relative transduction efficiency and optimizing transduction conditions in desired cells of interest.
 - » See dharmacon.gelifesciences.com/shrna/smartvector-lentiviral-controls/ for product options and catalog numbers.

- Polybrene (American Bioanalytical, Cat #AB01643)
- 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)
- Low range DNA Ladder
- Nucleotide mix dNTP set, 10 mM each (GE Life Sciences, Cat #28-4065-64 or similar)
- Blasticidin S (Fisher Scientific, Cat #BP2647-25; InvivoGen, Cat #ant-bl-1).
- HyClone™ Puromycin 2 HCl (GE Life Sciences, Cat #SV30075.01; InvivoGen, Cat #ant-pr-1)
- Base Medium: antibiotic-free cell culture medium (without supplements or serum)
- Growth Medium: antibiotic-free cell culture medium (with serum and/or supplements) recommended for maintenance of the cells
- Transduction Medium: the base cell culture medium containing lentiviral particles (with transduction additives and serum (if necessary).
- Selection Medium: Growth Medium supplemented with the appropriate concentration of blasticidin or puromycin
- Resazurin cell viability reagent or similar

5 Assay development and optimization

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

A. Selection of the optimal promoter for Cas9 expression

Expression levels of Cas9 nuclease can have a significant effect on gene knockout efficiency. Therefore, select the lentiviral Cas9 nuclease 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.

Edit-R Lentiviral Cas9 Nuclease Expression and sgRNA vectors do not contain a fluorescent reporter. Therefore, SMARTvector 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 (dharmacon.gelifesciences.com/uploadedFiles/Resources/smartvector-constitutive-manual.pdf).

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) may be added to enhance lentiviral particle binding to the cell surface. We recommend testing a range of concentrations, from 0-10 µg/mL, for identification of optimal transduction efficiency with minimal or no cell toxicity. If Polybrene is toxic to the cells of interest, DEAE-Dextran (1-10 µg/mL) or Protamine Sulfate (1-50 µg/mL) may be substituted.

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

C. Determination of functional titer

The functional titers are found on the Certificate of Analysis (C of A) provided with all Dhamacon lentiviral particle products. 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 of sgRNA.

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 (dharmacon.gelifesciences.com/uploadedfiles/resources/titer-crystal-violet-protocol.pdf) or genomic qPCR assay⁶ using Edit-R Lentiviral sgRNA Non-targeting Control particles. Alternatively, SMARTvector Non-targeting Control lentiviral particles (see Section 4.B. Additional materials required) containing either TurboGFP or TurboRFP reporter genes 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.

$$\text{Functional titer of Edit-R Lentiviral sgRNA Non-targeting Control in your cell line (TU/mL)} \div \text{Titer of Edit-R Lentiviral sgRNA Non-targeting Control particles stock reported on the C of A (TU/mL)} = \text{Relative transduction efficiency of your cell line}$$

Use the calculated relative transduction efficiency of your cell line to then calculate the relative functional titer for each batch of Edit-R Lentiviral Cas9 Nuclease particles and each lentiviral sgRNA pooled library using the following formula:

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

Calculation example

If the functional titer for the Edit-R Lentiviral sgRNA 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:

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

If the relative transduction efficiency of your cell line is 0.42 and the titer of a Edit-R 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:

$$0.42 \text{ (relative transduction efficiency)} \times 5.6 \times 10^8 \text{ TU/mL (titer reported on the C of A)} = 2.35 \times 10^8 \text{ TU/mL relative functional titer in your cell line}$$

D. Optimization of blasticidin and puromycin selection

The Edit-R Lentiviral Cas9 Nuclease Expression vectors contain the blasticidin resistance marker while the Edit-R Lentiviral sgRNA vector contains the puromycin resistance marker for selection of cells after transduction. Prior to treating cells with blasticidin or puromycin, 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 2-15 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 3-6 days under puromycin selection or 5-15 days under blasticidin selection.
5. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 3-15 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 instance, concentration or duration), and method of phenotypic selection (for instance, 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 abundance.⁷

Recommendations:

- Between 500 and 1000 independent integrations per sgRNA (*i.e.*, 500- 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 3). For lentiviral pooled screens, each cell will ideally express a single sgRNA. This ensures that the knockout 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.

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:

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

To calculate the number of cells you will need per sample at the time of transduction, first determine the proportion of cells with lentiviral integrants (Table 3), and then use the following formula:

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

Calculation example

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

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

At an MOI of 0.3, approximately 22% of seeded cells will undergo at least one lentiviral integration event. Therefore, calculate the number of cells needed at the time of transduction as follows:

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

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:

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

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

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

Table 4 provides examples of cell and plate numbers and plate sizes for various targeted sgRNA fold representations, assuming a pool size of 1000 sgRNAs and a cell line with optimal transduction density of 250 cells/mm².

Table 4. Number of cells required at the time of transduction and cell plating recommendations for indicated average fold representation assuming pools of 1000 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
500	5×10^5	2.3×10^6	1 plate × 100 mm
1000	1×10^6	4.5×10^6	2 plates × 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 each pool. All pooled libraries are supplied as multiple aliquots of 25 µL of lentiviral particles, at titers of $\geq 5 \times 10^8$ TU/mL.

