CELLECTA

USER MANUAL

RNAi Pooled Lentiviral shRNA Libraries

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Cellecta, Inc.

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1. RNAi Pooled Lentiviral shRNA Libraries

The basis of Cellecta's RNAi genetic screening technology is the stable suppression of specific genes on a large-scale using pooled shRNA libraries, allowing for loss-of-function screens in mammalian cell systems. Genetic screens with shRNA libraries can be utilized to investigate most aspects of biology that can be recapitulated in a cell culture model. As opposed to expressing and assaying the functional effects of an individual shRNA molecule, the development of complex shRNA libraries allows for simultaneous screening of thousands of different shRNA molecules on a target population. In general, genetic screens represent an unbiased approach to identify genes that act in specific cellular pathways. HT RNAi genetic screens have been proven to be an extremely potent and versatile tool to explore the molecular basis of cancer development and progression, and to discover genes essential for viability in cancer cells that can be used as targets for anticancer drug development.

The screening process introduces a lentiviral library encoding a highly heterogeneous population of barcoded shRNA constructs that are packaged in viral particles pseudotyped with the vesicular stomatitis virus glycoprotein G (VSV-g) into a population of cells under conditions where most of the cells only take up and express a single gene-specific shRNA. On completion of the screen, cells exhibiting the desired phenotypic changes are isolated, and the shRNA constructs, presumably inducing the phenotypes, are recovered by PCR and identified by Next-Generation Sequencing (NGS) of shRNA-specific barcodes.

Major advantages of VSV-g pseudotyped lentivectors for shRNA library delivery are broad spectrum transduction tropism, allowing efficient transduction of non-transfectable cell types and tissues, long-term silencing of target genes, presenting the possibility of screening functions (senescence, differentiation, growth in soft agar, etc.) that require weeks rather than days in vitro or ex vivo.

Genetic screens using pooled shRNA libraries have the requirement for recipient cells with desired phenotypic changes to be selected from a pool of unaffected cells. Selection may be based on cell survival, appearance of specific markers, induction of reporter constructs, changes in cell morphology or behavior, etc. The design of a selection strategy is the most critical arm of any genetic screen project. Repeated rounds of selection may be necessary for either secondary validation or to reduce the number of false positives thereby increasing the percentage of positive hits.

Cellecta's shRNA libraries also contain shRNA-specific barcodes. The barcodes can be read by NGS on the Illumina platform. Identified barcodes can be converted to lists of genes/shRNAs using our enumerated barcode data analysis software. The inclusion of clonal barcodes allows for identification of shRNAs without the need for amplification or sequencing of the hairpins themselves, which can be cumbersome due to the secondary structure present in them. For more information on how the clonal barcodes were built and how they are useful, please read the blog post Millions of Defined Sequenceable Barcodes for Clonal Cell Tracking.

Custom Pooled shRNA Libraries

For Custom Pooled shRNA Libraries, the structure of the vectors, barcodes, and shRNA sequences are specified by the customer. These details are present on the Product Certificate (PC or PAC) that is distributed along with the Custom shRNA Library. Please refer to this Product Certificate as necessary. The protocols below provide the instructions on how to package the plasmid form of the shRNA library into viral particles and guidelines for the preparation of barcoded probes for NGS and analysis of raw sequencing data sets. Please read the entire user manual before proceeding with your experiment. For a description of the theories behind using pooled shRNA lentiviral libraries, information on transduction of target cells, viral targeting, or for examples of positive and negative screens using pooled lentiviral libraries, please refer to the **Screening Recommendations** section.

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Human Genome-Wide Pooled Lentiviral shRNA Library

The Human Genome-Wide Pooled Lentiviral shRNA Library (hGW) targets virtually all (19,268) protein-encoding genes. It covers all genes in the human genome, and each gene is targeted by 8 hairpins. We have incorporated clonal barcodes to enable you to track growth, differentiation, or migration of specific cells containing a specific shRNA throughout your experiment. The hGW consists of three modules, each covering ~6,500 genes. Since each gene is targeted by 8 hairpins, there are a total of 50,000- 55,000 hairpins per module. The modules are made with non-overlapping barcodes so that they can be combined to form a complete genome-wide shRNA library. Each hairpin in the hGW includes a clonal barcode, which facilitates NGS data analysis, identification of functional shRNAs, and allows for tracking of specific shRNAs in individual cells. Each 55K hGW library module also includes a panel of internal controls. The control block consists of 2 shRNA sequences targeting PSMA1, 2 shRNA sequences targeting RPL30, and 4 shRNA sequences targeting Luciferase. Each shRNA sequence is replicated 5 times with 5 different barcodes. This 5-replicate internal control is useful for assessing internal noise, because the 5 replicates for each shRNA should elicit the same phenotype.

DECIPHER Pooled Lentiviral shRNA Libraries

The DECIPHER Pooled shRNA Libraries were Cellecta's first pooled shRNA libraries. There are 3 human modules, and 2 mouse modules of these libraries. Each modules targets ca. 5,000 genes with 5-6 shRNA to gene target. They cover much of the human and mouse gene set, but are not completely genome-wide. The barcodes in the DECIPHER libraries are composed of an 18-nt sequence. Although the barcodes are unique to each shRNA within a module, there is some overlap between barcodes in different modules, so the modules should not be mixed for screening or sequencing.

References and Product Citations for all Cellecta products can be found on the Cellecta website: https://cellecta.com/pages/citations-publications.

Please read the entire user manual before proceeding with your experiment. Also, please note that, when working with pseudoviral particles, you should follow the recommended guidelines for working with Biosafety Level 2 (BSL-2) materials.

Click the "Download as PDF" link located at the bottom of the left menu to download the PDF version of this user manual.

PLEASE NOTE: The purchase of all Cellecta Products is covered by Cellecta's standard Terms and Conditions of Sale as described on Cellecta's website, and selected Products containing particular technology or having certain features are also subject to restrictions on use as outlined in the Label License section of our website. Please review these Terms and Label License Restrictions before opening and using your Product and, if you are not able to abide by the restriction, contact Cellecta to return the item to Cellecta for a full refund.

NOTE: This User Manual replaces ALL previous shRNA library manuals—DECIPHER shRNA Libraries, hGW shRNA Libraries, Custom shRNA Libraries, and shRNA Screening Reference Manual.

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1.1. shRNA Library Barcodes and NGS Analysis

Unambiguous Sequenceable Barcodes

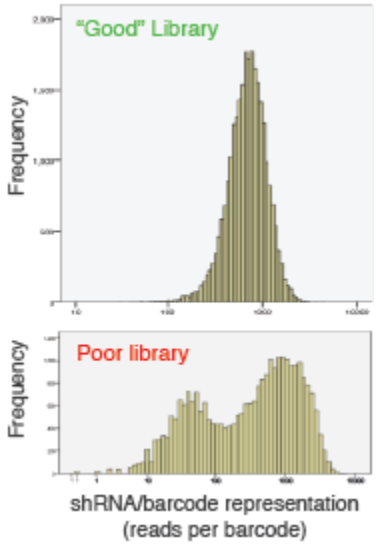
Quality Control of the libraries and the final screening representation analysis are greatly facilitated by the incorporation of easily sequenced barcodes in each shRNA construct. The barcodes enable unambiguous identification of each shRNA species with Next-Generation Sequencing (NGS). Depending on the shRNA library that you have chosen, the barcodes and flanking primers will vary. For example, the barcodes in the DECIPHER shRNA Libraries are 18 nucleotides long, while the barcodes in the Human Genome-Wide shRNA Library are 22 nucleotides long. Upon lentiviral transduction, barcodes integrate into the genomic DNA along with the shRNA expression cassette and are permanently present but not expressed in the cell. Lastly, some libraries contain clonal barcodes, which enable tracking of individual cell clones expressing specific shRNA sequences. These allow for a wider variety of screening protocols that involve cell proliferation, differentiation, migration, metastasis, or apoptosis in specific clones.

Please refer to the User Manual and Product Certificate (PC or PAC) for the specific library you are using for detailed information on the barcodes. An example barcode structure is shown below.

Representation Levels of Individual shRNA Sequences

Cellecta specifically designs and constructs pooled shRNA libraries using proven library construction procedures, not by re-amplifying and mixing pre-made individual shRNA constructs. As a result, it is possible to obtain a narrow representation of virtually all shRNA. The use of our optimized and unambiguous barcodes in combination with NGS enables Cellecta to ensure that more than 99% of shRNA encoding inserts are present in every library and that the representation frequencies of 80-90% of them fall within a 10-fold range.

In the shRNA Representation Histogram figure below, the upper panel shows a pooled library of 27,000 shRNAs with very good representation. Virtually all the shRNAs are seen between between 100 copies and 1,000 copies in 20 million reads. Thus, there is just a 10-fold difference between the most represented and least for about 90% of the shRNAs. The library has a relatively balanced representation of all shRNAs. On the other hand, the lower panel shows a poor library where almost half of the shRNAs are present at less than 100 copies whereas the others are very highly represented. Overall, the distribution is very broad. It is only possible to get readable signals for about half the shRNAs using the library in the lower panel.



