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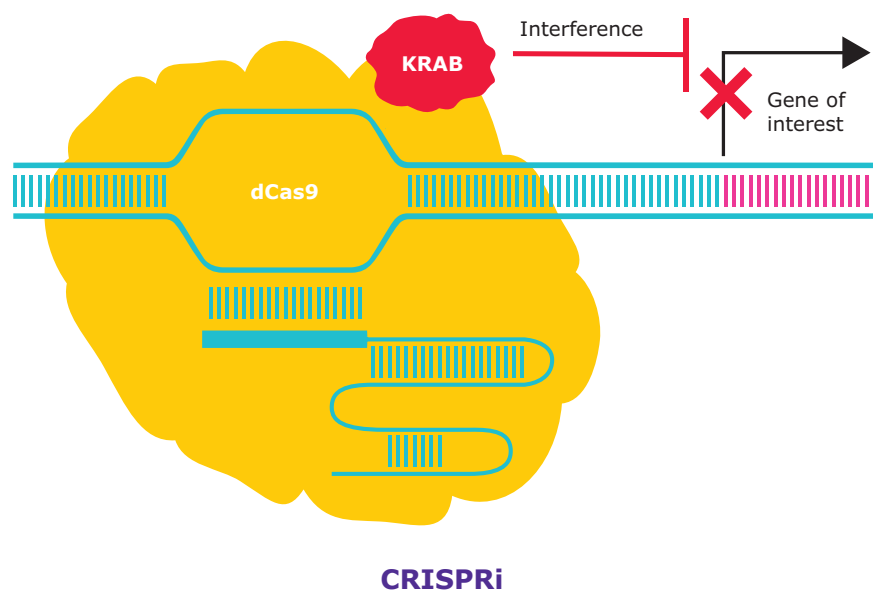
INSTRUCTIONS FOR USE

CRISPRi

Human Whole Genome and Long Non-Coding Library Screening User Manual

Interference

KRAB

Gene of interest

dCas9

CRISPRi

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Ordering Information

SigmaAldrich.com/CRISPRi

CRISPRi Available Libraries & Control Kits

CRISPRi Library Description	Target	Cat. No.	Number of Genes
Human Whole Genome & Non-Coding CRISPRi Library	Whole genome	CRISPRILIB-1KT	18,905
Control and Effector Kit: positive & negative control with dCas9	Coding and non-targeting	CRISPRICON-1KT	Coding gene RAB1A and a non-targeting control

CRISPR Sub Pool Descriptions	Target	Cat. No.	Number of Genes
10x Feature Barcode Optimization Kit	Coding	CRISPRI10X-1KT	Coding gene RAB1A and a non-targeting control
Druggable Genome Pool		CRISPRIS01-1KT	2,318
Cancer & Apoptosis Pool		CRISPRIS02-1KT	2,916
Stress Proteostasis Pool		CRISPRIS03-1KT	3,093
Mitochondria/Trafficking/Motility Pool		CRISPRIS04-1KT	2,219
Gene Expression Pool (Transcription Factors)		CRISPRIS05-1KT	2,292
Membrane Proteins Pool		CRISPRIS06-1KT	2,418
Unassigned Pool (Uncategorized Gene Function, Novel Targets)		CRISPRIS07-1KT	1,824
Common Cancer Cell Lines Pool & iPSC Pool: Common to 7 cell lines (5 cancer, iPSC, HFF)		CRISPRIS08-1KT	691
iPSC Pool	Long non-coding	CRISPRIS09-1KT	3,000
Common Cancer Cell Lines Pool (HeLa, HEK293T, MCF7, U87 cell lines)		CRISPRIS10-1KT	1,329

Storage Conditions

| Component	Temperature	Notes
CRISPRi Lentivirus Storage	−70 °C	Store CRISPRi in a non-frost-free freezer and avoid multiple freeze-thaw cycles. Store at −70 °C long term.

1. BEFORE YOU BEGIN

CRISPR/Cas9 gene editing has become an increasingly popular solution for introducing permanent loss-of-function (LOF) mutations by generating double-strand breaks at targeted genomic loci, after which endogenous cellular machinery repairs the cleaved DNA. LOF stems from the introduction of frameshift mutations during this repair process. The CRISPR/Cas9 system's adaptation expands the application of this technology, allowing CRISPR-mediated gene activation (CRISPRa) and repression (CRISPRi) without altering the DNA.

1.1. How CRISPRi Works

In contrast to regular CRISPR/Cas9 gene editing, CRISPRi employs a catalytically inactive form of Cas9 (dCas9). This Cas9 variant possesses point mutations in two amino acid residues, D10A and H840A, that deactivate the RuvC and HNH nuclease domains of Cas9, respectively.[1] Although dCas9 cannot cut DNA, it is still precisely recruited to the target DNA by the guide RNA (gRNA). Synthetic biologists have taken advantage of this property of dCas9 by constructing dCas9-based tools that expand the functionality of the CRISPR/Cas9 system.

dCas9 can be transformed into a transcriptional activator or repressor by fusing or otherwise recruiting the appropriate transcriptional effector domains to the inactive nuclease (**Figure 1**). The primary transcriptional repressor used in combination with dCas9 is the Krüppel associated box (KRAB) domain from KOX1.[3]
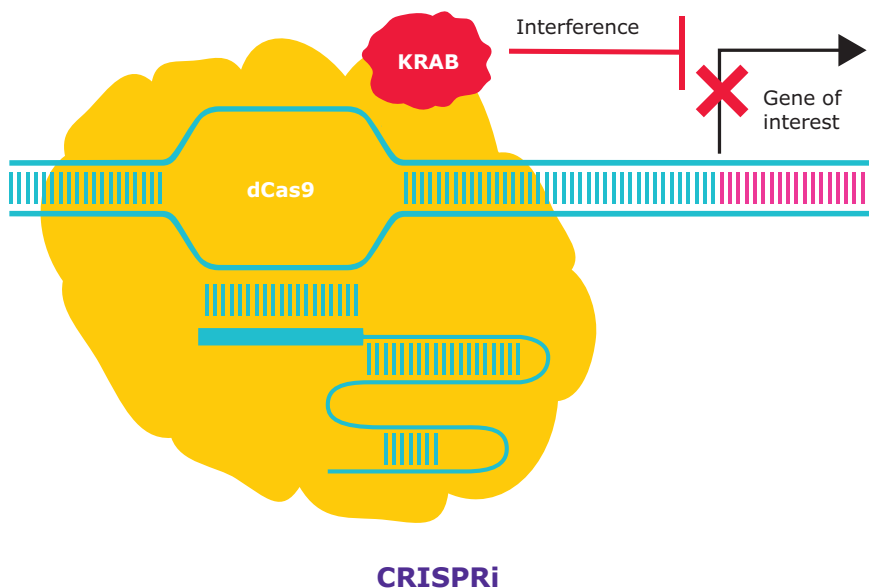
Figure 1. CRISPRi utilizes catalytically inactive Cas9 and transcriptional repressors to modulate gene expression.

1.2.   Considerations for Designing a CRISPRi Experiment

The design of gRNAs differs between CRISPR inhibition and traditional CRISPR knockout, where efficient gRNAs target early exons in protein-coding genes to prevent the expression of a truncated protein that may retain some function. A screen of all possible gRNAs surrounding the transcriptional start site (TSS) of 49 genes enabled the identification of the optimal targeting windows for CRISPRi.[6]

Consistent with its dual mechanism of action, CRISPRi using the dCas9-KRAB repressor was efficient for gRNAs falling in a window spanning from −50 to +300 base pairs (bp) from each transcriptional start site (TSS), with the best-performing gRNAs targeting the first 100 bp downstream of the TSS.[6]

Since the efficacy of a CRISPRi gRNA is impacted by the proximity to the TSS of the targeted gene, meaning gRNA design for CRISPRi is more complicated for poorly annotated genomes. The efficacy of gRNAs for CRISPRi is reduced by:

- Long protospacer lengths (>21 bp).
- The presence of nucleotide homopolymers (e.g., AAAA or GGGG) in the protospacer region.
- The chromatin environment surrounding the targeted genomic region can limit the nuclease accessing the site.[7]

Identifying highly active gRNAs is vital for constructing genome-scale CRISPRi libraries. Such libraries include 3–10 gRNAs per gene to ensure that screening hits are not because of inadequate gRNA efficacy.[8, 9] Pooled gRNA screens offer a robust and straightforward method for clarifying how various factors influence gRNA activity, as thousands of gRNAs can be tested with relatively minimal effort.[10]

Pooled gRNA screens also inform gRNA prediction algorithms. Observations, such as the effects of gRNA sequence, position, and accessibility, have enabled the iterative and comprehensive identification of highly active gRNAs.[11, 12] This has led to the development of highly active and compact (i.e., fewer gRNAs per gene) gRNA libraries for CRISPRi.[9] Ultimately, compact CRISPR libraries reduce the number of cells needed to perform genome-scale screens.