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

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

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

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

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

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

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

Calculation example

For example, at an MOI of 0.3 and a 500-fold average sgRNA representation at transduction, you will have prepared 2.3×10^6 cells at the time of transduction. Based on the above formula, calculate the number of lentiviral particles needed:

$$0.3 (\text{MOI}) \times 2.3 \times 10^6 \text{ cells} = 6.9 \times 10^5 \text{ TU of lentiviral particles are required for transduction}$$

If you have determined that you need 6.9×10^5 TU and you have a functional titer of 1.0×10^8 TU/mL in your cell line, you can calculate the volume of lentiviral particles needed as follows:

$$6.9 \times 10^5 \text{ TU} \div 1.0 \times 10^8 \text{ TU/mL} = 0.007 \text{ mL of lentiviral particles}$$

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

$$0.007 \text{ mL of lentiviral particles} \times 2 \text{ biological replicates} = 0.014 \text{ mL of lentiviral particles}$$

Table 5. Volume of lentiviral particles at 5×10^8 TU/mL in HEK293T cells required for the indicated fold representation and relative transduction efficiencies. The volume of lentiviral particles is indicated for one pool of 1000 sgRNA with one biological replicate at an MOI of 0.3.

Fold representation	Relative transduction efficiency			
	1	0.5	0.2	0.1
500	1.2 µL	2.4 µL	6 µL	12 µL
1000	2.4 µL	4.8 µL	12 µL	24 µL

6 Primary screen

The following sections describe the primary screening workflow (Figure 4). Please use the [Laboratory protocols and calculation tracking worksheet](#) (see Appendix A) as a guide.

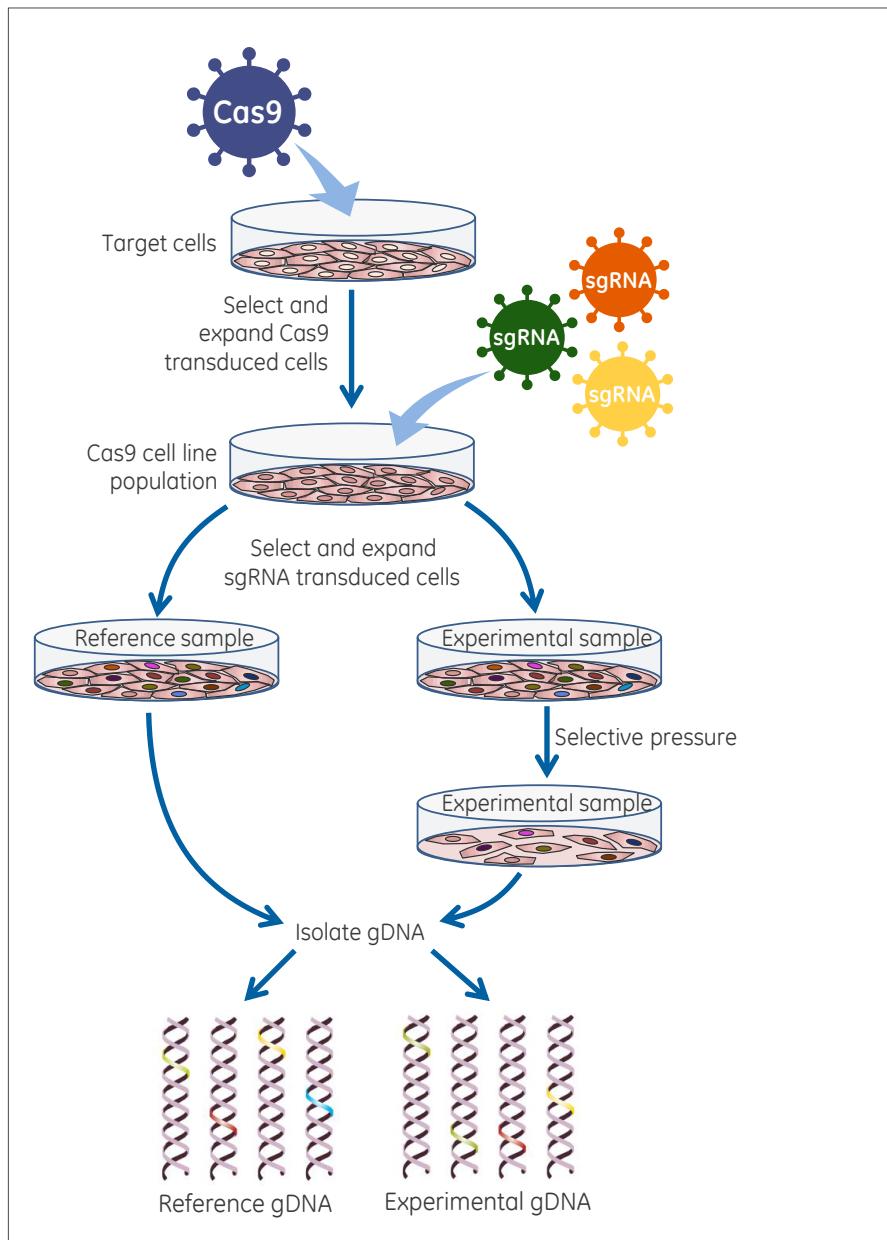


Figure 4. Primary screening workflow.

A. Cell transductions and selection screen

The experimental conditions described here, and in Figure 5, 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.