Representation histograms of a "good" and a "poor" pooled shRNA library

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This definitive representation data at the start of a screening provides a starting point for the analysis to find shRNAs that significantly increase or decrease during screening, indicating relevant targets. With a poorly defined distribution, it is difficult to differentiate signal vs. noise in any screening assays—or even which shRNA is actually missing in the screen. In other words, you need this data to know what is truly being screened.

Quantifiable Next-Generation Sequencing (NGS)

Next-Gen Sequencing (NGS) significantly outperforms the hybridization-based approach for identification of individual shRNA species based on the high-quality “digital” expression data generated by using barcodes. Even using optimized barcode sequences, array hybridization suffers from a limited dynamic range of approximately 2 orders of magnitude which results in a loss of as much as 30% of the signals that fall outside their effective range. Also, spot-to-spot cross hybridization on arrays results in significant noise that does not occur with NGS where virtually every shRNA in the population is detected and counted, from those present in only a few copies to those present in several million. Differences in shRNA species between control and test populations are very easily detected and statistically analyzed, so that hits can be confidently identified.

shRNA Sequence Design (Purposeful Mismatches)

Cellecta has developed its own in-house shRNA design algorithm that makes use of internal studies primarily focused on the most functionally effective structural features (e.g. length, loop size, mismatches, etc.), combined with published information regarding sequence preferences, and known sequences that have been shown effective for a particular target.

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2. Materials Provided with Cellecta shRNA Libraries

Cellecta's Human Genome-Wide (hGW) and DECIPHER shRNA Libraries are provided with the following materials:

- **For plasmid library purchase:** 200 µg of each plasmid library in the pRSI12-U6-(sh)-HTS4-UbiC-TagRFP-2A-Puro for DECIPHER or in the pRSI16cb-U6-(sh)-HTS6cb-UbiC-TagRFP-2A-Puro for the hGW shRNA Library.
- **For viral library purchase:** Aliquots of virus at the ordered amount of transduction units (TU), plus a small amount of packaged library for titering purposes. Exact amount and titer are indicated on the Product Certificate (PC or PAC).

With Cellecta's custom libraries, usually 500 µg of the plasmid library is provided, and varying amounts of the viral packaged library can be optionally purchased in addition.

You will receive the following resources and documentation with any Cellecta shRNA Library purchase:

- List of shRNA and barcode sequences
- NGS QC data of plasmid libraries
- Link to Library Configuration Files required for the Cellecta Alignment Software

Finally, the map, sequence, and shRNA cassette diagrams for Cellecta library vectors can be downloaded from the Cellecta website: https://cellecta.com/pages/cloning-and-expression-vector-information.

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2.1. Additional Materials for Production of Lentivirus

- 293T Cell Line (e.g. 293T/17, ATCC, Cat.# CRL-11268™ or 293FT, Thermo Fisher, Cat.# R70007)
- Ready-to-use Lentiviral Packaging Plasmid Mix (Cellecta, Cat.# CPCP-K2A). Libraries can be packaged into lentiviral particles with nearly any 2nd or 3rd generation HIV-based lentiviral packaging mix. Cellecta's 2nd generation lentiviral packaging mix contains two plasmids: psPAX2 and pMD2.G, pre-mixed in a ratio optimized for production of lentivirus.
- Dulbecco's Modified Eagle Medium (D-MEM) (1X) (Corning cellgro™, Cat.# 15-013-CV)

> **NOTE:** ADD FRESH GLUTAMINE (1X) at the time a sealed bottle of D-MEM is opened, even if the label indicates glutamine has already been added. Glutamine in solution at 4°C has a half-life of 1-2 months, so glutamine(+) D-MEM purchased "off-the-shelf" from a supplier is to be regarded as glutamine(-). In our experience, the addition of glutamine increases titer approximately 2-fold. If D-MEM comes supplemented with stable L-Alanyl-L-Glutamine dipeptide, addition of fresh glutamine is not necessary.

- Glutamine (L-Alanyl-L-Glutamine, Dipeptide L-glutamine) (Corning glutagro™, Cat.# 25-015-CI)
- Fetal Bovine Serum (Recommended: Corning, Cat.# 35-010-CV)
- Trypsin-EDTA, 1X (Corning, Cat.# 25-052-CI)
- Tissue Culture Plates
  - 150 × 25mm (15-cm) Tissue Culture Plates (Corning, Cat.# 430599)
  - 100 × 20mm (10-cm) Tissue Culture Plates (Corning, Cat.# 430167)
- Related Tissue Culture Supplies
- Lipofectamine® Transfection Reagent (Thermo Fisher, Cat.# 18324020)
- PLUS Reagent (Thermo Fisher, Cat.# 11514015)
- DNase I, RNase-free (Epicentre-Illumina, Cat.# D9905K)
- HEPES pH 7.2-7.6, 1M solution (Corning, Cat.# 25-060-CI)
- $MgCl_2$, 1M solution

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- Nalgene™ Rapid-Flow™ Sterile 500 ml, 0.2 µm PES filter units (Thermo Fisher Cat.# 569-0020)
- LentiFuge™ Viral Concentration Reagent (Cellecta, Cat.# LFVC1) *(Optional, for concentration of virus)*
- D-PBS, 1X (Corning, Cat.# 21-031-CV)

Other than the specific reagents and instruments listed above, the protocols assume the user has access to standard materials (e.g., polypropylene tubes, pipette tips), equipment (table top centrifuges, pipettes, scales), and common reagents (e.g., TE buffer, ethanol) and buffers used in a typical life science laboratory.

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2.2. Additional Materials for Transduction of Cells

The materials below are based on the transduction protocol in this manual which has been optimized for HEK293 and K-562 cells. Depending on the characteristics of your specific cells, the choice of media or other cell culture specifics may be different.

- LentiTrans™ Transduction Reagent (Cellecta, Cat.# LTDR1)
- Puromycin, 10 mg/ml (Sigma-Aldrich, Cat.# P9620-10ML)
- Dulbecco's Modified Eagle Medium (D-MEM) (1X) (Corning cellgro™, Cat.# 15-013-CV)
- Glutamine (L-Alanyl-L-Glutamine, Dipeptide L-glutamine) (Corning glutagro™, Cat.# 25-015-CI)
- Fetal Bovine Serum (Recommended: Corning, Cat.# 35-010-CV)
- Trypsin-EDTA, 1X (Corning, Cat.# 25-052-CI)
- Tissue Culture Plates and Related Tissue Culture Supplies
- HEPES pH 7.2-7.6, 1M solution (Corning, Cat.# 25-060-CI)
- $MgCl_2$, 1M solution
- Doxycycline, Ready Made Solution, 100 mg/ml (Sigma-Aldrich, Cat.# D3072-1ML) *(Optional, for tet-inducible vectors)*

Other than the specific reagents and instruments listed above, the protocols assume the user has access to standard materials (e.g., polypropylene tubes, pipette tips), equipment (table top centrifuges, pipettes, scales), and common reagents (e.g., TE buffer, ethanol) and buffers used in a typical life science laboratory.

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2.3. Additional Materials for Genomic DNA Extraction

- QIAamp DNA Micro Kit (QIAGEN, Cat.# 56304) — **for <1M cells**
- DNeasy Blood and Tissue Kit (QIAGEN, Cat.# 69504) — **for 1M-10M cells**
- Falcon® 15ml PP Screw-Cap Centrifuge Tubes (12,000 RCF rated, P:CHCl3-resistant) (Corning, Cat.# 352196)
- Buffer P1 (50mM Tris-HCl pH 8.0, 10mM EDTA) (QIAGEN, Cat.# 19051)
- Sodium Dodecyl Sulfate (SDS), 10% solution (Thermo Fisher, Cat.# 24730020)
- RNase A (QIAGEN, Cat.# 19101)
- Sonicator, for shearing Genomic DNA
- Proteinase K from Tritirachium album (Sigma-Aldrich, Cat.# P4850)
- Phenol:Chloroform:Isoamyl Alcohol 25:24:1 pH 8.0 (Sigma-Aldrich, Cat.# P3803)
- Sodium Acetate, 3M solution (Sigma-Aldrich, Cat.# 71196- 100ML)
- Isopropanol (2-Propanol) (Sigma-Aldrich, Cat.# I9516-500ML)
- Linear Acrylamide (5 mg/ml) (Thermo Fisher, Cat.# AM9520) — **for <5M cells**

Other than the specific reagents and instruments listed above, the protocols assume the user has access to standard materials (e.g., polypropylene tubes, pipette tips), equipment (table top centrifuges, pipettes, scales), and common reagents

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(e.g., TE buffer, ethanol) and buffers used in a typical life science laboratory.