Superior algorithm placement and gRNA scaffold improvements enhance your CRISPRi screening experiments. The Sigma-Aldrich® suite of functional genomics solutions includes optimized, whole-genome gRNA libraries for CRISPRi as well as custom gRNA design services for individual targets and large-scale libraries.

1.3.   CRISPRi Compared to Alternate Technologies

The mechanisms of CRISPRi differs from other gene perturbation technologies, such as RNA interference (RNAi) and CRISPR KO (**Figure 2**). These differences offer advantages and challenges that should be taken into consideration when selecting which technology to implement.
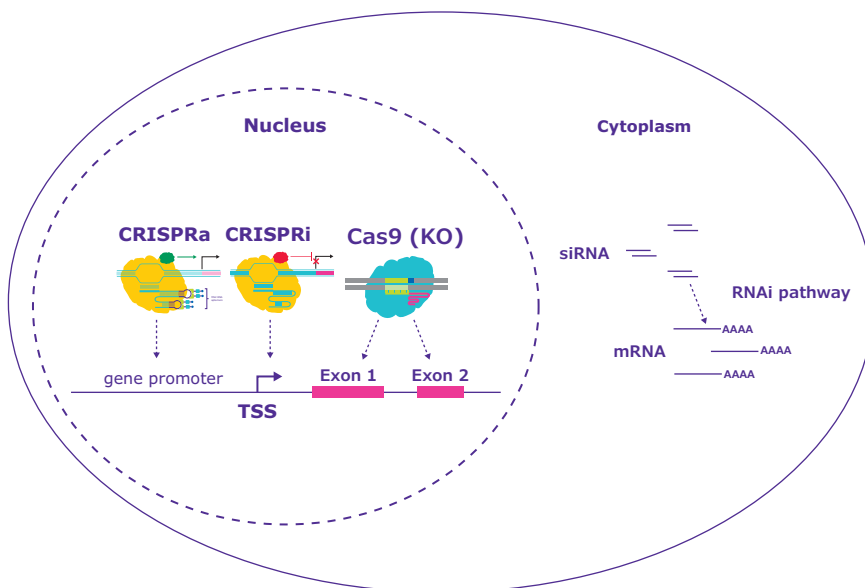
Figure 2. Mechanism of CRISPRa and CRISPRi compared to RNAi and CRISPR KO.

1.4. CRISPRi vs. RNAi

The mechanism of CRISPRi differs from other gene perturbation technologies, such as RNA interference (RNAi). RNAi inhibits gene expression by utilizing a conserved RNA processing pathway to degrade targeted mRNAs in the cytoplasm. The cytoplasmic location of the endogenous machinery co-opted by RNAi prevents it from efficiently targeting nuclear RNA, such as ncRNA.[13] RNAi can also induce off-target effects by displacing endogenous small RNAs (e.g., microRNAs) from RISC.[14] CRISPRi avoids these unwanted effects because all of the components are from exogenous sources. Finally, CRISPRi outperforms RNAi in large-scale screening applications by generating more robust phenotypes with fewer off-target effects.[6]

1.5. CRISPRi vs. CRISPR KO

Although CRISPR gene editing is a powerful method for generating LOF mutants, several disadvantages preclude its use in all LOF studies. The double-strand breaks caused by Cas9 can induce cytotoxicity and genomic instability, particularly in cancer cell lines.[15, 16] Also, roughly one-third of indels will not cause the necessary frameshift to knock out targeted genes. Non-coding regions (e.g., long non-coding RNAs) can be difficult to target by Cas9 because they require edits at two sites to create a sufficiently large mutation to generate LOF.[17] CRISPR KO is also a suboptimal solution for investigating the effects of genes that are essential for cell survival beyond their identification in genome-wide screens as cells can tolerate a partial knockdown, but not a complete knockout, of such genes.[10] By not cutting DNA, CRISPRi circumvents the disadvantages of CRISPR KO, making it a superior choice for perturbation of non-coding genes or applications that call for a reversible or titratable solution.

1.6. Combinatorial Screening Methods

CRISPRi and CRISPRa add to the existing suite of genome engineering technologies by offering complementary insights into complex biological processes. Unlike CRISPR KO, which provides binary and permanent outcomes, CRISPRa and CRISPRi can be used to reversibly titrate gene expression levels within a broad, dynamic range (up to and exceeding 1,000-fold).[2] Also, large-scale LOF and GOF screens can identify unique, yet functionally related, hits under otherwise identical screening parameters. The identification of SPI1 (by CRISPRa) and GATA1 (by CRISPRi) as regulators of cell growth in the K562 human myeloid leukemia cell line highlights the usefulness of this parallel use of CRISPRi and CRISPRa.[6] Both manipulations increased cell growth in K562 cells, confirming SPI1's known inhibitory effects on GATA1 activity.[19] Therefore, while individual CRISPR screens are independently powerful tools, when used in combination, CRISPRi, CRISPRa, and traditional CRISPR KO screens offer robust validation and strengthen findings, as well as provide a more detailed understanding of gene pathways.

1.7. How to Perform a CRISPRi Experiment: Overview

A typical CRISPRi experiment involves three necessary steps:

- Ideally, the generation of a stable cell line that expresses the modified Cas9 with the appropriate CRISPRi constructs.
- Delivery of the gRNA.
- Analysis of the results.

Below is a brief overview of these steps

Generation of a Helper Cell Line

Transient transfection of all components is sufficient for cell lines that are efficiently transfected (e.g., HEK293 cells), while lentiviral transduction provides more consistent and robust results across a variety of cell lines. For large-scale screening applications, creating a stable "helper" cell line that expresses dCas9-KRAB at the outset permits rapid validation at the end of the screening process. Sigma-Aldrich® lentiviral vectors are available for helper cell line generation that are optimized to improve functional viral titers.

gRNA Delivery

Lentiviral transduction of gRNAs followed by antibiotic selection ensures consistent and robust activation for both small and large-scale applications. Lentiviral delivery is essential for pooled CRISPR screens because genomically-integrated gRNA sequences serve as barcodes for downstream NGS deconvolution. As such, pooled CRISPR libraries should be delivered at a low (<0.3) multiplicity of infection to ensure each cell receives only a single CRISPR copy.[8]

Analysis

For small-scale applications, analysis involves measuring changes in mRNA and protein levels using quantitative polymerase chain reaction (qPCR) and western blotting, respectively, as well as the functional consequences of those changes. Pooled CRISPR screens use next-generation sequencing to analyze changes in the representation of lentivirally transduced gRNAs following a selection process.

1.8.   Applications of CRISPRi

CRISPRi is particularly amenable to genome and subgenome-scale screening applications, including identifying drug targets[20] and cellular factors that mediate drug resistance,[5] due to the relative ease with which gRNA libraries can be synthesized, cloned, and packaged in lentivirus.[6, 5] Arrayed screening is also possible with CRISPRi. Sigma-Aldrich® webinars provide more insight into CRISPR screening including the benefits of a whole-genome gain-of-function screening and the differences between CRISPRa, CRISPRi and CRISPR KO screens.

Although pooled CRISPR screening has many advantages over arrayed screening, it is typically limited to studying crude phenotypes such as cell growth and viability. By contrast, virtually any type of analysis can be done in an arrayed screen because CRISPR perturbations occur in individual wells of microtiter plates. The advent of single-cell RNA sequencing, however, provided a high-content transcriptomic analysis that could be integrated into pooled CRISPR screen workflows. This is because each CRISPR perturbation, including its transcriptomic and phenotypic effects, is confined to an individual cell.[21] Thus, as long as individual cells can be separated, CRISPR perturbations can be linked to a transcriptomic readout as well as a phenotypic outcome. CRISPR single-cell screening in partnership with 10x Genomics is now available from the Sigma-Aldrich® portfolio.

CRISPRi is also able to distinguish between different transcript variants, unlike CRISPR KO and RNAi, as long as the TSS for each variant is mapped.

1.9.   Considerations for sample preparation and NGS

Adequate representation of the complex cell population should be maintained during library preparation. Genomic DNA is isolated from a number of cells that is 500-fold the number of different sgRNAs in the screened library.