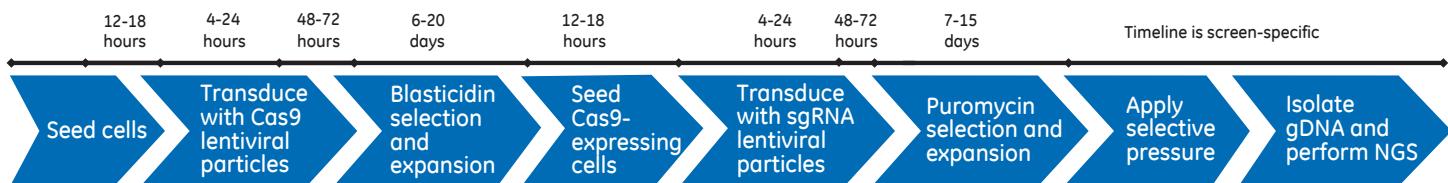


Figure 5. Timeline of primary screen.

i. Transduction of cells with Edit-R Lentiviral Cas9 Nuclease particles

Prior to performing the screen, a population of stable Cas9-expressing cells is first generated using Edit-R Lentiviral Cas9 particles (with the most active cellular promoter). If a clonal Cas9-expressing cell line is preferred, isolate and characterize clonal cell lines using protocols appropriate for your cells of interest.

Day 1:

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 (as determined previously, see Section 5.B. Optimization of lentiviral transduction) with the appropriate amount of Edit-R Lentiviral Cas9 Nuclease particles so that the cells are just covered.
2. After the appropriate transduction time (as determined previously, see Section 5.B. Optimization of lentiviral transduction), add additional Growth Medium to your cells such that the cells can be incubated for 48-72 hours.

Days 4-14:

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 blasticidin and the minimum number of days required to kill non-transduced cells as determined by a blasticidin kill curve (see Section 5.D. Optimization of blasticidin and puromycin selection).

Once a pure population of transduced cells has been obtained (5-10 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 Cas9-expressing cell line by estimating gene editing events with sgRNA positive control lentiviral particles (see Section 4.B. Additional recommended materials for catalog numbers) before performing the screen.

ii. Transduction of Cas9-expressing cells with Edit-R Lentiviral sgRNA Pooled Screening Library

Day 1:

1. Seed the Cas9-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. Optimization of lentiviral transduction) with the appropriate amount of lentiviral particles (Section 5.H. Calculation of volume of lentiviral particles needed for transduction) 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. Optimization of lentiviral transduction), 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. Optimization of blasticidin and puromycin selection).

Once a pure population of puromycin-resistant cells has been obtained (3-6 days), begin screening. Split cells into at least two populations: one as a reference and another (experimental) for application of selective pressure and/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 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.

To ensure high DNA quality and yield, do not use more than the manufacturer's recommended cell number and be sure that your gDNA is fully solubilized. If multiple purification columns are required to maintain representation of your sample, combine gDNA isolations after elution.



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

- 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 (Figure 6) 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 identification.⁷



The Edit-R Pooled sgRNA Indexing PCR primers have been designed for use with Illumina sequencing platforms. They are not compatible with other high-throughput sequencing systems.