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2.4. Additional Materials for NGS of sgRNA or Barcodes

- PCR and NGS primers for sgRNA/barcode amplification and sequencing (IDT): See NGS Cassette Diagram, provided as a link or attachment in email upon shipment of product or in Product Certificate of Library NGS Prep Kit.

> For your convenience, we also offer **Library NGS Prep Kits** which provide primers and reagents to prepare samples screened with your library. The NGS Prep Kit and Supplementary Primer Set each contain 12 unique indexing primers that can be used to multiplex up to 24 screening samples on a flow cell or lane.

- Titanium Taq DNA Polymerase with PCR buffer (Clontech-Takara, Cat.# 639242)
- dNTP Mix (10 mM each) (Thermo Fisher, Cat.# 10297018)
- QIAquick PCR Purification Kit (QIAGEN, Cat.# 28106)
- QIAquick Gel Extraction Kit (QIAGEN, Cat.# 28706)
- PhiX Control (Illumina, Cat.# FC-110-3001)
- NGS Reagent Kits, for NextSeq and HiSeq (Illumina):

Platform	Kit Type	Illumina Cat.#	Description
NextSeq® Single Read (SR) or Paired End (PE)	Sequencing & Cluster Gen.	20024906	NextSeq 500 v2.5 Kit (75 cycles, High-Output)
HiSeq® 2000/2500 **Single Read (SR)**	Sequencing	GD-401-3001	TruSeq SBS Kit v3 – HS (50 cycles)
	Cluster Gen.	FC-401-3002	TruSeq SR Cluster Kit v3-cBot-HS
HiSeq® 2000/2500 **Paired End (PE)**	Sequencing	GD-401-3001	TruSeq SBS Kit v3 – HS (50 cycles)
	Cluster Gen.	PE-401-3001	TruSeq PE Cluster Kit v3-cBot-HS
HiSeq® 3000/4000 **Single Read (SR)**	Sequencing	FC-410-1001	HiSeq 3000/4000 SBS Kit (50 cycles)
	Cluster Gen.	GD-410-1001	HiSeq 3000/4000 SR Cluster Kit
HiSeq® 3000/4000 **Paired End (PE)**	Sequencing	FC-410-1001	HiSeq 3000/4000 SBS Kit (50 cycles)
	Cluster Gen.	PE-410-1001	HiSeq 3000/4000 PE Cluster Kit

> **NOTE:** See Illumina website for information on kits for other Illumina instruments (e.g. NovaSeq, NextSeq 1000/2000, MiSeq).

Other than the specific reagents and instruments listed above, the protocols assume the user has access to standard materials (e.g., polypropylene tubes, pipette tips), equipment (table top centrifuges, pipettes, scales), and common reagents (e.g., TE buffer, ethanol) and buffers used in a typical life science laboratory.

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2.5. Related Products and Services from Cellecta

Related Products

Description	Cat.#	Quantity

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NGS Prep Kit for shRNA Libraries	Various	Kit for 6-48 samples (48 preps of 50 µg each)
Supplementary Primer Set (12 Additional Index Primers)	Various	48 Reactions (12 Index Primers x 4 reactions each)
Ready-to-Use Lentiviral Packaging Plasmid Mix	CPCP-K2A	250 µg (25 transfections in 10-cm plates)
LentiFuge™ Viral Concentration Reagent	LFVC1	1 ml of 1000X solution
LentiTrans™ Transduction Reagent (Polybrene for Transduction)	LTDR1	1 ml of 1000x
LentiPrep™ Lentiviral Reagent Set with GFP Control for Packaging and Transduction	LTSET-G	CPCP-K2A x 1 LFVC1 × 1 LTDR1 × 1 GFP Ctrl
LentiPrep™ Lentiviral Reagent Set with RFP Control for Packaging and Transduction	LTSET-R	CPCP-K2A x 1 LFVC1 × 1 LTDR1 × 1 RFP Ctrl

Related Services

- Lentiviral Packaging Services
- NGS Service for Samples from Genetic Screens
- Custom shRNA Libraries
- Custom Lentiviral shRNA Constructs

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3. Recommended Pilot Experiments

In order to obtain reliable data from your library screen or individual construct experiment, we suggest appropriate planning beforehand. We recommend the pilot studies below in your cell system of choice (the cell system that will be used in your experiment). Cell-type specific data from these pilot experiments will provide you more confidence in your results.

Using the following procedures, confirm the cell doubling time, antibiotic efficacy on the target cells, and sensitivity of the target cells to the LentiTrans Transduction Reagent.

- Cell Doubling Time
- Calculating a Kill Curve
- Check Toxicity of LentiTrans Transduction Reagent

Visit the Cellecta Vector Information web page for details on our most common sgRNA, shRNA, and barcode lentiviral vectors.

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3.1. Check Toxicity of LentiTrans Transduction Reagent

The LentiTrans™ Transduction Reagent used during the transduction of target cells with lentivirus contains Polybrene, a transduction enhancement reagent. Some cell lines may be sensitive to this reagent. Before doing large-scale transduction, we recommend checking the toxicity of the LentiTrans Reagent using the procedure below:

1. In six wells of a 12-well plate, grow cells for 24 hours in complete culture medium without the LentiTrans Reagent, and with LentiTrans added at 0.25 µl/ml, 0.5 µl/ml and 1:1000 (1 µl/ml).

2. Replace old medium with LentiTrans-free complete culture medium and grow cells for an additional 72 hours.

3. Check for toxicity by counting viable cells.

For your experiments, use the highest concentration of LentiTrans Reagent that results in less than 10% cell toxicity compared to the no-LentiTrans culture (typically, a 1:1000 is recommended). For a few cell types, you may not be able to use LentiTrans Reagent.

Last modified: 29 December 2023

3.2. Calculating a Kill Curve

Most of the lentiviral vectors used in making Cellecta's libraries express an antibiotic resistance marker. Regardless of the antibiotic selection marker of the library, you need to know the concentration of antibiotic that kills non-transduced cells within a given amount of time in order to successfully select transduced cells. In order to avoid the enrichment of double integrants and loss of single-integrants, we recommend avoiding over-selection: calculate an antibiotic kill curve in your cell line beforehand and choose the lowest antibiotic concentration which gives 90%-95% killing. For fine-tuning of the antibiotic concentration, if the lentivirus carries both an antibiotic selection marker and a fluorescence marker, follow the procedure described in the Antibiotic Selection of Library Transduced Cells section.

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3.3. Cell Doubling Time

The doubling time is the time it takes your cells to double in number. It is useful to know the doubling time of your cells so that you can plate the appropriate number for transduction with a lentiviral library or construct.

1. Start with cells that have already been growing for a few weeks, rather than using cells that have just been thawed from a frozen state. To calculate the doubling time, trypsinize your cells as if you were going to split them.

2. Count them using a hemacytometer or cell counter, and keep track of the number that you replate onto the cell culture plates. The starting number of cells (at the beginning) is ***Xb***.

3. Propagate the cells as you normally do, replacing media as necessary.

4. The next time they are ready to be split, trypsinize them as usual and count them again using a hemacytometer or cell counter. The number of cells at the end is referred to as ***Xe***.

> **NOTE:** The cells should be in the log phase of growth to calculate doubling time properly, so it is important to not let the cells become confluent.

To calculate the doubling time, use the following formula:

> *Doubling Time* = [***T*** × (ln2)] / [ln (***Xe*** / ***Xb***)]
> where ***T*** = Time in any units

Example

> Let's say that on Day 0, you count 2 × 10^6 cells. Three (3) days later, you count 16 × 10^6 cells.
>
> ***Xb*** = 2 × 10^6
> ***T*** = 3 days
> ***Xe*** = 16 × 10^6
>
> Doubling Time = [3 × (ln2)] / [ln(16,000,000 / 2,000,000)]
> = [3 × (0.69)] / [ln(8)]
> = 2.08 / 2.08 = **1 day**

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4. Screening Recommendations

Researchers are often interested in using a pooled shRNA library for genome-wide RNAi screening to cast a very “wide and unbiased net” to identify any and all genes functionally involved in some pathway. Although it is not difficult to make an shRNA library targeting all human or mouse genes, it is difficult to comprehensively screen using such a library. Careful consideration of starting cell numbers and handling of cells during propagation is essential to ensure thorough screening of pooled shRNA expression libraries, minimize false negatives, and obtain consistent and reproducible results.

Library Complexity and Number of Cells

First, there is an issue of library complexity since it is necessary to have several shRNAs designed to target each gene. The effectiveness of validated shRNA varies from cell-to-cell. For these reasons, it is necessary to incorporate several shRNAs for each gene to ensure reasonable knockdown of a high percentage of targets. Cellecta typically designs 5-10 shRNAs against each target gene depending on the design of the library, so at least 25,000 shRNAs are required to target 2,500-5,000 genes. A library targeting the entire human genome of approximately 20,000 genes requires approximately 115,000 individual shRNA constructs. While it is not particularly difficult to construct libraries of this complexity, this number of unique shRNA sequences can produce technical challenges with some types of screens.