In the PCR-amplified material, the sgRNA-encoding region is highly enriched compared with its abundance in pre-PCR genomic DNA. Therefore, it is critical to avoid cross-contamination of pre-PCR material with any post-PCR material. Ideally, the pre-PCR steps should be performed using separate equipment (e.g., pipettes and vacuum manifolds) from that used for post-PCR steps.

Although growth and selection conditions must clearly be optimized for each screen and cell type, several general principles should guide screen design:

- Selection should be set up so that a sufficient dynamic range between treated and untreated conditions is detected.

It is extremely important to avoid population bottlenecks at each stage of the experiment: lentiviral infection, screen selection and preparation of the sequencing sample. Seek to maintain 500–1,000-fold representation of each library sgRNA on average at each stage. However, it is often difficult to conduct the infection at 1,000-fold coverage (as the target infection rate is 20–50%), so we infect at around 300-fold coverage, select, and then harvest "T0" post-selection. Any noise introduced with this infection bottleneck is largely ignored as it occurs pre- T0.

- For example, for a screen of an sgRNA library with 50,000 different sgRNAs, the cell population should be maintained at 50 million cells or more. In particular, when designing a screen, care should be taken to avoid selective pressures that are so high that a large fraction of the cell population is killed. We aim for selective pressures that do not kill more than 50% of the population at any time point during the screen.

- It can be more challenging to confidently detect sgRNAs that cause cells to drop out of the screen, especially if they also affect growth under standard conditions, as phenotypes for such sgRNAs are then calculated on the basis of low numbers of reads in the sequencing data, which inherently will increase statistical noise. Therefore it is recommended to follow the above recommendations of cell coverage and non-targeting control (NTC) for statistical analysis.

1.10. CRISPRi Summarized

CRISPRi is a potent tool for modulating gene expression in a reversible and titratable manner. This technology uses dCas9 to act as a synthetic transcription factors by recruiting endogenous transcription repressor complexes to gene promoters and enhancers, resulting in down regulated gene transcription. CRISPRi achieves LOF phenotypes without the limitations of RNAi and CRISPR KO, although CRISPRi usually relies on delivery by lentivirus, this technology offers new possibilities for genome engineering. CRISPRi can be used individually to target areas of the genome that are inaccessible by other gene perturbation technologies (e.g., non-coding regions) or in conjunction to uncover gene-regulatory networks underlying discrete phenotypes. Finally, pooled CRISPRi screening can be paired with single-cell RNA-sequencing methods to enable high-dimensional characterization of CRISPR gene perturbation.

2. PROTOCOL SECTION

2.1. Antibiotic concentrations for CRISPR library selection: Kill Curve

A kill curve is a dose-response experiment where cells are subjected to a range of antibiotic concentrations to determine the minimum concentration of antibiotic needed to kill all cells over a desired period of time. This step is suggested for each new cell line tested as well as when a new selection antibiotic is used. High concentrations of antibiotic are undesirable as they can lead to toxicity, even in successfully transduced populations.

Required Reagents:

- Target cells in culture
- Antibiotic
  - Puromycin (10 mg/mL solution), Cat. No. P9620
  - Blasticidin S hydrochloride (lyophilized), Cat. No. 15205
- Complete growth media specific for the target cells

Protocol:

- Plate cells according to normal passaging protocols for the cell line in use.
- The next day, replace the media with media containing serial dilutions of the antibiotic to be used during selection:
  - Puromycin: 0.1–10 mg/mL
  - Blasticidin: 2.5–50 µg/mL
- Examine the cells daily for visual signs of toxicity.
- Replace the media containing antibiotic every 2–3 days. Culture for 3–15 days depending on growth rate of the cell type and length of time that cells would typically be under selection during a normal experimental protocol.

> The minimum concentration of antibiotic that causes complete cell death after the length of time that cells would typically be under selection should be used for that cell type and experiment. Some cells require passage or expansion into a larger vessel in order to avoid senescence or differentiation and continue antibiotic selection.

2.2. Multiplicity of Infection (MOI)

Multiplicy of infection (MOI) represents the number of viral particles per cell. For example, an MOI of 0.5 means you have 0.5 viral particles per cell, or one viral particle for every two cells. When establishing a stable cell line using lentivirus, it is generally important to ensure no more than one viral particle per cell. For single constructs or single CRISPR guides, we recommend an MOI of less than 0.7–1 viral particles per cell. Preventing incorporation of more than one guide becomes critical when transducing cells with large pools of guides. NGS deconvolution and analysis of biological relevance of top hits is easily confounded by having multiple guides in a single cell. For pooled library screens, we recommend an MOI of <0.3. With an MOI of 0.3, you would expect 70% of your cells to die off during selection, assuming equal growth rates between cells. To ensure an accurate MOI, we recommend determining the functional viral titer in your cell line (see section 3.2).

2.3. Preparatory Work

- Perform a 3–15 day kill curve in each target cell stock for each antibiotic used. Selection timeline varies with cell type.
- For CRISPRILIB-1KT and KRAB-dCas9 the, the sensitivities to puromycin and blasticidin must be determined.
- Determine the Multiplicity of Infection (MOI) for each target cell type

2.4. Generation of Stable dCas9+KRAB Helper Cell line

Day 1 — Seeding Cells

- We generally recommend seeding cells in smaller volumes such as a 6-well cell culture plate with enough cells for 10% confluency.
- Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 2 — Transduction

- Remove the medium from each well of the plate(s) prepared on day 1.
- Add 8 µg/mL polybrene-containing medium to each well. Gently swirl the plate to mix.
- Transduce the cells with the helper construct(s): using the equation below, determine and add the appropriate volume of lentiviral particles for each construct. The desired MOI is <0.7. Leave at least one well for selection of non-transduced cells, one well for cells with virus but without selection, and one well for cells without virus or selection agent.

$$Volume\ of\ Viral\ Particles\ (\mu L) = 1000\ \mu L \times \frac{Number\ of\ Cells \times Desired\ MOI}{Viral\ Titer\ (TU/mL)}$$

- Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 3 — Medium Change

- Remove the virus-containing medium from each well of the plate(s). Sterilize discarded virus medium and contaminated supplies using a 10% bleach solution for 24 hours.
- Add fresh growth medium (without polybrene) to each well.
- Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 4 — Antibiotic Selection

- Remove the medium from each well.

- Feed cells with fresh growth medium containing the appropriate concentrations of antibiotic(s).

Days 5–10 — Continued Antibiotic Selection

- Maintain cells under antibiotic selection for the duration of the screen to maintain cells actively expressing CRISPR components.

- Replace the antibiotic-containing medium as necessary during the selection process (usually every other day). The non-transduced control cells should die completely after 7-day selection with antibiotic.

- Following selection and subsequent expansion, split selected cells; bank a frozen stock for future culture (CryoStor® cell cryopreservation media, Cat. No. C2874) and grow the rest for transduction with the gRNA library.

2.5. Transduction of the gRNA Library

Prior to transduction of your library or pool, functional viral titer must be determined. See section 3.2 for guidance.

Day 1 — Seeding Cells

- Determine the appropriate number of cells to seed in each well using the equation below. The desired MOI should be <0.1–0.3 to ensure that no cells receive more than one guide. The desired coverage is 300–500x. *For example, assuming 24,000 guides per subpool, a coverage of 500x, and a desired MOI of 0.2, you would require 60,000,000 cells for transduction of 1 subpool.*

$$Number\ of\ cells = \frac{Number\ of\ Guides \times Desired\ Coverage}{Desired\ MOI}$$

- Seed the appropriate number of cells per well in fresh medium. We recommend seeding enough wells for 2–4 biological replicates per sample from independent library transductions.

- Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 2 — Transduction

- Remove the medium from each flask prepared.

- Add 8 µg/mL polybrene-containing medium to each flask. Gently rock the flask to mix.

- Using the equation below, determine and add the appropriate volume lentiviral particles. *For example, transduction of 60,000,000 at an MOI of 0.2 would require 80 µL of virus with a CFU functional titer of 1.5E+08.* Be sure to leave some wells as negative controls for selection, and remember to transduce 2–4 biological replicates for each sample.

$$Volume\ of\ Viral\ Particles\ (µL) = 1000\ µL \times \frac{Number\ of\ Cells \times Desired\ MOI}{Viral\ Titer\ (TU/mL)}$$

- Incubate at 37 °C with 5% CO_2 for 24 hours.