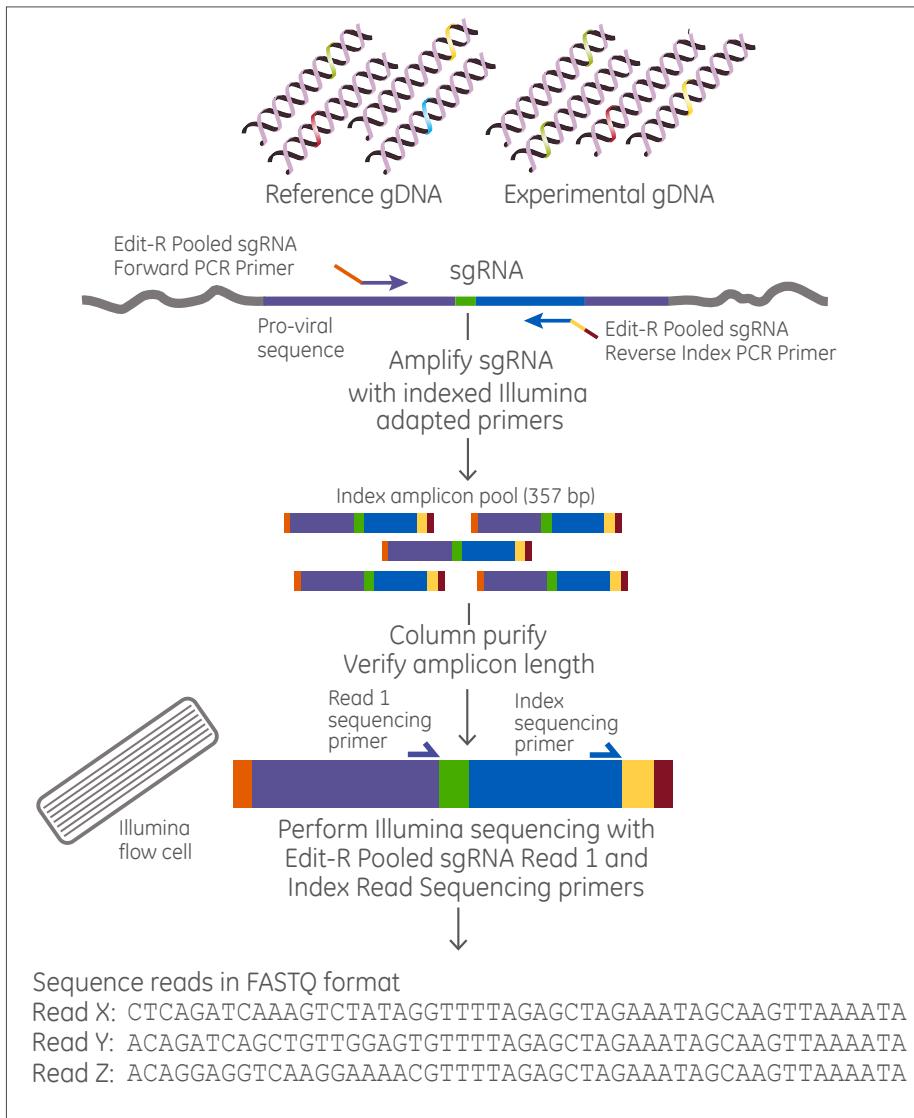


Figure 6. PCR amplification and Illumina high-throughput sequencing workflow.

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:

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



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

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:

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

* Round up the value obtained to the next whole number

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

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

sgRNA fold representation	Input gDNA (μg)	Number of PCR reactions
500	3.3	4
1000	6.6	8

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

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

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

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

Calculation example

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

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

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

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

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

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

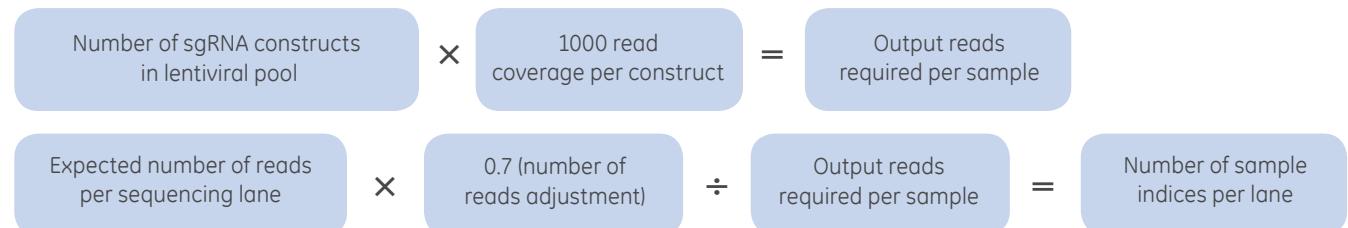
To perform 16 PCRs you need the following amount of Phusion Hot Start II DNA Polymerase:

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

ii. Multiplexing of high-throughput sequencing samples

Most high-throughput sequencing platforms provide a sufficiently high number of sequence reads to allow multiplexing of samples in sequencing runs (running multiple samples in one lane). For accurate hit identification, we recommend obtaining a minimum of 1000 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.

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



Calculation examples

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

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

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

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

Edit-R Pooled sgRNA Indexing PCR primers are provided as 50 µM forward and reverse indexed primers. The index tags are shown in Table 7.

Table 7. The index tags on Edit-R Pooled sgRNA Indexing PCR primers are compatible with Illumina TruSeq LT indices.

Item	Index	Index Sequence
Kit A		
Edit-R Pooled sgRNA Reverse Index PCR Primer 2	2	CGATGT
Edit-R Pooled sgRNA Reverse Index PCR Primer 4	4	TGACCA
Edit-R Pooled sgRNA Reverse Index PCR Primer 5	5	ACAGTG
Edit-R Pooled sgRNA Reverse Index PCR Primer 6	6	GCCAAT
Edit-R Pooled sgRNA Reverse Index PCR Primer 7	7	CAGATC
Edit-R Pooled sgRNA Reverse Index PCR Primer 12	12	CTTGTA
Edit-R Pooled sgRNA Reverse Index PCR Primer 13	13	AGTCAA
Edit-R Pooled sgRNA Reverse Index PCR Primer 14	14	AGTTCC
Edit-R Pooled sgRNA Reverse Index PCR Primer 15	15	ATGTCA
Edit-R Pooled sgRNA Reverse Index PCR Primer 16	16	CCGTCC
Edit-R Pooled sgRNA Reverse Index PCR Primer 18	18	GTCCGC
Edit-R Pooled sgRNA Reverse Index PCR Primer 19	19	GTGAAA