Number of Starting Cells and Representation

Pooled shRNA library screens require quantification of changes in the fraction of cells bearing each shRNA sequence in selected vs. control cells (or starting library). A “hit” occurs when selected cells have significantly more or fewer cells bearing a particular shRNA sequence. Whether one is looking at enrichment of shRNA sequences in the selected cell population vs. control (positive selection) or depletion of shRNA sequenced in selected cell population vs. control (negative selection), it is critical that the screens begin with sufficient numbers of cells expressing each shRNA to ensure measured changes in the fraction of cells bearing any given shRNA sequence are statistically significant. This means that, if there are very low numbers of cells bearing specific shRNA sequences at the start of the screen, small random changes in a drifting population may be difficult to differentiate from significant trends.

Simply put, a loss of 2 cells is a 20% change if there are only 10 initially vs. 2% if there are 100. For this reason, a least a few hundred cells need to be infected with each shRNA to initiate a good screening. This is demonstrated in the Reproducibility of Triplicate figure below where starting with a smaller population of just 50 cells per shRNA (third bar) leads to significantly more variation than starting with a population of 200 cells per shRNA (first bar).

To ensure adequate representation of the whole library in the initial population, start a screen by infecting at least 200 times more cells than the complexity of the library. For a library with 25,000 shRNAs, the starting population should be at least 5 million infected cells, and for a library with 55,000 shRNAs, the starting population should be at least 11 million infected cells.

Multiplicity of Infection

For pooled shRNA screens, it is important to have 2-3 times more cells than infecting viral particles (i.e., a multiplicity of infection [MOI] of 0.3-0.5) to ensure that the majority of cells are only infected with one shRNA-carrying virus, so you need to have 2-3 times more cells than the number targeted for infection. Thus, 6-8 million cells are needed to start a screen with libraries of 25,000 shRNAs, and a whole genome library of 150,000 shRNAs would require 60-90 million cells. Since each screen should be done in duplicate, or better, triplicate, the number of cells needed makes a full genome screen with a redundant shRNA library challenging.

The lower the MOI, the more cells you need to start the screen so it is tempting to use a high MOI. However, you should

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consider that a higher MOI produces a higher percentage of infected cells bearing two or more different shRNA constructs. For most RNAi screens, we recommend optimizing conditions and performing genetic screen transductions at no more than 0.5 MOI (ca. 40% transduction efficiency) which balances these two considerations. Please note that to accurately calculate the MOI, it is critical to determine the library titer directly in your target cells prior to beginning your experiment. Once conditions are established to achieve ~40% transduction efficiency in the titering assay, scale up all conditions proportionately to accommodate the larger amount of transduced cells needed for the genetic screen.

Representation and Cell Propagation Techniques

Finally, to ensure a comprehensive screen, it is not simply sufficient to start with the right amount of cells. During the screening process, incorrect propagation of the cells can completely undercut the representation set up at the initiation of the screen. This is especially true for a negative selection screen, such as a viability screen where one is interested in identifying shRNA that kill or inhibit proliferation of cells, and, therefore, drop out of the population. It is critical to maintain the full library representation that was initially used at the start of the screen.

If a portion of propagating cells are removed during propagation (e.g. cells are split), the representation of the library can be skewed in the sample which introduces significant random noise. This effect is readily seen in the second bar in the Reproducibility of Triplicates figure where the effect of starting with sufficient cells (i.e. 200-fold library complexity) is completely undercut by splitting cells during propagation so that that the final count of cells after 10 days is the same as the initial number of transduced cells (i.e. 200- fold library complexity). The correlation between triplicates falls dramatically when the cells are split to this degree. For this reason if cells are ever to be discarded or samples split at any time during the screen, the number of remaining cells in each sample should always exceed the complexity of the library by at least 1,000-fold, as shown in the first bar of the figure. For example, keep at least 27 million cells after every splitting step, for a 27K library. Also, before splitting or discarding, make sure you first pool all cells from the same replicate together.

Modular Approach to Genome-Wide Screening

Library representation is often overlooked, especially when the desire is for large-scale unbiased screens. However, without careful consideration in designing screening procedures that reflect the complexity of the library, results of these large-scale screens can produce relatively meaningless data with anecdotal results at best. So, what about genome-wide screening? Our approach is to provide modules, each targeting approximately 5,000 genes with 27,500 shRNA in our Three- or Two-Module DECIPHER Human and Mouse libraries, or targeting 6,500 with 55,000 shRNA in our Three-Module Human Genome-Wide (hGW) library. These modules enable comprehensive genome-wide screens with manageable numbers of cells for negative selection screens. For cases where it is practical to work with larger numbers of cells, for example some positive screens, the modules of the hGW library can be combined to make a larger library since they contain non-overlapping barcodes (with the exception being Modules 1 and 3 of the DECIPHER Human shRNA Library.

Last modified: 10 January 2019

4.1. Recommendations for Positive Screens

Positive Selection Screens (aka. Rescue Screens)

Find genes required to produce a response to added factors or compounds, for example, genes necessary for trigging apoptosis or cell death in response to FAS, PUMA or other effectors. Positive screens are also known as enrichment screens. Many positive screens use FACS to look for modulators of signaling molecules like NF-κB, p53, c-myc, HSF-1, HIF-1α using fluorescent reporter cell lines, or cells expressing specific antibody-detectable markers, such as specific receptors.

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Length of the Screen

A positive screen involves a selection that eliminates most of the cells. With this sort of screen, the goal is to isolate a small population of cells with shRNAs that enable the cells to pass through the selection step. The critical factor here then is the nature of the selection, which ultimately determines the screen procedure. In most cases, it is advisable to wait about 1 week after library transduction before carrying out the selection step. The 1 week wait period is needed to allow for knockdown of genes encoding for long half-life mRNAs and proteins, and the development of the resistant phenotype before applying selection. Cells should then be harvested as soon as positive selection is completed. Growing and expanding clones after positive selection is not advised.

For a positive selection screen, the puromycin or other antibiotic selection of transduced cells it is not essential, but provides a way to reduce the total number of cells before positive selection and makes cell culture handling more manageable.

MOI of Transduction

A positive screen involves isolation of a small population of cells with shRNA sequences that will be over-represented or enriched when compared to the starting library shRNA counts. As with any screen, to ensure reproducible and reliable results, it is critical that you transduce enough cells to maintain sufficient representation of each shRNA construct present in the library. The number of cells stably transduced with the shRNA library at the time of transduction should exceed the complexity of the shRNA library by at least 200-fold. For a library with 27,500 shRNAs, the starting population should be at least 5.5 million infected cells, and for a library with 55,000 shRNAs, the starting population should be at least 11 million infected cells.

Maintenance of the Cells

A positive selection screen often involves the comparison of two types of samples: selected and unselected (control) samples. After transduction and before selection, it is best practice not to discard any cells. However, this is often not practical. If cells have to be discarded or split before selection, the number of remaining cells in each sample should always exceed the complexity of the library by at least 1,000-fold (e.g. keep at least 2.7×10^7 cells after every splitting step, for a 27K library).

After the selection step, all the cells in the selected samples should be collected for genomic DNA purification and barcode PCR amplification. For the control samples, follow the aforementioned 1,000-fold rule in the sense that you should collect enough cells to equal 1,000- fold the complexity of the library. Similarly, when amplifying barcodes from isolated DNA, you should always use all the genomic DNA recovered from cell samples, up to the amount corresponding to 1000X cells the library size. For diploid cells, 25-30 million cells ~150-180 µg of genomic DNA.

Baseline Controls for Positive Selection Screens

In order to calculate the enrichment-fold of the shRNA sequences present in the selected population, a baseline control is needed. Depending on the screen, the plasmid library itself can be used as baseline, or pre-selection cells, or mock-selected cells.

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4.1.1. Example Positive Screens

Positive Selection Screen #1

Aim: Identifying genes that when knocked down confer resistance to cytotoxic Compound X. Cytotoxic Compound X kills 95%-99% cells within 48 hours when administered at a 1 nM concentration.

shRNA library used: 55K hGW shRNA Library Module 1

Procedure:

Day 1. 5 × 10^7 cells transduced at MOI 0.5 (40% transduction efficiency expected)

Day 2. Media change

Day 4. Actual transduction efficiency checked by flow cytometry. Start of puromycin selection (1 µg/ml)

Day 7. End of puromycin selection

Day 8. Culture split 50:50 into 2 samples (>1 × 10^8 cells/sample)

- Sample A: cytotoxic Compound X added at a 1 nM concentration
- Sample B: untreated

Day 10. Both samples harvested for genomic DNA isolation and NGS of integrated barcodes. For the untreated sample, 400 µg of genomic DNA is used for PCR and NGS of barcodes. For the treated sample, all of the recovered genomic DNA is used.