*Functional titer can be quantitated via CFU assay or FACS. Visit SigmaAldrich.com/LentiProtocols for additional information

Day 3 — Medium Change

- Remove the virus-containing medium from each flask.
- Sterilize discarded virus medium and contaminated supplies using a 10% bleach solution for 24 hours.
- Add fresh medium (without polybrene).
- Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 4 — Antibiotic Selection

- Remove the medium.
- Feed cells with fresh medium containing the appropriate concentration of puromycin.

Days 5–10 — Continued Antibiotic Selection

- Continue to select for transduced cells for the duration of the screen.
- Replace the antibiotic-containing medium as necessary during the selection process (usually every other day). The non-transduced control cells should die completely after 4–7 day selection with antibiotic.
- Following selection and subsequent expansion, we recommend splitting your cells to bank a frozen stock of selected, transduced cells for future culture (CryoStor® cell cryopreservation media, Cat. No. C2874), in case you need to restart the experiment.

Day 11 — Enrichment Screen

Expand and maintain enough cells for at least 3 samples, per replicate, at 300-500x coverage. Harvest one group of cells representing time-point zero (T0) for guide representation analysis by deep sequencing (frozen cell pellet is acceptable). The remaining two samples are cultured for your control and experimental conditions.

Culture enough cells during each passage to maintain an average guide coverage of 300–500x.

Perform your enrichment screen using the selection agent or phenotypic assay of your choice.

2.6. Harvesting Genomic DNA for Screening Analysis

Harvest cells using AccuMax™ solution (Cat. No. A7089) instead of Trypsin to reduce the carry-over of cell-free DNA and reduce noise on your sequencing reads. Capture genomic DNA for each sample using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Cat. No. G1N70).

2.7. PCR Amplification of gRNA cassettes for NGS

Nested amplification is the preferred method for this type of library generation. Direct amplification from integrated vector sequence in genomic DNA can lead to many nonspecific bands, often close in size to the intended target. Additionally, amplification of cell-free DNA carried over during gDNA harvesting can result in significant NGS read noise. Therefore, an initial outer PCR should be performed using primers detailed in **Table 1**. While library generation can be accomplished with a single round of amplification, this may require extensive PCR optimization and could lead to wasted reads in the sequencing reaction. Additionally, the length and cost of the final PCR primers that include any NGS platform-specific sequences, phase-shifting diversity sequences, and unique demultiplex barcodes, along with the number of PCR reactions required to consume the harvested unique genomes, would increase the cost of direct amplification step.

On both rounds of PCR, cycle number should be kept to a minimum. This is an important principle, since PCR-introduced errors and biases should be strictly avoided. To compensate for the resulting low yield of PCR amplicons, a large number of PCR reactions should be performed, and the final products combined, purified, and concentrated. Perform enough PCR reactions such that all the harvested gDNA is amplified. Each 50 µL reaction can hold up to 2.5 µg of gDNA.

Example Methods adapted from Joung et al., 2017 Nature Protocols

1. For all reactions of a single sample, make a single master mix (see **Table 2**, below) including outer PCR primers (see Table 1), template DNA, and JumpStart™ Taq ReadyMix™ reagent (Cat. No. P2893).

2. Aliquot reactions into each PCR well and amplify using the cycle conditions detailed in **Table 3**.

Table 1: Outer CRISPRi PCR Primers

Primer (all per sample)	Sequence 5'–3'
CRISPRi NGS-outer-fwd	AAATTCAAAATTTTCGGGTTTATTACAGGGACAGC
CRISPRi NGS-outer-rev	TACACGACATCACTTTCCCAGTTTACC

Table 2: Outer PCR Reaction Mix

Reagent	1X (multiplied by N reactions)
2X JumpStart™ Taq ReadyMix™ reagent	25 µL
5 µM NGS-outer-fwd Primer	2.5 µL
5 µM NGS-outer-rev Primer	2.5 µL
gDNA Template	2.5 µg
H$_2$0	to volume
	50 µL Reaction Total

Table 3: Outer PCR Reaction Conditions

95 °C	2 min	
95 °C	30 sec	
55 °C	30 sec	**25x**
72 °C	20 sec	
72 °C	2 min	

3. Purify the ~650–700 bp outer PCR amplicons using GenElute™ PCR Clean-Up Kit (Cat. No. NA1020). This will be the template for the inner nested PCR reaction.

4. Perform PCR amplification of the guide cassette from purified PCR amplicons, adapted from the guidelines outlined in Joung et al., 2017 Nature Protocols.

5. Combine each of the 10 Forward Diversity Primers (see **Table 4**, next page) for every sample. (e.g. 95 µL H$_2$0 + 0.5 µL each 100 µM fwd primer = 5 µM fwd primer mix).

6. For all reactions of a single sample, make a single master mix (see **Table 6**, next page), including template DNA, forward primer mix, and corresponding reverse primers (see **Table 5**, next page).

7. Aliquot reactions into each PCR well and amplify using the conditions detailed in **Table 7**.

Table 4: Example CRISPRi Inner PCR Forward Primers (with sample adapters and diversity sequences)

Primer (all per sample)	Sequence 5' – 3' (vector sequence underlined)
NGS-CRISPRi-Lib-Fwd-1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTAAGTAGAGGCTTTATATATCT<u>CTATAAGTATCCCTTGGAGAACCACCTTGTT</u>
NGS-CRISPRi-Lib-Fwd-2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCATGCTTAGCTTTATATATCT<u>CTATAAGTATCCCTTGGAGAACCACCTTGTT</u>
NGS-CRISPRi-Lib-Fwd-3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGATGCACATCTGCTTTATATATCT<u>CTATAAGTATCCCTTGGAGAACCACCTTGTT</u>
NGS-CRISPRi-Lib-Fwd-4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATTGCTCGACGCTTTATATA TCT<u>CTATAAGTATCCCTTGGAGAACCACCTTGTT</u>
NGS-CRISPRi-Lib-Fwd-5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGATAGCAATTCGCTTTATAT ATCT<u>CTATAAGTATCCCTTGGAGAACCACCTTGTT</u>
NGS-CRISPRi-Lib-Fwd-6	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCGATAGTTGCTTGCTTTATA TATCT<u>CTATAAGTATCCCTTGGAGAACCACCTTGTT</u>
NGS-CRISPRi-Lib-Fwd-7	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCGATCCAGTTAGGCTTTAT ATATCT<u>CTATAAGTATCCCTTGGAGAACCACCTTGTT</u>
NGS-CRISPRi-Lib-Fwd-8	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATCGATTTGAGCCTGCTTTA TATATCT<u>CTATAAGTATCCCTTGGAGAACCACCTTGTT</u>
NGS-CRISPRi-Lib-Fwd-9	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACGATCGATACACGATCGCTTT ATATATCT<u>CTATAAGTATCCCTTGGAGAACCACCTTGTT</u>
NGS-CRISPRi-Lib-Fwd-10	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTACGATCGATGGTCCAGAGCTT TATATATCT<u>CTATAAGTATCCCTTGGAGAACCACCTTGTT</u>

Table 5: Example CRISPRi Inner PCR Reverse Primers (with sample adapters)

Primer (one per sample)	Sequence 5'-3' (demultiplex barcode in bold, vector sequence underlined)
NGS-Lib-CRISPRi-Rev-1	CAAGCAGAAGACGGCATACGAGAT**TCGCCTTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT<u>TTCAAGTTGATAACGGACTAGCCTT</u>
NGS-Lib-CRISPRi-Rev-2	CAAGCAGAAGACGGCATACGAGAT**ATAGCGTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT<u>TTCAAGTTGATAACGGACTAGCCTT</u>
NGS-Lib-CRISPRi-Rev-3	CAAGCAGAAGACGGCATACGAGAT**GAAGAAGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT<u>TTCAAGTTGATAACGGACTAGCCTT</u>
NGS-Lib-CRISPRi-Rev-4	CAAGCAGAAGACGGCATACGAGAT**ATTCTAGG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT<u>TTCAAGTTGATAACGGACTAGCCTT</u>
NGS-Lib-CRISPRi-Rev-5	CAAGCAGAAGACGGCATACGAGAT**CGTTACCA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT<u>TTCAAGTTGATAACGGACTAGCCTT</u>
NGS-Lib-CRISPRi-Rev-6	CAAGCAGAAGACGGCATACGAGAT**GTCTGATG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT<u>TTCAAGTTGATAACGGACTAGCCTT</u>
NGS-Lib-CRISPRi-Rev-7	CAAGCAGAAGACGGCATACGAGAT**TTACGCAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT<u>TTCAAGTTGATAACGGACTAGCCTT</u>
NGS-Lib-CRISPRi-Rev-8	CAAGCAGAAGACGGCATACGAGAT**TTGAATAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT<u>TTCAAGTTGATAACGGACTAGCCTT</u>