Item	Index	Index Sequence
Kit B		
Edit-R Pooled sgRNA Reverse Index PCR Primer 1	1	ATCACG
Edit-R Pooled sgRNA Reverse Index PCR Primer 3	3	TTAGGC
Edit-R Pooled sgRNA Reverse Index PCR Primer 8	8	ACTTGA
Edit-R Pooled sgRNA Reverse Index PCR Primer 9	9	GATCAG
Edit-R Pooled sgRNA Reverse Index PCR Primer 10	10	TAGCTT
Edit-R Pooled sgRNA Reverse Index PCR Primer 11	11	GGCTAC
Edit-R Pooled sgRNA Reverse Index PCR Primer 20	20	GTGGCC
Edit-R Pooled sgRNA Reverse Index PCR Primer 21	21	GTTTCG
Edit-R Pooled sgRNA Reverse Index PCR Primer 22	22	CGTAGC
Edit-R Pooled sgRNA Reverse Index PCR Primer 23	23	GAGTGG
Edit-R Pooled sgRNA Reverse Index PCR Primer 25	25	ACTGAT
Edit-R Pooled sgRNA Reverse Index PCR Primer 27	27	ATTCCG

iii. PCR from genomic DNA

Once you have determined the number of PCR reactions you need per sample and available multiplexing capabilities, 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 µL reactions using 96-well PCR plates. PCR conditions have been optimized to amplify 825 ng of gDNA per 50 µL reaction. As described above, calculate the number of reactions needed for the amplification of total gDNA necessary to maintain the fold representation used at transduction. Table 8 provides the components and volumes needed for one 50 µL PCR amplification reaction.

Table 8. PCR components for amplification of genomic DNA.

Component	Volume per reaction (µL)	Final concentration
5x Phusion HF Buffer	10	1x
10 mM dNTPs	1	200 µM each
Edit-R Pooled sgRNA Forward PCR Primer (50 µM)	0.5	0.5 µM
Edit-R Pooled sgRNA Reverse Index PCR Primer (50 µM)	0.5	0.5 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	

2. To PCR amplify the Edit-R sgRNA constructs, use the cycling conditions outlined in Table 9. The conditions indicated in this protocol ensure that amplification is in the exponential phase when using Phusion Hot Start II DNA Polymerase; if using another polymerase, the optimal cycle conditions should be determined empirically.

Table 9. PCR cycle parameters

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

3. Confirm that a 357 base pair amplicon is obtained from each sample by running 10 µL of PCR product on a 2% agarose gel.



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

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



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

7 Illumina platform sequencing

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

Please follow the manufacturer's instructions for Illumina platform sequencing. Quantify the library using the quality standards that are recommended for your Illumina sequencing platform and then follow Illumina's instructions for dilution and denaturation of the library.

For the Illumina MiSeq Desktop Sequencer, we recommend spiking the library with 10% PhiX Control to serve as an internal control and loading the library onto the flow cell at 7 to 10 pM using standard loading volumes. After loading your sample into the Illumina MiSeq Reagent Cartridge, add 5 µL of the 100 µM Edit-R Pooled sgRNA Read 1 Sequencing Primer stock to the "Read 1 Primer mix" reservoir and 5 µL of the 100 µM Edit-R Pooled sgRNA Index Read Sequencing Primer stock to the "Index Primer Mix" reservoir on the Illumina MiSeq reagent cartridge (**please check the MiSeq System User Guide to confirm the inlet positions**).



PhiX Control contains a balanced representation of A, T, G and C nucleotides, thus aiding with sequencing of unbalanced or low diversity libraries that may result during a pooled lentiviral library screen.

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

At minimum, 19 base single-end reads are required to identify the sgRNA target sequence, as outlined in Figure 7.



The Edit-R Pooled sgRNA Reverse Index PCR Primers are designed for the Illumina sequencing platforms and they use the same indices as the Illumina TruSeq LT Index sequences, A001 to A027 (see Table 7).

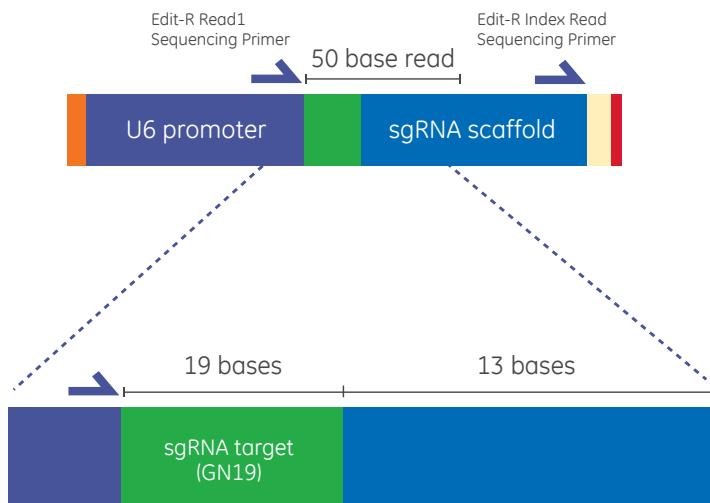


Figure 7. Position of Edit-R Pooled sgRNA Read 1 and Index Read Sequencing primers and contents of 50 base sequencing read, including the sgRNA target sequence.

8 Hit identification and follow up

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

Alignment of sequence files and hit identification can be performed 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 [Edit-R Lentiviral sgRNA Pooled Screening Library](#) webpage under the Resources tab. Samples should be de-multiplexed on the sequencer to bin each index tag with the appropriate sample. The first 19 bases of each read correspond to the sgRNA target sequence and are sufficient for differentiating among unique sgRNAs. The remaining bases in the read include the sgRNA vector backbone and can be ignored in the subsequent analysis (Figure 7). Using an alignment tool, such as Bowtie 2,⁸ 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 DESeq,⁹ to determine primary hits. Hits can be confirmed and studied further using individual Edit-R Lentiviral sgRNAs.