- shRNAs are evaluated based on barcode enrichment in Sample A vs. Sample B
- Gene hits are identified based on evaluation of targeting shRNAs

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Positive Selection Screen #2

Aim: Identifying genes required for the transactivation of Promoter X by Compound Y. GFP is expressed from Promoter X and accumulates in cells within 24 hours of stimulation by Compound Y at a 10 nM concentration.

shRNA library used: 55K hGW shRNA Library Module 1

Procedure:

Day 1. 5 × 10^7 cells transduced at MOI 0.5 (40% transduction efficiency expected)

Day 2. Media change

Day 4. Actual transduction efficiency checked by flow cytometry. Start of puromycin selection (1 µg/ml)

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Day 7. End of puromycin selection

Day 8. Compound Y added at 10 nM concentration.

Day 9. 1×10^8 cells sorted by FACS into two samples:

- Sample A: bottom 1% dimmest GFP cells
- Sample B: top 50% brightest GFP cells

Both samples are harvested immediately after sorting for genomic DNA isolation and NGS of integrated barcodes. For the top 50% population, 400 µg of genomic DNA is used for barcode PCR and NGS. For the bottom 1% population, all of the recovered genomic DNA is used.

- shRNAs are evaluated based on barcode enrichment in Sample A vs. Sample B
- Gene hits are identified based on evaluation of targeting shRNAs

Last modified: 2 November 2020

4.2. Recommendations for Negative Screens

A standard dropout viability screen (negative selection screen) relies on the fact that some of the shRNAs in the screen are either cytotoxic or cytostatic (presumably by interfering with an essential target gene). Cells with shRNAs that do not inhibit growth, then, grow normally populating the culture in which the cells with the lethal shRNA do not propagate. The endpoint analysis involves looking for shRNA sequences that are underrepresented or depleted in the sample population relative to the original library.

Length of the Screen

For a dropout viability screen to work, the cells need to be cultured long enough for the cells with unaffected growth to significantly increase their proportion relative to the affected cells. For this to occur, the cells need enough time in culture for multiple doublings. The length of any particular screen may need to be altered depending on the specifics (e.g. cell growth rates, types of targets of interest, if additional compounds are used). However, typically, we find allowing for ~10 population doublings after transduction to be optimal.

If the screen is not run long enough, all the shRNA counts will be in a narrow range and it will be difficult to identify significantly depleted shRNA sequences from background variability. If the screen is run too long, the range of representation of shRNA sequences will become broader due to the natural growth variance in different cells in the population. This phenomena, often referred to as genetic drift, will increase the background variance of the screen and, if the spread becomes too broad, can also make it difficult to identify significantly depleted shRNAs from background variability. For optimal results, it is important to run the screen long enough that shRNAs that have an effect on cell growth/viability will be distinct from background variance but not to the point where background variance becomes large enough to confound the ability to call hits.

For a negative selection screen, the puromycin or other antibiotic selection it is not essential, but provides a way to reduce the total number of cells and makes cell culture handling more manageable. In a typical screen about 30%-40% cells are transduced, the remaining 60%-70% of cells without virus are not needed. Unless you want to maintain a larger than needed cell population throughout the screen, it makes sense to perform antibiotic selection to get rid of unwanted cells.

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MOI of Transduction, Number of Cells, Amount of DNA

As mentioned above, the number of cells stably transduced with the shRNA library at the time of transduction should exceed the complexity of the shRNA library by at least 200-fold. For a library with 27,500 shRNAs, the starting population should be at least 5.5 million infected cells, and for a library with 55,000 shRNAs, the starting population should be at least 11 million infected cells. The MOI of transduction should be kept at or below 0.5, to ensure that the majority of transduced cells carry only one integrated provirus. After transduction, the ideal is to never discard any cells at any time during the experiment (e.g. at treatment, harvesting, DNA purification, etc.). However, this is often not practical— especially for a negative screen where most of the cells propagate normally. If the number of cells becomes too large and you are forced to discard a fraction, the number of remaining cells should always exceed the complexity of the library by at least 1,000-fold (e.g. keep at least 27 million cells after every splitting step, for a 27K library). Similarly, when amplifying barcodes from isolated DNA, you should always use all the genomic DNA recovered from cell samples, up to the amount corresponding to 1,000 cells × the number of shRNA in the library.

Baseline Controls for Negative Screens

In a simple screen aimed at identifying shRNAs which are cytotoxic in a given cell line, we typically use the library itself as the baseline control, since the shRNA frequency distribution in plasmid and packaged lentiviral library is virtually identical. The plasmid library has already been sequenced as part of the QC when we made the library, so it is not necessary to re-sequence the library at this point. If you would also like to use transduced cells as a baseline control, typically we recommend harvesting and sequencing genomic DNA from them by about 18 hours post-transduction so that the cells have not had too much time to proliferate and express shRNAs to affect the library distribution. In more complex experiments, aiming at identifying differential toxicity between isogenic cell lines, or between compound-treated and non-treated cells, other baselines will be needed.

Last modified: 10 January 2019

4.2.1. Example Negative Screens

Negative Selection Screen #1

Aim: Identifying shRNAs that are differentially cytotoxic in Cell Line A vs. Cell Line B.

shRNA library used: 55K hGW shRNA Library Module 1

Cell Lines A and B each have a doubling time of ~36 hours.

Procedure:

Day 1. 5×10^7 cells per cell line transduced at MOI 0.5 (40% transduction efficiency expected).

Day 2. Media change.

Day 4. Actual transduction efficiency checked by flow cytometry. Start of puromycin selection (1 µg/ml).

Day 7. End of puromycin selection, ~3×10^8 cells/sample. In some cases, it can be appropriate to continue puromycin selection throughout the experiment. All cells from each sample are pooled together and re-plated at 6×10^7 cells/sample.

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Day 10. All cells from each sample are pooled together and re-plated at 6 × 10^7 cells/sample.

Day 13. All cells from each sample are harvested for genomic DNA isolation and NGS of integrated barcodes. 400 µg genomic DNA per sample is used for PCR and NGS of barcodes.

- shRNAs are evaluated based on barcode depletion in Sample A vs. Sample B.
- Gene hits are identified based on evaluation of targeting shRNAs.

Negative Selection Screen #2

Aim: Identifying shRNAs that synergize with cytotoxic effect of Compound X in Cell Line A. Compound X has IC_{50} = 1 nM when administered continuously for 7 days.

shRNA library used: 55K hGW shRNA Library Module 1

Procedure:

Day 1. 5 × 10^7 cells transduced at MOI 0.5 (40% transduction efficiency expected).

Day 2. Media change.

Day 4. Actual transduction efficiency checked by flow cytometry. Start of puromycin selection (1 µg/ml).

Day 7. End of puromycin selection, ~3 × 10^8 cells. In some cases, it can be appropriate to continue puromycin selection throughout the experiment. Cells are pooled and re-plated into 2 separate samples, 6 × 10^7 cells/sample. Sample A is treated with 1 nM Compound X, and Sample B is mock-treated.

Day 10. All cells from each sample are pooled and re-plated at 6 × 10^7 cells/sample. Sample A is still treated with 1 nM Compound X, and Sample B is still mock-treated.

Day 13. All cells from each sample are harvested for genomic DNA isolation and NGS of integrated barcodes. 400 µg genomic DNA per sample is used for barcode amplification and NGS.

- shRNAs are evaluated based on barcode depletion in Sample A vs. Sample B.
- Gene hits are identified based on evaluation of targeting shRNAs.

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5. Library Packaging and Transduction of Target Cells

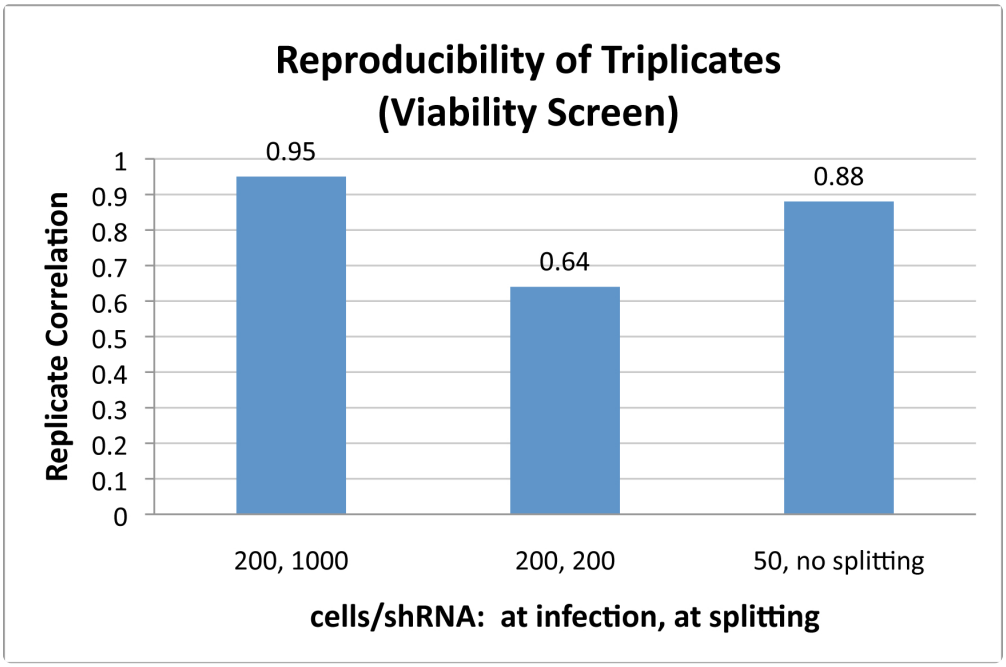
To package your lentiviral sgRNA, shRNA, or barcode library as VSV-G pseudotyped lentiviral particles, follow the general lentiviral packaging protocol. However, when packaging a library for screening, it is critical to package sufficient amount of the library to ensure representation of all the library constructs. The chart below indicates the number of 15-cm plates to use depending on the library complexity (i.e. number of constructs in the library). We do not recommend scaling down the lentiviral packaging protocol due to risk of compromising the representation of the library.