Table 6: Inner PCR reaction mix

Reagent	1X (multiplied by N reactions)
2X JumpStart™ Taq ReadyMix™ reagent	25 µL
5 µM Fwd Primer Mix	2.5 µL
5 µM Rev Primer	2.5 µL
Purified outer PCR product	100 ng
H$_2$0	to volume
	50 µL Reaction Total

Table 7: Inner PCR reaction conditions

95 °C	2 min	
95 °C	30 sec	
55 °C	30 sec	**20x**
72 °C	20 sec	
72 °C	2 min	

8. Purify the 250–280 bp inner PCR amplicons using GenElute™ PCR Clean-Up Kit (Cat. No. NA1020). Quantify the PCR product and run 2 µg of product on a 2% (wt/vol) agarose gel. Perform gel extraction of the proper band using GenElute™ Gel Extraction Kit (Cat. No. NA1111). Elute in nuclease-free water (Cat. No. W4502). Quantify the DNA.

9. Follow the NGS sequencing preparation and data deconvolution guidelines outlined by Joung et al., 2017 Nature Protocols, or by the manufacturer's protocol specific to your NGS platform.

2.8. Validation of Candidate Genes for Screening Phenotype

Order custom guide RNA constructs or lentiviral particles and transduce gRNA constructs for each candidate gene or gRNA to be validated from the 'top hits' determined by statistical analysis of the screen data. A parsimonious candidate list will give priority to genes that show a similar trend for 2–5 guides per gene. Inhibition of expression by CRISPRi is reported by Gilbert et al., 2013 to be visible 3 days post transduction, and strong at 6 days.

Day 1 — Seeding Cells

- We generally recommend seeding cells in smaller volumes such as a 6-well cell culture plate with enough cells for 10% confluency.
- Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 2 — Transduction

- Remove the medium from each well of the plate(s) prepared on day 1.
- Add 8 µg/mL polybrene-containing medium to each well. Gently swirl the plate to mix.
- Transduce the cells with a single guide construct: using the equation below, determine and add the appropriate volume of lentiviral particles for each construct. The desired MOI is <0.7-1. Leave at least one well for selection of non-transduced cells, one well for cells with virus but without selection, and one well for cells without virus or selection agent.

$$\textit{Volume of Viral Particles (µL)} = 1000 \text{ µL} \times \frac{\textit{Number of Cells} \times \textit{Desired MOI}}{\textit{Viral Titer (TU/mL)}}$$

- Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 3 — Medium Change

- Remove the virus-containing medium from each well of the plate(s). Sterilize discarded virus medium and contaminated supplies using a 10% bleach solution for 24 hours.
- Add fresh growth medium (without polybrene) to each well.
- Incubate at 37 °C with 5% CO_2 for 24 hours.

Day 4 — Antibiotic Selection

- Remove the medium from each well.
- Feed cells with fresh growth medium containing the appropriate concentrations of either puro or zeo.

Days 5 – 10 — Continued Antibiotic Selection

- Continue to select for transduced cells using concentrations and timelines based on the kill curve prior to transduction (usually 4-7 days). Maintain cells under antibiotic selection for the duration of the screen to maintain cells actively expressing CRISPR components.

- Replace the antibiotic-containing medium as necessary during the selection process (usually every other day). The non-transduced control cells should die completely after 4- or 7-day selection with antibiotic.

- Following selection and subsequent expansion, split selected cells; bank a frozen stock for future culture (CryoStor® cell cryopreservation media, Cat. No. C2874), and grow the rest for screen validation.

Days 10+ Screen Validation

- Subject cells to enrichment screen to validate survival/death/expression/phenotype.

Analysis of Gene Expression

- Harvest total RNA from selected cells using GenElute™ Mammalian Total RNA Miniprep Kit (Cat. No. RTN70). Elute in nuclease-free water.

- Perform qRT-PCR analysis to determine relative gene expression changes compared to controls, using Quantitative RT-PCR ReadyMix™ kit (Cat. No. QR0200).

2.9. Suspension cells: Lentivirus transduction and general screening plan: Adapted from University of California San Francisco (UCSF) Protocols

Prepping for screening

- Ensure you have enough media for screen

- If using spinner flasks, wash carefully and autoclave prior to infection day (can also grow up un-infected cells in these flasks)

- Test small scale (~6-well plate size) transfection and infection to estimate infectivity. Goal infection rate is 20–50% infected cells, as measured by % BFP+ 2–3 days after infection.

- If using a drug/toxin selection, test concentrations and dosing protocols in as close to the screen conditions as possible (e.g. test in small spinner flasks if planning to use, as these can affect dosing significantly).

- Scale up culture to reach the number of cells you need on infection day.

- The quantities listed below are typical numbers we use in K562 cells. In addition, these quantities are for a single replicate. We typically do screens in duplicate, maintaining two independent cultures starting from the infection.

Day 0 Infection

- Centrifuge a number of cells equal to ~1000x the size of the library and aspirate media.

- Resuspend cells in 0.5 mL virus-containing media per million cells. Add 8 µg/mL (final conc.) polybrene. For an example genome-scale screen, resuspend 240 million cells in 120 mL of virus-containing media and then add polybrene stock to get final concentration.

- Aliquot cell/virus mixture to 6-well plates (~5–6 mL per well), and spin 1000x g for 2 hr at 33 °C.

- Resuspend cells from the 6-well plates, centrifuge cells, and aspirate virus-containing media.

- Resuspend cells carefully with 2 mL fresh media per million cells instatic or spinner flask.

Day 1

Check cell number and split or expand as necessary (see Day 5), maintaining cells at 0.5×10^6/mL each day. Some BFP fluorescence may be visible but will not accurately represent infection rate.

Day 2 Start puromycin selection

Check infection by BFP % using flow cytometry. Split or expand cells as necessary, then start puromycin selection with final concentration of 0.75 µg/mL (concentration will vary depending on the responsiveness of your specific cell line to different selection compounds.) Typically, 2 days of puro selection at this dose will enrich the population to 80–90% infected, the target range for T0. This allows you to monitor the fraction of uninfected cells throughout the course of the screen.

Day 3

Continue puro selection, assuming the dose from Day 2 has been fully metabolized and applying a fresh dose at 0.75 µg/mL final concentration.

Day 4

Recover cells by diluting with puro-free media or by centrifuging cells and resuspending in fresh media.

Day 5 "T0"

To start the screen, you will need to harvest cells, with the number of infected cells equal to 1000x the size of the library, for each of the following groups:

- The untreated/DMSO-treated control culture
- Each drug-treated condition
- Freezer stocks for sequencing sample prep.

At least two stocks are recommended to insure against any issues that may arise in sample prep. For a genome-scale screen with one drug condition, this requires 240×10^6 cells infected at 85% (for 200×10^6 infected cells) each for two cultures and two freezer stocks, or almost 10^7 cells total.

For the screening cultures, seed cells into flasks and dilute to 0.5×10^6/mL. Throughout the screen, maintain cells at this number of infected cells at a minimum and at the same volume if possible. For any drug-treated conditions, begin treatment today.

For freezer stocks, centrifuge enough cells for at least two 1000x stocks, aspirate media, and resuspend in freezing media (FBS with 10% DMSO). Aliquot cells into cryotubes (either multiple 1 mL tubes or one 5 mL tube per stock) and slow- freeze at –80 °C.

Days 6+

Each day, measure cell concentration, viability (just for your reference, not used for growth calculations), and BFP% for each culture, and split to maintain cells at 0.5×10^6/mL. You can freeze the remaining cells from splits as midpoint samples if you want to measure cell responses at multiple points of the screen or to protect against contaminations arising late in the screen.

With drug-treated cells, the day-to-day culturing depends on your dosing protocol and cell health—recently treated cells may not require any splitting, while cell populations that have recovered from treatment will return to the untreated growth rate and can be then treated again or harvested depending on the selection pressure exerted by preceding treatments (see below).

Ending the screen

There is no one optimal time for ending a screen. As the screen progresses, there is a trade-off between increasing the selective pressure on the population (thus improving signal-to-noise) and saturating the dynamic range. Each day also increases reagent cost. Our general rule of thumb is to measure cell doublings as above and aim for at least 6 doubling differences between treated and untreated cells, or 10 cell doublings if only culturing an untreated sample (i.e. for essential gene screens). Treated cells should be allowed to recover to almost untreated growth rates before harvesting.