9 Appendix

A. Laboratory protocols and calculation tracking worksheet

Dharmacon™ Edit-R™ Lentiviral sgRNA Pooled Screening Library Laboratory protocols and calculation tracking sheet

This laboratory protocols and calculation tracking sheet is provided as a benchtop guide for using this product. We strongly recommend that you thoroughly read through the Technical Manual before using this guide. The steps in this lab protocol and calculation tracking sheet are numbered & lettered to correspond to the relevant sections in the Technical Manual.

INPUT your values into light blue boxes OUTPUT values are displayed in light grey boxes

4 Required materials for lentiviral sgRNA pooled library screening

Determine materials required

5 Assay development and optimization

A. Selection of the optimal promoter for Cas9 expression

Select the promoter which is most active in your cells for optimal levels of Cas9 expression. Make note of this selection.

B. Optimization of lentiviral transduction

Transduction conditions should be determined for each cell line and screen. Input those parameters here:

Transduction medium: % FBS (0.5-2% recommended)

Transduction duration: hours (4-24 hours recommended)

Transduction medium additives: µg/mL Polybrene (0-10 µg/mL recommended)

Cell density at transduction: cells/mm²

C. Determination of functional titer

Edit-R Lentiviral sgRNA Pooled Library titer as provided in Certificate of Analysis (C of A), e.g., 5e8: TU/mL

Edit-R Lentiviral sgRNA Non-targeting Control titer as provided in Certificate of Analysis (C of A), e.g., 2e7: TU/mL

Determine the lentiviral titer in your cell line of choice using the Edit-R Lentiviral sgRNA Non-targeting Control Particles in a functional titration protocol such as limiting dilution with crystal violet staining or genomic qPCR (see Technical Manual for further information).

Functional titer of non-targeting control in your cell line: TU/mL functional titer

Relative transduction efficiency of your cell line:

$$\frac{\text{Functional titer of Edit-R Lentiviral sgRNA Non-targeting Control in your cell line (TU/mL)}}{\text{Titer of Edit-R Lentiviral sgRNA Non-targeting Control particles stock reported on the C of A (TU/mL)}} = \text{Relative transduction efficiency of your cell line}$$

$$\text{TU/mL functional} \div \text{TU/mL} = \text{relative transduction efficiency of your cells}$$

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

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

$$\text{relative transduction efficiency} \times \text{TU/mL} = \text{TU/mL relative functional titer of library in your cells}$$

D. Optimization of blasticidin and puromycin selection

Generate an antibiotic kill curve to determine the optimal concentration of each antibiotic and the minimum number of days under selection required to kill 100% of non-transduced cells.

For many mammalian cells, the blasticidin concentration range is 2-15 µg/mL in 5-15 days of selection; for puromycin it is 0.5-10 µg/mL in 3-6 days.

Blasticidin concentration: µg/mL blasticidin

Duration of antibiotic selection: days

Puromycin concentration: µg/mL puromycin

Duration of antibiotic selection: days

E. Determination of assay-specific screening conditions

Assay-specific conditions, such as application of selective pressure and phenotypic selection, should be determined and optimized before beginning the screen. Wherever possible, optimize your assay(s) using a positive control sgRNA against a known gene target.

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

Critical parameters to decide upon include average sgRNA fold representation and the number of biological replicates. The Technical Manual provides details on how to determine these factors.

Average sgRNA fold representation during transduction: fold representation (> 500 recommended)

Number of biological replicates: replicates (> 1 recommended)

Number of sgRNAs per pooled library: sgRNAs

G. Calculation of number of cells needed for transduction**Desired number of cells with lentiviral integrants (calculate for each lentiviral pooled library):**

$$\begin{array}{l} \boxed{\text{Number of lentiviral sgRNAs in the pool}} \times \boxed{\text{Desired fold representation}} = \boxed{\text{Desired number of cells with lentiviral sgRNA integrations}} \\ \text{sgRNAs} \times \text{fold representation} = \text{cells with lentiviral integrations} \end{array}$$

Desired MOI: _____ (to ensure single integration events, MOI should always be < 1, 0.3 is recommended)

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

$$\begin{array}{l} \boxed{\text{Desired number of cells with lentiviral sgRNA integrations}} \div \boxed{\text{Proportion of cells with lentiviral sgRNA integrations}} = \boxed{\text{Number of cells required at the time of transduction}} \\ \text{cells with lentiviral integrations} \div \text{proportion of cells with integrations} = \text{cells required at transduction} \end{array}$$

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

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

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

$$\begin{array}{l} \boxed{\text{Number of plates required per sample}} \times \boxed{\text{Number of biological replicates}} = \boxed{\text{Number of plates required per pool}} \\ \text{plates required per sample} \times \text{biological replicates} = \text{plates per pool} \end{array}$$

H. Calculation of volume of lentiviral particles needed for transduction**Transducing units of lentiviral particles (calculate for each lentiviral pooled library):**

$$\begin{array}{l} \boxed{\text{Desired MOI}} \times \boxed{\text{Number of cells required at the time of transduction}} = \boxed{\text{Required number of lentiviral particles (TU)}} \\ \text{MOI} \times \text{cells required at transduction} = \text{TU (transducing units)} \end{array}$$

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

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

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

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

6. Primary screen**A. Cell transduction and selection screen****i. Transduction of cells with Edit-R Lentiviral Cas9 Nuclease particles****Day 1**

1. Seed cells in normal Growth Medium. Incubate overnight.

Day 2

2. Remove the Growth Medium and add medium with _____ % FBS, _____ µg/mL Polybrene and the appropriate volume of **Edit-R Lentiviral Cas9 Nuclease Expression** particles so that the cells are just covered.
3. _____ hours post-transduction, add additional Growth Medium to your cells such that the cells can be incubated for 48-72 hours.