Library Complexity	Min. Number of Plates to use	Estimated Yield of Virus
6K	2 × 15-cm plates	$6 \times 10^7 – 3 \times 10^8$ TU
12K	4 × 15-cm plates	$1.2 \times 10^8 – 6 \times 10^8$ TU
24K	8 × 15-cm plates	$2.5 \times 10^8 – 1.25 \times 10^9$ TU
50K	16 × 15-cm plates	$5 \times 10^8 – 2.5 \times 10^9$ TU
80K	24 × 15-cm plates	$7.5 \times 10^8 – 3.75 \times 10^9$ TU

Pooled library screens require quantification of changes in the fraction of cells bearing each sgRNA/shRNA sequence in selected vs. control cells (or starting library). A “hit” is identified when selected cells have significantly more or fewer cells bearing a particular sgRNA/shRNA sequence. Whether one is looking at enrichment of sgRNA/shRNA sequences in the selected cell population vs. control (positive selection) or depletion of sgRNA/shRNA sequenced in selected cell population vs. control (negative selection), it is critical that the screens begin with a sufficient number of cells expressing each sgRNA/shRNA to ensure that measured changes in the fraction of cells bearing any given sgRNA/shRNA sequence are statistically significant. This means that if there are very low numbers of cells bearing specific sgRNA/shRNA sequences at the start of the screen, random changes in a drifting population may be difficult to differentiate from significant trends.

Simply put, a loss of 2 cells is a 20% change if there are only 10 initially vs. 2% if there are 100. For this reason, at least a few hundred cells on average need to be infected with each sgRNA to initiate a good screening. This is demonstrated in the figure below which shows the effect on reproducibility of different infection and splitting ratios. A smaller population of just 50 cells per shRNA (third bar) leads to significantly more variation than starting with a population of 200 cells on average for each shRNA (first bar). For this reason, for example, at least 25 million cells (500 × library complexity) are needed to start a screen with libraries of 50,000 sgRNAs.

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Reproducibility is adversely affected if starting infected cell populations have fewer than 200 cells per shRNA (third bar). Allowing representation to drop below 1000X representation at any time is even more detrimental to reproducibility, as shown in the middle bar, when splitting causes the representation to drop to 200 cells per shRNA.

For experiments with pooled lentiviral libraries of shRNA, sgRNA, or barcodes, it is important to have more cells than infecting viral particles. For sgRNA/shRNA screens, typically, 2-3 times more cells than infecting viral particles is needed (i.e., a multiplicity of infection [MOI] of 0.3-0.5) to ensure that the majority of cells are only infected with one sgRNA/shRNA-carrying virus, so you need to have 2-3 times more cells than the number targeted for infection. For barcode labeling of cells, lower MOIs may be desirable to ensure almost all transduced cells only pick up a single barcode.

The lower the MOI, the more cells you need to start the screen so it is tempting to use a high MOI. However, you should consider that a higher MOI produces a higher percentage of infected cells bearing two or more different sgRNA/shRNA constructs.

For most loss-of-function screens, we recommend optimizing conditions and performing genetic screen transductions at no more than 0.5 MOI (a 40% transduction efficiency), which balances these two considerations. Please note that to accurately calculate the MOI, it is critical to determine the library titer directly in your target cells prior to beginning your experiment. Once conditions are established to achieve ~40% transduction efficiency in the titering assay, scale up all conditions proportionately to accommodate the larger number of transduced cells needed for the genetic screen.

To ensure a comprehensive screen, it is not simply sufficient to start with the right amount of cells. During the screening process, incorrect propagation of the cells can completely undercut the representation set up at the initiation of the screen. This is especially true for a negative selection screen, such as a viability screen, where one is interested in identifying sgRNAs or shRNAs that kill or inhibit proliferation of cells, and, therefore, drop out of the population. It is critical to maintain the full library representation that was initially used at the start of the screen.

If a portion of propagated cells is removed during propagation (e.g., cells are split), the representation of the library can be skewed in the sample, which introduces significant random noise. This effect is readily seen in the middle bar in the above figure where the effect of starting with sufficient cells (i.e., 200-fold library complexity) is completely undercut by splitting cells during propagation so that the final count of cells after 10 days is the same as the initial number of transduced cells (i.e., 200-fold library complexity). The correlation between triplicates falls dramatically when the cells are split to this degree.

Once you have calculated the titer of the packaged sgRNA or shRNA library and you know the MOI and starting number of

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cells you need to maintain representation, transduce your target cells (Cas9- or dCas9-positive cells if performing a CRISPR screen) using the appropriate number and size of plates and the correct amount of virus.

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5.1. General Lentiviral Packaging Protocol

The following protocol describes the general procedure for generation of pseudoviral packaged lentiviral constructs using ThermoFisher's Invitrogen Lipofectamine™ and PLUS Reagent (see **Additional Materials for Production of Lentivirus**). Other transfection reagents may be used, but the protocol should be adjusted to fit the manufacturer's protocol. This protocol can be used to package individual lentiviral plasmid constructs expressing shRNA, sgRNA, Cas9/dCas9, barcodes, cDNA, promoter reporters, and sgRNA, shRNA, or barcode libraries in 3rd generation lentiviral vectors.

- The yield of recombinant lentiviral particles produced under these optimized conditions is typically between 1×10^6 TU/ml and 5×10^6 TU/ml for individual lentiviral constructs with a viral transcript less than 7kb (i.e. the region from the beginning of 5'LTR to the end of 3'LTR).

Plate Size	Surface Area	Volume of Media	Approximate Virus Yield *
10 cm	55 cm^2	10 ml	2×10^7 TU
15 cm	150 cm^2	30 ml	6×10^7 TU

> *** NOTE: The yield of lentiviral particles will be *significantly lower* for lentiviral constructs and libraries with viral transcripts larger than 7kb (e.g., approximately 1×10^5 TU/ml for Cas9 constructs with viral transcripts around 8kb).**

- If using tissue culture plates or flasks of other sizes, please scale amounts in the protocol based on surface area.

Cellecta offers Lentiviral packaging services. Please contact us at sales@cellecta.com or visit Cellecta's Lentiviral Packaging Services web page.

2-3 Days Prior to Packaging

1. Start growing 293T cells in D-MEM medium plus glutamine* supplemented with 10% FBS without antibiotics, and expand until you have sufficient cells to package at the scale desired.

> * **NOTE:** ADD FRESH GLUTAMINE (1X) to Dulbecco's Modified Eagle Medium (D-MEM) at the time a sealed bottle of D-MEM is opened, even if the label indicates glutamine has already been added. Glutamine in solution at 4°C has a half-life of 1-2 months, so glutamine(+) D-MEM purchased "off-the-shelf" from a supplier is to be regarded as glutamine(-). If D-MEM comes supplemented with stable L-Alanyl-L-Glutamine dipeptide, addition of fresh glutamine is not necessary. In our experience, the addition of glutamine increases titer approximately 2-fold.

Day 0 – Plate Cells

1. Twenty four (24) hours prior to transfection, plate 12.5×10^6 293T cells per **15-cm plate** (or 150 cm^2 flask) and use

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30 ml of media per plate. If you are using **10-cm plates**, plate 4 × 10^6 cells and use 10 ml of media per plate. Disperse the cells and ensure even distribution.

2. Incubate at 37°C in a CO_2 incubator for 24 hours.

> **NOTE:** The goal is to have the 293T cells reach ~80% confluency by Day 1. You may want to calculate the number of cells seeded empirically since cell counts can vary.

Day 1 – Transfection into 293T Cells

1. Using the volumes in the table below, mix Ready-to-use Lentiviral Packaging Plasmid Mix and your Plasmid Lentiviral construct in a sterile, appropriately-sized polypropylene tube. Add the D-MEM medium without serum or antibiotics to the plasmid mixture, then mix. Add the PLUS Reagent, mix, and incubate at room temperature for 15 minutes.