Freeze at least two 1000x library cell stocks as above, expanding the culture prior to the endpoint day if necessary to have enough cells.

3. Additional Lentivirus Information

3.1. Methods and tips for successful use of lentivirus products

Successful targeting relies on optimizing key sensitive steps in the process, including lentiviral transduction. Below are some helpful handling and titration tips from our R&D lentiviral experts. As always, contact our technical support if you have any questions about your genome modification project visit **SigmaAldrich.com/CRISPR**
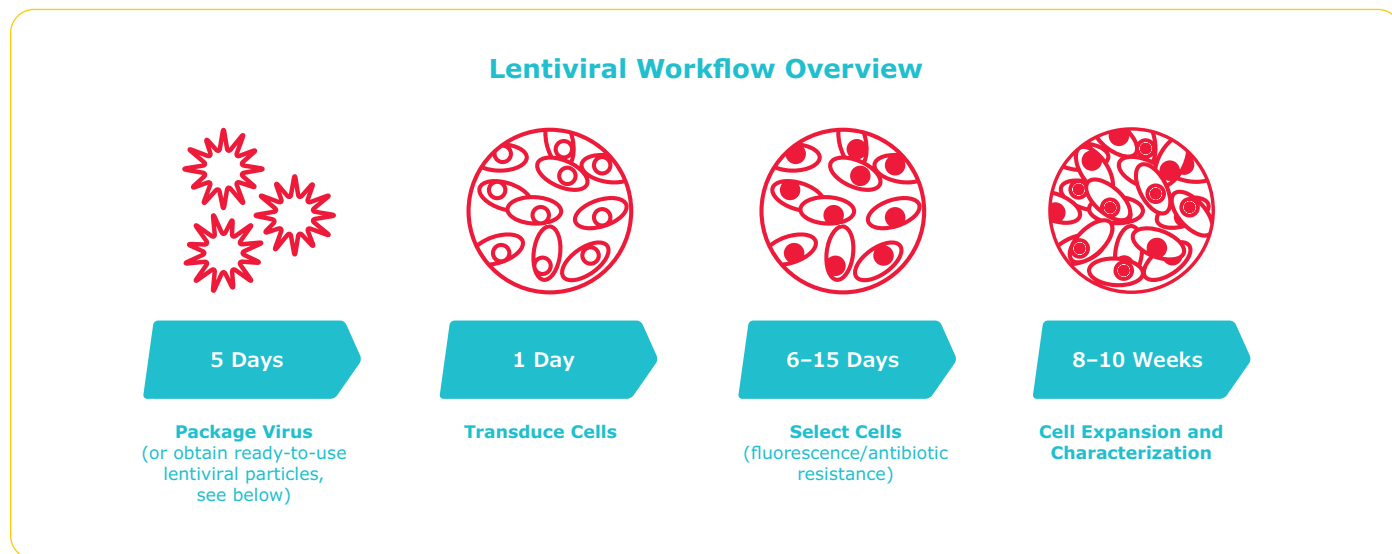

Figure 3: Lentivirual Workflow

Key Terms:

- MOI — Multiplicity of Infection — MOI is the number of transducing lentiviral particles that should be added to each cell in order to ensure successful integration into the cell. It is highly recommended that for each new cell type to be transduced with a specific vector, a range of MOI be tested.

- VP — Viral Particle — the extracellular infective form of a virus, consisting of an RNA or DNA core inside a protein envelope

- TU — Transducing Unit — the number of functional viral particles in a solution that are capable of transducing a cell and expressing the transgene

3.2. Determination of Functional Viral Titer via CFU Assay:

Functional viral titer is affected by many factors, including target cell characteristics; it is important to determine the infection rate for every target cell type and stock used. The infection rate is equal to the number of Colony Formation Units (CFU) that results from transducing 1 mL of a control lentivirus sample (CFU = transforming units per mL), TU/mL); it is highly recommended that the control lentivirus be in the same vector as that used in the actual experiment. This infection rate, or lentiviral titer, can be used to calculate the number of transducing lentiviral particles per cell, or the Multiplicity of Infection (MOI). Ultimately, the titer and MOI will be used to calculate the volume of lentivirus to use in transduction for each experimental.

Notes:

- Lentiviruses are quite labile. Multiple freeze-thaw cycles and prolonged exposure to ambient temperatures will decrease the lentiviral titer.
- Other methods are available to determine functional viral titer. For example, if a fluorophore is present in your system FACS may be used.

Materials Required

- Target cells in culture
- 2 mg/mL Hexadimethrine bromide (Polybrene, Cat. No. H9268)
- 10 mg/mL Puromycin (Cat. No. P9620)
- Crystal Violet Solution (Cat. No. HT90132)
- Dulbecco's Phosphate Buffered Saline (Cat. No. D8662)

Methods

Day 1 — Seeding Cells

- Seed 10^5 cells per well in fresh medium on three 6-well plates. It is recommended to use cells at low passage number for transduction experiments.
- Place the plates in an incubator set to 37 °C with 5% CO_2 for 24 hours.

Day 2 — Transduction

- Thaw one vial of Sigma-Aldrich® library lentiviral particles (25 mL) on ice. Mix by gently tapping the tube several times. Keep them stored on ice when not in use.

- Prepare 25 mL of 8 mg/mL polybrene-containing complete growth medium.

- Prepare 2.5 mL 10-fold serial dilutions of virus over a range of 10^{-2} to 10^{-9} in 15 mL conical vials; use steps a–h, below, as a guide. Mix gently by inverting the tubes 3 to 4 times.

  a. Mix 25 mL of lentivirus with 2,475 mL polybrene-containing medium to achieve a 10^{-2} dilution.

  b. Mix 250 mL of 10^{-2} dilution with 2,250 mL of polybrene-containing medium to achieve a 10^{-3} dilution.

  c. Mix 250 mL of 10^{-3} dilution with 2,250 mL of polybrene-containing medium to achieve a 10^{-4} dilution.

  d. Mix 250 mL of 10^{-4} dilution with 2,250 mL of polybrene-containing medium to achieve a 10^{-5} dilution.

  e. Mix 250 mL of 10^{-5} dilution with 2,250 mL of polybrene-containing medium to achieve a 10^{-6} dilution.

  f. Mix 250 mL of 10^{-6} dilution with 2,250 mL of polybrene-containing medium to achieve a 10^{-7} dilution.

  g. Mix 250 mL of 10^{-7} dilution with 2,250 mL of polybrene-containing medium to achieve a 10^{-8} dilution.

  h. Mix 250 mL of 10^{-8} dilution with 2,250 mL of polybrene-containing medium to achieve a 10^{-9} dilution.

- Remove the medium from each well of the three 6 well plates prepared on Day 1.

- Add 1.0 mL of polybrene-containing medium to two wells on one 6 well plate — these will be used as negative controls.

- Add 1.0 mL of each of the lentivirus dilutions to the remaining wells of the three plates. Duplicate the transduction for each lentiviral dilution.

- Place plates in an incubator set to 37 °C with 5% CO_2 for 24 hours

Day 3 — Medium Change

- Remove the medium containing the lentiviral particles from each well.

- Add 2.0 mL of fresh medium (without polybrene) to each well.

- Place the plates in an incubator set to 37 °C with 5% CO_2 for 24 hours

Day 4 — Antibiotic Selection

- Remove the medium from each well.

- Feed cells with fresh medium containing the appropriate concentration of puromycin determined in the puromycin kill curve.

Days 5–8 — Continued Antibiotic Selection

- Observe each well for cell death daily.

- Replace the puromycin-containing medium as necessary during the selection process (usually every 2 to 3 days). The non-transduced control cells should die completely after 4-day selection with antibiotic.

Day 9 — Staining and Counting Colonies

- Remove the medium and gently wash each well with 3.0 mL of Dulbecco's phosphate-buffered saline (PBS).

- Add 1.0 mL of crystal violet solution per well and incubate 10 minutes at room temperature.

- Remove the crystal violet solution and wash each well twice with 3.0 mL of PBS per wash.

- Invert the plates on paper towels and let them dry for about 1 hour.

- Count the blue-stained colonies (see **Figure 4**) using a microscope at a magnification of 40´. Only count wells that contain 10–300 colonies; any counts with more or less are not significant.