Days 4-14

4. At 48-72 hours post-transduction begin **blasticidin** selection to remove non-transduced cells. Monitor the cells daily.
5. Every 2-3 days, replace with fresh Selection Medium containing **blasticidin**. Passage the cells as needed.

Once a pure population of transduced cells has been obtained, test for gene editing with a positive sgRNA control and expand the cells for transduction with the lentiviral sgRNA pooled library.

ii. Transduction of Cas9-expressing cells with Edit-R Lentiviral sgRNA Pooled Screening Library**Day 1**

1. Seed cells in normal Growth Medium. Incubate overnight.

Day 2

2. Remove the Growth Medium and add medium with _____ % FBS, _____ µg/mL Polybrene and the appropriate volume of **Edit-R Lentiviral sgRNA Pooled Library** particles so that the cells are just covered.
3. _____ hours post-transduction, add additional Growth Medium to your cells such that the cells can be incubated for 48-72 hours.

Days 4-18

4. At 48-72 hours post-transduction begin **puromycin** selection to remove non-transduced cells. Monitor the cells daily.
5. Every 2-3 days, replace with fresh Selection Medium containing **puromycin**. Passage the cells as needed.

Once a pure population of puromycin-resistant cells has been obtained, begin selection screening.

Split cells into at least two populations: one as a reference and another for application of selective pressure and phenotypic selection.

Maintain your desired sgRNA fold representation in the library at each cell passage.

B. Genomic DNA isolation

1. Collect cells for gDNA isolation by trypsinizing and counting.
Use at least the number of cells that corresponds to the desired number of lentiviral integrants.
Follow manufacturer's protocol for gDNA isolation.
Combine gDNA isolations after elution, as needed.
2. Assess DNA purity and quantify the isolated gDNA by spectrophotometry.

C. PCR amplification of sgRNA from genomic DNA**i. Number of PCR reactions****Amount of gDNA required to maintain fold representation (calculate for each lentiviral pooled library):**

Number of cells with lentiviral integrations	\times	Nanograms per genome	=	Mass of gDNA required to maintain representation of each sgRNA (ng)
---	----------	-------------------------	---	--

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

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

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

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

Number of PCR reactions per sample	\times	Number of samples per pool	=	Number of PCR reactions per pool
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PCRs per sample \times samples per pool = PCR reactions per pool

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

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

ii. Multiplexing of high-throughput sequencing samples**Sequencing reads per sample**

Number of unique sgRNAs in lentiviral pool	\times	Read coverage per sgRNA	=	Output reads required per sample
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$\text{sgRNA} \times 1000 \text{ reads/sgRNA} = \text{output reads required per sample}$

Sample indices per sequencing lane

Expected deep sequencing read output	\times	0.7 (number of reads adjustment)	\div	Output reads required per sample	=	Number of sample indices per lane
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reads $\times 0.7 \div$ output reads per sample = sample indices per lane

iii. PCR from genomic DNA**1. PCR components for sgRNA amplification (calculate Master Mix for each sample)**

Component	Volume per reaction (μL)	Final Concentration	Master Mix (μL)	# of reactions per sample (rounded up to nearest whole number)
5x Phusion HF Buffer	10	1 \times		
10 mM dNTPs	1	200 μM each		
Edit-R pooled sgRNA Forward PCR Primer (50 μM)	0.5	0.5 μM		
Edit-R pooled sgRNA Reverse Index PCR Primer (50 μM)	0.5	0.5 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			

2. PCR cycling conditions

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

3. Combine reactions amplifying the same gDNA sample into a single 1.5 mL tube.Confirm that a 357-base pair amplicon is obtained from each sample by running 10 μL of PCR product on a 2% agarose gel.**4. Purify PCR products.****5. Evaluate purified amplicons using the quality standards recommended by your Illumina platform.**

7. Illumina platform sequencing

Follow the manufacturer's instructions for Illumina platform sequencing.

Load Illumina flow cell with amplicon samples (we recommend 7-10 pM using standard loading volumes).

Obtain at least 19 base single-end reads with the provided **Edit-R Pooled sgRNA Read 1 Sequencing Primer**.

Perform index read with **Edit-R Pooled sgRNA Index Read Sequencing Primer**.

8. Hit identification and follow up

Bin each index tag.

Trim sequences to 19 base pairs.

Align sequence reads with FASTA files provided with your lentiviral pooled library.

Count the number of alignments for each sequence that correspond to a gene-targeting sgRNA.

Perform differential expression analysis to determine primary hits.

B. Stability and storage

Edit-R Lentiviral sgRNA Pooled Screening Libraries are shipped on dry ice as 25 µL aliquots and must be stored at -80 °C. Under these conditions, lentiviral particles are stable for at least 12 months. Repeated freeze-thaw cycles should be avoided, as this is expected to negatively affect titer. When setting up a 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

Edit-R 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 pool master library glycerol stocks are sequenced using 1 × 50 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. A Certificate of Analysis (C of A) with specified lentiviral titers is included with each shipment.