1 × 10-cm plate	1 × 15-cm plate	4 × 15-cm plates	8 × 15-cm plates	16 × 15-cm plates	24 × 15-cm plates	Component
20 µl	60 µl	240 µl	480 µl	960 µl	1440 µl	Packaging Plasmid Mix (0.5 µg/µl)
2 µl	6 µl	24 µl	48 µl	96 µl	144 µl	Plasmid / Library (1 µg/µl)* *see **NOTE***
1,000 µl	1,200 µl	4,800 µl	9,600 µl	19,200 µl	28,800 µl	D-MEM, no FBS, no antibiotics
20 µl	60 µl	240 µl	480 µl	960 µl	1,440 µl	PLUS Reagent
1,042 µl	**1,326 µl**	**5,304 µl**	**10,608 µl**	**21,216 µl**	**31,824 µl**	**Total volume**

> **NOTE:** The volume of plasmid DNA assumes the DNA is suspended at a 1 µg/µl concentration. For plasmid DNA at other concentrations, adjust the volume accordingly (e.g., for 0.5 µg/µl, use twice the indicated µl).

2. Add Lipofectamine Reagent to the D-MEM medium without serum or antibiotics in order to make a master mix according to the table below. Mix gently.

1 × 10-cm plate	1 × 15-cm plate	4 × 15-cm plates	8 × 15-cm plates	16 × 15-cm plates	24 × 15-cm plates	Component
1,000 µl	1,200 µl	4,800 µl	9,600 µl	19,200 µl	28,800 µl	D-MEM, no FBS, no antibiotics
30 µl	90 µl	360 µl	720 µl	1,440 µl	2,160 µl	Lipofectamine Reagent
1,030 µl	**1,290 µl**	**5,160 µl**	**10,320 µl**	**20,640 µl**	**30,960 µl**	**Total volume**

3. Add the diluted Lipofectamine (from Step 2) to the DNA / PLUS Reagent complex (from Step 1 above), mix gently by flicking the tube or vortexing and incubate at room temperature for 15 minutes.

4. Add 2.5 ml of the resulting DNA / PLUS Reagent / Lipofectamine complex to each plate (from the previous step **Day 0 – Plate Cells**), and mix complexes into the medium with gentle rotation. Take care not to dislodge cells from the plate.

5. Incubate at 37°C in the CO_2 incubator for 24 hours.

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Day 2 – DNase I Treatment

1. At 24 hours post-transfection, replace the medium containing complexes with 30 ml (for 15-cm plates) or 10 ml (for 10-cm plates) of fresh D-MEM medium supplemented with 10% FBS, DNase I (1 U/ml), $MgCl_2$ (5 mM), and 20mM HEPES, pH 7.4.

2. Continue incubation in the CO_2 incubator at 37°C overnight.

> **NOTE:** Overnight DNase I treatment before harvesting virus does not negatively affect lentiviral titer or infectivity and helps prevent undesirable carryover of plasmid DNA into the virus prep.

> ***IMPORTANT:*** Failure to change the media the day after transfection results in large carryover of plasmid (free and/or Lipofectamine-bound) into your lentiviral prep. This may cause problems with most downstream molecular biology applications, especially whenever there is a PCR step involved such as during NGS sample preparation after a pooled library screen.

Day 3 – Collect Lentiviral Supernatant

1. At 48 hours post-transfection, collect the entire virus-containing medium from each plate and filter the supernatant (~30 ml per 15-cm plate or ~10 ml per 10-cm plate) through a Nalgene 0.2 µm PES filter (a low protein-binding filter) to remove debris and floating packaging cells. Failure to filter supernatant could result in carry-over of cells into your lentiviral prep.

> **NOTE:** Usually, the peak of virus production is achieved at 48 hours post-transfection. Supernatant can also be collected again at 72 hours post-transfection—replace the collected 48-hour supernatant with 30 ml (for 15-cm plates) or 10 ml (for 10-cm plates) of fresh D-MEM medium supplemented with 10% FBS and 20mM HEPES pH 7.4, and continue incubation in the CO_2 incubator at 37°C for 24 hours. Then, repeat Step 1 above for 72 hours post-transfection.

NOTE: Freezing and thawing lentivirus results in some loss of titer with each cycle.

Concentration of Lentivirus (Optional)

The following procedure was optimized to concentrate virus harvested at 48 hours with high recovery. Although concentrating virus is optional, it is recommended if any of the following conditions applies:

- Very high titer virus stock is needed to achieve desired MOI in hard-to-transduce target cells.
- The virus needs to be suspended in a different media (besides D-MEM/10%FBS) that is optimal for sensitive target cells.
- If packaging an shRNA, sgRNA, or barcode library for screening, because the concentration minimizes problems that might arise from genomic DNA carryover.

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Virus Concentration Protocol

1. Aliquot lentiviral supernatant in clear, sterile centrifuge tubes.

2. *(Recommended)* Add Cellecta's LentiFuge™ Viral Concentration Reagent (see **Additional Materials for Production of Lentivirus**) according to the protocol described in the LentiFuge User Manual.

3. Centrifuge at 15,000 × g for at least 1 hour at 4°C. Mark the tubes to identify the location where the pellet will be. At the end of centrifugation, you may or may not be able to see a pellet—assume it is at the location of the mark.

4. Immediately discard the supernatant by aspirating.

5. Place the tubes on ice, resuspend the pellet (which may not be visible) in PBS, PBS/10%FBS, or PBS/1%BSA, make aliquots, and freeze at -80°C. 100-fold concentration is recommended (e.g., resuspend in 1 ml PBS if starting from 100 ml supernatant).

Last modified: 29 October 2024

5.2. General Lentiviral Transduction Protocol

This section provides the general protocol for transduction of mammalian cells with VSV-G packaged lentiviral particles. This protocol was developed and optimized using HEK293 and K-562 cells, and has been successfully used with many other common cell types. However, each cell is different and, depending on the characteristics of your specific cells, some optimization may be necessary.

Lentiviral transductions are performed by mixing cells and virus in culture media. For both adherent and suspension cells, transductions are initiated in suspension and carried out overnight. Adherent cells are allowed to adhere to substrate during transduction and are transduced at a cell density that allows for 2-3 population doublings before reaching confluence. Suspension cells are typically transduced at a higher density than standard growth density, and then they are diluted to standard growth density 18-24 hours after transduction. Do not let cells become too dense or let the medium become yellow at any point.

Before transduction, seed and expand cells from frozen stocks. Cells should be actively growing.

> ! **CAUTION:** Only open the tube containing the lentiviral particles in the laminar flow hood. Please refer to the **Safety Guidelines** and check with your institution regarding the use of lentiviruses.

Day 0—Inoculate Cells

1. Quickly thaw the lentiviral vector particles in a water bath at 37°C. Transfer the thawed particles to a laminar flow hood, gently mix by rotation, inversion, or gentle vortexing, and keep on ice. Unused viral particles can be aliquoted, refrozen at -80°C, and used again for subsequent experiments. There will be some loss of viral activity (usually 10-20%) with each refreeze.

2. Suspend sufficient cells for transduction in appropriate complete media supplemented with 1 µl/ml LentiTrans™ Transduction Reagent—if cells are sensitive to the LentiTrans Reagent, use a lower concentration or omit it. For HEK293 cells, we usually suspend at a density of 1×10^5 cells/ml in D-MEM supplemented with 10% FBS and LentiTrans Reagent. For K-562 cells, we usually suspend at a density 2×10^6 cells per ml in RPMI/10%FBS

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supplemented with 20mM HEPES, pH 7.4 and LentiTrans Reagent.

3. Aliquot cells into wells or plates. For small scale transductions or titering assays, cells may be plated into multiwell microtiter plates (e.g., 0.5 ml/well for 24-well plates or 1 ml/well for 12-well plates). To transduce larger numbers of cells, use larger plates and scale up the volume accordingly

4. To each plate, add an appropriate amount of lentivirus. The amount of virus will depend on your viral titer and your experiment. Refer to the Transduction Guidelines or Assay Procedures section of the product manual for application specific recommendations.

5. Close the plate and mix by gentle agitation:
    - For adherent cells, place the plate into the CO_2 incubator and grow cells under standard conditions for 16-24 hours.
    - For suspension cells only, “spinoculate” by wrapping the perimeter with parafilm, placing the plate into the centrifuge with an appropriate balance, and spin the cultures at 1,200 × g at +25°C for 2 hours. Following centrifugation, remove plate(s) from centrifuge, carefully remove parafilm, and place in incubator. After 3 hours, “feed” cells with 0.5 ml additional complete medium per well (no LentiTrans Reagent).

Day 1—Change Media

Between 16 to 24 hours post-transduction, remove media and replace with fresh complete media without LentiTrans Reagent. For suspension cells, spin down and resuspend cells in complete media at 1-5 × 10^5 cells/ml. Place in incubator and grow for an additional 24-48 hours. Avoid confluency or too high a density of cells during and after transduction. If necessary, replate.