- Calculate the infection rate by multiplying the number of colonies per well by the dilution factor. This value is the lentiviral titer, given in units of TU/mL

Dilution: Ctr 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2}
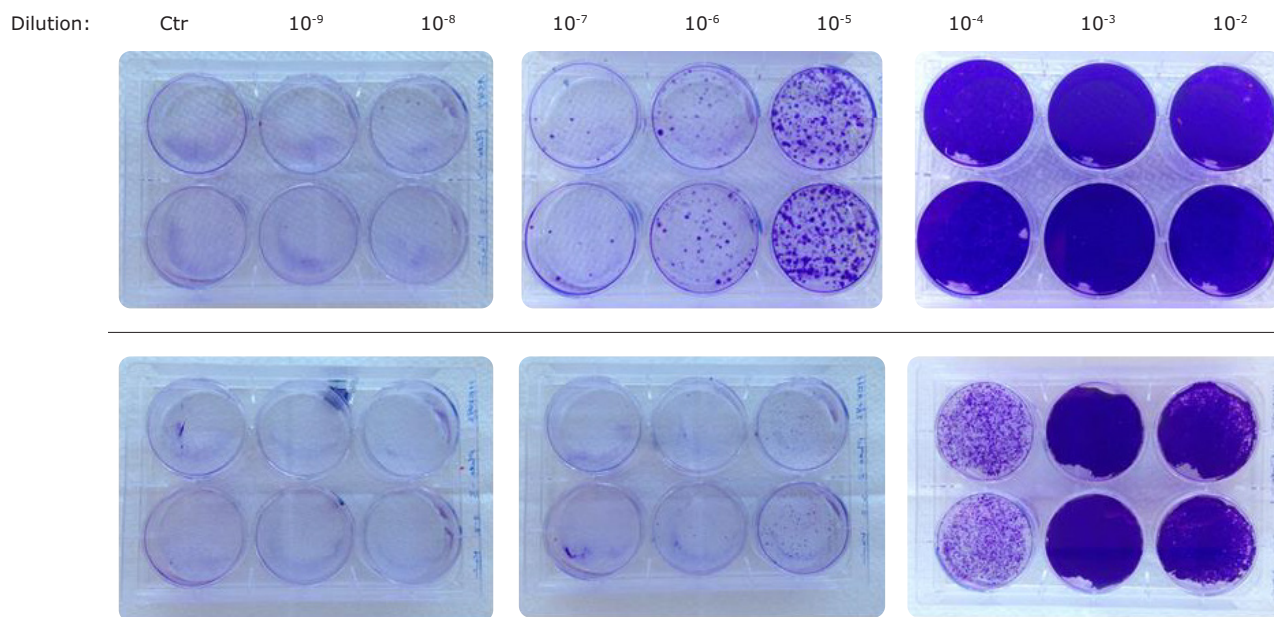
** Upper row: **A549 cells**; Lower row: **HEK293 cells**

Figure 4: Example data for colony staining and counting to determine lentiviral TU/mL based on puromycin resistance.

CFU count data

Plate Replicate	Dilution Factor	# colonies	Titer	Average	Stdev
1	1.00E+02	DNC	DNC	1.50E+08	5.59E+07
	1.00E+03	DNC	DNC		
	1.00E+04	DNC	DNC		
	1.00E+05	DNC	DNC		
	1.00E+06	189	1.89E+08		
	1.00E+07	11	1.10E+08		
	1.00E+08	DNC	DNC		
	1.00E+09	DNC	DNC		
2	1.00E+02	DNC	DNC	1.84E+08	4.95E+06
	1.00E+03	DNC	DNC		
	1.00E+04	DNC	DNC		
	1.00E+05	DNC	DNC		
	1.00E+06	187	1.87E+08		
	1.00E+07	18	1.80E+08		
	1.00E+08	DNC	DNC		
	1.00E+09	DNC	DNC		
plates 1 and 2 combined				1.67E+08	3.79E+07

Note: If there are too many or too few colonies to count in a given well, record DNC in the # colonies field.

3.3.   Safe Handling of Lentivirus

- Lentiviruses are sensitive to extreme temperature shifts. Multiple freeze-thaw cycles and prolonged exposure to ambient temperatures will decrease the functional lentiviral titer.

- Thaw lentiviral particles on wet ice. Keep them stored on ice while performing lentiviral transductions.

- Lentivirus should be aliquoted into smaller working volumes upon first thaw. Return all aliquots not used in your initial experiment immediately to –70 °C. Alternatively, order your lentivirus in working volume aliquots and only thaw the volumes you will need for your transductions.

- Soak all virus and any tech ware and tips that touch virus or virus-containing cells in 10% bleach for 20 minutes before discarding into biohazardous waste.

Our lentiviral vector systems have been developed with enhanced safety features. Numerous precautions are in place in the design of our lentiviruses to prevent replication, including eliminating virulence genes and splitting key genes among different vectors. Still, good handling practices are a must. Read on to understand more about lentivirus safe usage FAQs.

To make lentivirus safe, four viral accessory genes have been deleted leaving the minimum number of genes necessary to make replication-deficient lentiviral particles. These have been separated across multiple plasmids: 3 for 2nd generation packaging systems and 4 for 3rd generation packaging systems. This means 3 or 4 recombination events would need to occur simultaneously to generate replication competent lentiviruses.

3.4.   Vectors for Lentiviral Production

Feature	Second Generation	Third Generation
Plasmids	3	4
U3 Deletion in 3'LTR - Self-INactivation (SIN)	No	Yes
5' LTR	wild type 5'LTR	Chimeric RSV
Packaging plasmids	1	2
Accessory genes: vif, vpr, vpu, nef	All absent	All absent
tat and rev genes	On a single packaging plasmid	Tat is eliminated. Rev is on its own packaging plasmid
gag and pol genes	Together on same plasmid as tat and rev	Together on one plasmid
Pseudotyped or WT envelope Protein	Pseudotyped (VSV-G)	Pseuodotyped (VSV-G)
Recombination events needed to generate Replication Competent Lentiviruses (RCL)*	3 recombinations	4 recombinations
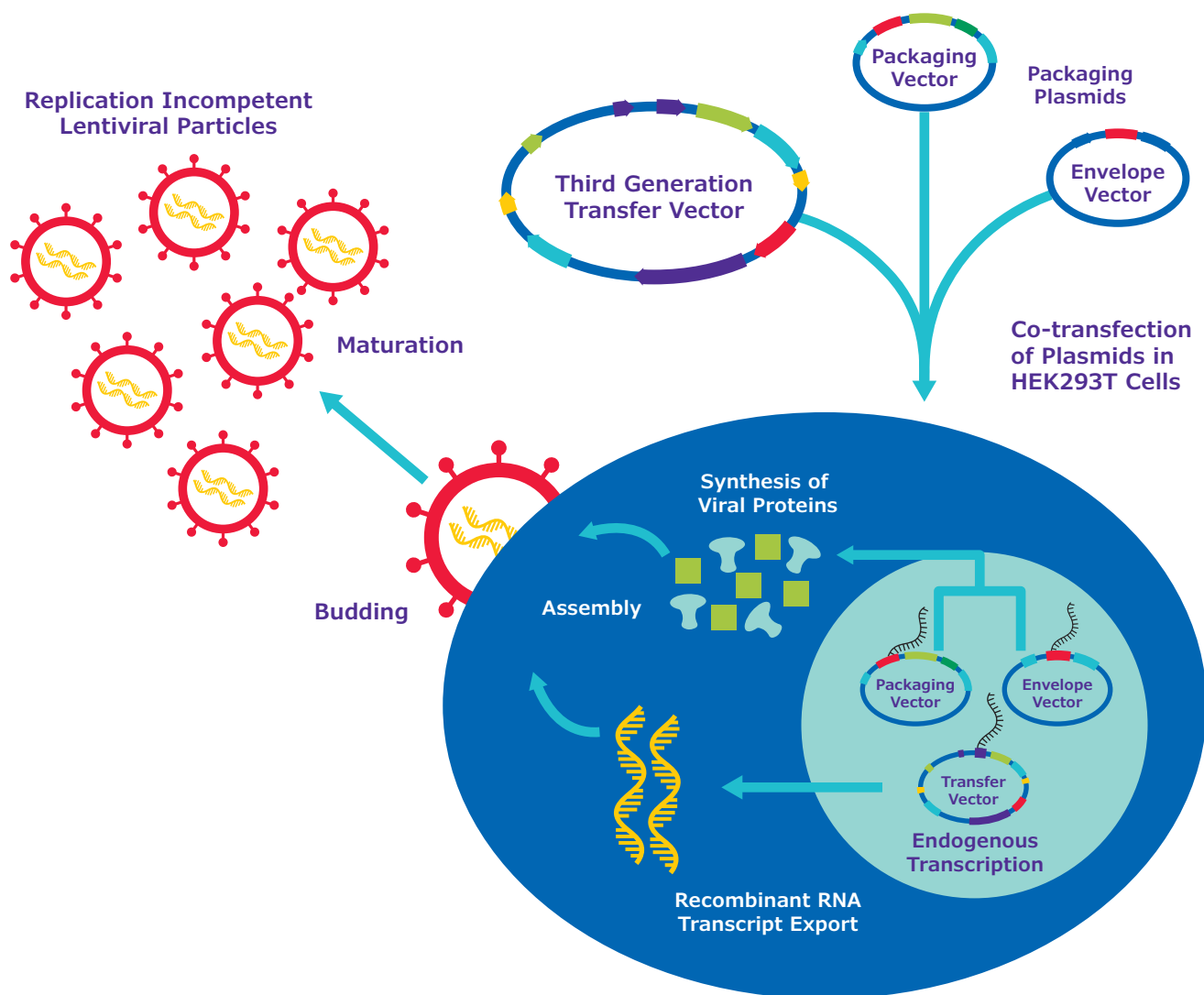
Figure 5. Lentiviral Packaging