10 Frequently asked questions

How do I choose between the various Edit-R Lentiviral Cas9 Nuclease promoter options?

Choose the promoter option that has been demonstrated, either by your own experimental observations or through references in the published literature, to actively express a transgene in your cells of choice. For optimal experimental confidence or if such information is not available, consider using multiple lentiviral promoter-Cas9 constructs or selecting the best promoter empirically using the Dharmacon SMARTchoice Promoter Selection Plate Cat #SP-001000-01 (dharmacon.gelifesciences.com/shrna/smartchoice-shrna-promoter-selection-plate/).

How many screens can I perform with my pools?

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

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

How do I know if my target cell type can be transduced with Edit-R Lentiviral Cas9 Nuclease and sgRNA particles?

Edit-R Lentiviral Cas9 Nuclease and sgRNA vectors are pseudo-typed with the VSVg envelope protein to allow broad tropism across many cells. Therefore, most cell types are transduced, albeit, with varying degrees of efficiency. However, the efficiency of target gene regulation is not only a factor of successful transduction, but is also dependent upon the efficiency of transgene expression as driven by the selected promoter.

Can Edit-R Lentiviral Cas9 Nuclease or sgRNA particles be further propagated in the lab?

No. Edit-R lentiviral products are engineered for biosafety and are provided as replication-incompetent lentiviral particles.

I am using human cell lines in my research; should I always choose a human promoter for best Cas9 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 around the world without incident. Handling of lentiviral products requires extensive experience with cell culture techniques. It is vital that the protocols provided by Dharmacon and the safety guidelines described for appropriate handling and storage are fully understood and followed precisely (see Section 12, Lentiviral particle product safety level information).

How are Dharmacon lentiviral particle products manufactured?

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

How are lentiviral particle products shipped?

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

How should lentiviral particle products be stored?

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

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

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

Can lentiviral particle products be kept at 4 °C?

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

Where can I find titer information for my pools?

The titer of your lentiviral pools will be indicated on the Certificate of Analysis (C of A) and can also be requested from Technical Support.

Where can I find the targeting sequences for sgRNAs included in my pool(s)?

The sgRNA target sequences are in the data files sent with your library shipment. Please contact Technical Support with your Purchase Order number 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 10) below.



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

Table 10. Constants needed for calculating gDNA input.

Constants	Value
Mass of a base pair	660 g/mol/bp
Base pairs per diploid human genome	6×10^9 bp
Base pairs per diploid mouse genome	5.6×10^9 bp
Avogadro constant	6.02×10^{23} mol ⁻¹

Example:

Calculate the mass of human genome:

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

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

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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 technical 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 (<http://osp.od.nih.gov/office-biotechnology-activities/biosafety/biosafety-guidance>);
2. The U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories (BMBL);
3. The NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines) (<http://osp.od.nih.gov/office-biotechnology-activities/biosafety/nih-guidelines>).

In the EU:

For the EU directives, please consult the following:

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

In Germany:

Required Containment Measures: The containment requirements as stated in the German Genetic Safety Ordinance (Gentechnik-Sicherheitsverordnung) of Safety Level 2* or higher have been assigned to the handling of the above-mentioned lentiviral vector particles. Please note a higher Security Level might be required if the lentiviral vector particles are used for genetic engineering operations with other products which require a higher Security Level.

*Safety Level 2: activities of low risk for human health and the environment by the state of scientific knowledge (Stand der Wissenschaft).

For the German regulations, please consult the following:

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

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Name Inventor	Title	Patent No.	Application No.	
Graham/Rice	Control of Gene Express	ZA 2000/4507* AU2001100608* SG75542* US 6,573,099* GB2353282* AU 743316* NZ 506648* US 10/346853* PCT/AU/99/00195* BR PI9908967-0* CA 2323726* CB 99804255-2* CZ PB2000-3346* EP 99910039.9* US10/646,070 US 10/759,841* EP 04015041.9* AU2005211538	HK 01105904.3* HU PO101225* IN 2000/00 169/DEL* JP P2000-537990 KR 7010419/2000* MX008631* PL P.343064* SK PV 1372-2000* AU 35647/02* NZ 525941* SG200205122-5* US 09/646807* PP2492/98* AUPP2499/98* US 10/821,710* US 10/821,726* AU 2005209648	
Graham/Rice/M/R	Genetic Silencing	WO 01/70949 GB 237722 AU PQ6363	SG 91678 ZA2002/7428 AU2001240375 AUPR2700	
Grahm, et al.	Double-Stranded Nucleic Acid	AU 2003906281 AU 2003906281 US 10/861,191	AU 2004902279 PCT/AU04/000759	

OXFORD TECHNOLOGY

PCT Application (filing date)	Priority Application(s) (filing date)	National Application	Issued Patents
PCT/US91/05699 (9 Aug 1991)	US 07/586,603 (21 Sept 1990)	US 08/361,839	5,817,491
	US 07/658,632 (19 Feb 1991)	EP 91915104.3 JP 3-514518 AU 84302/91 CA 2,104,396	0572401 3547129 663470 2,104,396
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