For enhanced safety, the structural and accessory genes necessary to produce viral particles are separated onto multiple plasmids. All wild-type virulence and replication genes are deleted. All Sigma-Aldrich® lentiviral transfer vectors contain a modified, self-inactivating 3' long terminal repeat (SIN/LTR) which renders the resulting lentiviral particles replication incompetent. Lentiviral particles are packaged in producer cell lines such as HEK293T cells. Upon co-transfection of the plasmids, all required sequences are available to produce and package a viral particle containing the transgene of interest. Only the region between the viral LTRs of the transfer vector is packaged within the viral capsid.

3.5. Frequently asked Lentivirus Safety Questions

1. **How serious are lentiviral biosafety concerns? Is there a realistic risk of HIV infection?**
   Viral vector systems have been developed with enhanced safety features as described above. Resulting particles are replication-incompetent and deletion in the U3 portion of the 3' LTR eliminates the promoter-enhancer region, further negating the possibility of viral replication. The system has also removed virulence genes which are not necessary for viral packaging. In combination, these features have improved biosafety and handling. It is recommended to use a third-generation transfer vector for its enhanced biosafety features. While there is no documented occurrence of third generation transfer vectors producing replication competent virus, it is important to monitor for replication competency when performing routine lentiviral packaging. NIH guidelines recommend replication-incompetent lentiviral particles be handled as Risk Group-Level 2 (RGL2). Additional precautions may be required based upon local, state, or country regulations.

2. **Can lentivirus pass the blood-brain barrier (BBB)?**
   Given the size of the particles and the current VSV-G pseudotype, it is not expected that lentivirus will pass through the BBB (although we have no data to support this claim). Multiple researchers targeting areas of the brain have shown that direct cranial injection is optimal.

3. **How can I minimize environmental impact and regulatory risks?**
   Good laboratory practices are necessary to prevent any forms of contamination that may result in an opportunity for a replication-competent virus to form. Replication-competent virus testing is necessary. Because viral structural genes have been placed on different genetic units, multiple recombination events must occur before a replication competent helper virus is generated. Furthermore, areas of homology among the units expressing the helper virus proteins have been minimized. Also, heterologous promoters for the helper virus proteins are used. CDC-directed guidelines for BSL-2 + practices should govern the handling of large amounts of virus and concentrated virus. Our Sigma-Aldrich® production facilities routinely conduct standardized procedures to test for the spontaneous production of a replication-competent lentivirus (RCV). In over ten years of manufacturing on site, we have never observed replication competent virus; data available on request.

4. **What should I do with material that has been in contact with lentivirus?**
   Soak all virus and any techware and tips that touch virus or virus-containing cells in 10% bleach for 20 minutes before discarding into biohazardous waste. Please consult with your institution's biosafety officer for specific instructions.

Please contact us with any other questions you may have about the safe usage of lentiviral vectors and in the development of your gene editing project.

4. Troubleshooting

Problem	Cause	Solution
Low transduction efficiency	Cell density was not optimized: too low or too high.	To determine optimal density and transduction efficiency of tested cells, first use one aliquot of lentivirus included in the kit (start with one of the helper constructs). When adding cells to the wells make sure they are mixed well. Keep mixing them periodically to ensure uniform number of cells in each well.
	Cell culture conditions were not optimized.	Use low passage number cells. Confirm that cells are 90% viable upon transduction.
	Cells are sensitive to polybrene.	Test cells for sensitivity by including control cells incubated with medium containing polybrene (8 µg/mL). If cells are sensitive to polybrene, omit this reagent. Cells can still be transduced but with lower efficiency.
	Polybrene was not included during transduction.	Transduce in the presence of polybrene when possible since it enhances the transduction efficiency of most cell types.
	Selection concentration is not optimal for cell selection.	For each new cell type used, it is recommended to perform a selection agent kill curve to determine the lowest concentration of antibiotic needed to efficiently select transduced cells.
Too many guides mapped in NGS results (High NGS noise)	Carryover of cell-free DNA	To reduce the amount of cell-free genomic DNA carried over from dead cells, passage and harvest cells using AccuMax or another Trypsin-alternative possessing DNase activity.
		Perform a nested PCR on harvested gDNA to prevent amplification of guides in cell-free DNA protected by a nucleosome.

5. Additional Required Materials

Instrumentation and Equipment

- Incubator, CO_2
- Hemocytometer
- Thermocycler
- Benchtop centrifuge
- Next generation sequencer
- Bioinformatic Analysis

Related Molecular Biology and Cell Culture Reagents

Product #	Description
15205	Blasticidine S hydrochloride
SHP002	CRISPR & MISSION® Lentiviral Packaging Mix
G8168	G418 disulfate salt solution 50 mg/mL in H_2O, sterile-filtered, BioReagent, suitable for cell culture
A7089	Accumax™ solution
HGECKO2G	Gecko2 Human Whole Genome CRISPR Pool, gRNA Only Lenti Particles (Gecko2 vector)
MGECKO2G	Gecko2 Mouse Whole Genome CRISPR Pool, gRNA Only Lenti Particles (Gecko2 vector)
H9268	Hexadimethrine bromide ≥94% (titration)
SHM01	MISSION® ExpressMag® Super Magnetic Kit Increases transduction efficiency
SHPHLIBR	MISSION® LentiPlex® Complete Human Pooled shRNA Library For Rapid, Convenient Genome-wide shRNA Screens
SHPMLIBR	MISSION® LentiPlex® Complete Mouse Pooled shRNA Library For Rapid, Convenient Genome-wide shRNA Screens
SHCLNV	MISSION® shRNA Lentiviral Particles
ORF	MISSION® TRC3 Human LentiORF Collection
ORFPOOLWG	MISSION® TRC3 Human Whole Genome Lentiviral ORF Pool
P9620	Puromycin dihydrochloride Ready Made Solution, from Streptomyces alboniger, 10 mg/mL in H_2O, 0.2 μm filtered
HSANGERV	Sanger Arrayed Whole Genome Lentiviral CRISPR Library Human, Virus Format
MSANGERV	Sanger Arrayed Whole Genome Lentiviral CRISPR Library Mouse, Virus Format
HWGCRISPR	Sigma Whole Human Genome Lentiviral CRISPR Pool
CRISPR	CRISPR/Cas9 Products and Services Design and order CRISPR gRNA, Cas9, screening libraries, controls and companion products. Formats include plant, lentivirus, IVT-RNA, plasmid, synthetic, and protein.

6. Further Reading

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Additional Resources and Protocols

Sigma-Aldrich® CRISPRi technology was originally investigated and developed by Gilbert et. al. at UCSF and Weissman et. al.

SigmaAldrich.com/CRISPRi and **SigmaAldrich.com/LentiProtocols**

Gilbert Lab Protocols:

https://www.gilbertlabucsf.com/protocols

Weissman Lab Protocols:

https://weissmanlab.ucsf.edu/CRISPR/CRISPR.html

For a list of our worldwide offices please go to:

www.sigmaaldrich.com/site-level/corporate/worldwide-offices.html

For customer support please go to:

www.sigmaaldrich.com/customer-service.html

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