Day 3 or 4—Harvest or Split Cells

At about 72 hours after adding virus, you may expand cells as normal or harvest cells for an assay. To continue growing cells, split the cells 1:4 to 1:8 (or as appropriate, depending on the type of cells) as the culture approaches confluence, and add complete medium. As required by your experiments add antibiotics, other factors (tetracycline), etc., and expand as normal.

NOTE: Typical doxycycline concentration for induction is 0.5 ug/ml. However, some cell lines can be more sensitive than others, so you may want to optimize the concentration.

Last modified: 29 December 2023

5.3. Assessing Transduction Efficiency by Flow Cytometry or Antibiotic Selection

For lentiviral constructs with a fluorescent marker or antibiotic resistance marker, transduction efficiency (i.e., % infected cells) can be determined from the fraction of fluorescent or antibiotic resistant cells in the population. To calculate the viral titer, it is also important to know the total number of cells at the time of transduction.

To determine viral titers, transductions should be set up as a serial dilution range of different amounts of virus in 12-well plates, 100,000 cells/well. For example, when titering standard virus from Cellecta, we recommend aliquoting 0, 0.1, 0.3, 1, 3.3 of concentrated stock to 5 different wells (or, for non-concentrated virus, scale up volumes ca. 100-fold). If you are using

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cells that transduce with low efficiency (e.g., primary cells, some suspension cell lines, etc.) you may need to use 10 µl, or even 30ul, of concentrated virus stock for titering. Always be sure to include the initial well of cells only (no virus) as a negative control.

Option 1: Use flow cytometry with a fluorescent protein marker to determine % infected cells:

> **IMPORTANT:** Do not use fluorescence microscopy to assess the percentage of transduced cells. A significant number of transduced cells will be missed, so transduction efficiency will be underestimated.

1. About 72 hours after adding virus to the cells, spin down and resuspend cells in plate in 1X D-PBS.

2. If trypsin is used, block it with FBS/media, then centrifuge.

3. Determine the percentage of transduced (RFP/GFP/BFP-positive) cells by flow cytometry. For detection of Cellecta's RFP-, GFP-, and BFP-positive cells use the following settings:

Flow Cytometry Settings for TagRFP	
Excitation:	561nm (yellow laser) [530nm laser is still acceptable]
Emission:	590/20nm band-pass filter, or similar
Flow Cytometry Settings for TagGFP2	
Excitation:	488nm (blue laser)
Emission:	530/20nm band-pass filter, or similar
Flow Cytometry Settings for TagBFP	
Excitation:	405nm (violet laser)
Emission:	470/20nm band-pass filter, or similar

4. Use the formula below to calculate the percentage of transduced cells:

> (Fluorescent cells / Total number of cells) x 100 = **% infected cells** (use this for the titer calculation)

Option 2: Use antibiotic selection to determine % infected cells:

1. About 72 hours after adding virus to cells following the standard transduction protocol, split each transduction dilution (including the no-virus control) into 2 separate twin wells using 1:8 split ratio.

2. Add an appropriate amount of antibiotic to one twin well for each viral dilution (including the no-virus control).

3. Incubate all cultures for 2-5 additional days to allow antibiotic time to kill >99% cells in the no-virus control. Do not let the cultures reach confluence (split if needed).

4. After the antibiotic has had time to kill the cells, remove media from cells and replace with fresh media containing a 1/10 total volume alamarBlue stock solution. Include a media-only sample (no cells) for a background alamarBlue reading.

5. Incubate 1 hour at 37°C. Read the fluorescence intensity of the alamarBlue staining using a plate reader. Use the alamarBlue fluorescence values in the following calculation to determine the percent transduced cells in each antibiotic/non-antibiotic pair:

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> ([antibiotic-selected cells – media-only] / [non-selected {no antibiotic} cells – media-only]) x 100 = **% infected cells** (use for titer calculation)

Last modified: 18 January 2024

5.4. Lentiviral Titer Calculation

This section describes how to calculate the titer of packaged lentiviral particles. To calculate the number of transducible viral particles in the viral stock, you need to know the number of cells transduced at a specific dilution factor of the stock virus. This can be assessed by counting RFP-positive cells on a flow cytometer or using staining to assess cells with antibiotic resistance (e.g., puromycin).

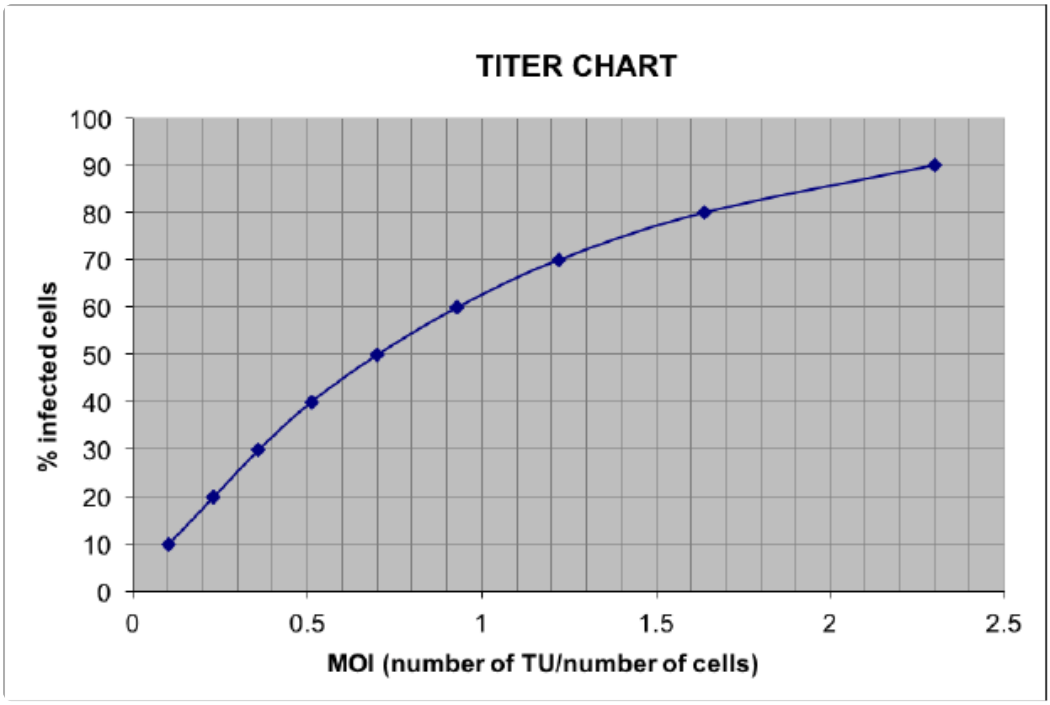
- It is important to use an amount of virus sufficient to only transduce a fraction of the target cells so that you can accurately assess the number of transductions occuring. You cannot accurately assess the number of transductions if the whole population is transduced (i.e., fluorescent, or die out due to antibiotic selection). Ideally, it is best to aim for transducing less than half the cells so that most of the cells have been transduced with a single viral particle. For this reason, it is typical to titer several dilutions of the viral stock to obtain a culture with enough transduced cells for the calculation but not to the point where there are more viral particles than cells.

- To calculate the titer of a viral stock, it is necessary to have the following information:
    - The number of cells at transduction.
    - The percent of transduced cells after transduction (i.e., the Transduction Efficiency).
    - The volume of the viral stock used to transduce the cells.

Calculate the Transduction Units at Infection.

Lentiviral titer is measured as Transduction Units per ml (TU/ml). One TU produces one integration event in target cells. To calculate the viral titer, it is first necessary to determine the number of Transduction Units (TU) used to infect the cells. When the percentage of infected cells is at or below 20%, the number of integrations is approximately equal to the number of transduced cells. However, at higher transduction levels, the fraction of transduced cells with multiple integrations increases, so that the percentage of transduced cells relative to integration events per cell is no longer linear. Using the chart below, the number of integrations per cell, or **MOI** (Multiplicity Of Infection), can be accurately estimated for cultures with up to 75% transduced cells (i.e., MOIs in the range of ~0.2-1.5).

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% Infected Cells	MOI
10	0.10
20	0.23
30	0.36
40	0.51
50	0.70
60	0.93
70	1.22
80	1.64
90	2.30

Estimation of percentage of cells infected based on Multiplicity Of Infection (MOI).

Calculate the Stock Titer

To calculate the titer of the original viral stock, apply the formula below:

> TU/ml = (# of cells at Transduction) × [MOI / (ml of Lentiviral Stock used at Transduction)]

- # of cells at Transduction = Total number of cells in the culture when viral particles were added
- MOI = Derived from the chart above based on the percentage of transduced cells.
- ml of Lentiviral Stock used for Transduction = The volume in ml of the virus added to the cells. Include any dilution of the viral stock.

Example Calculations:

> **IF:**
> The original # of cells at Transduction was 100,000, and
> The volume of virus stock used was 10 µl, and
> The observed % of transduced cells (RFP+ or antibiotic resistant) is 25%,
>
> **THEN:**
> The calculated MOI is 0.3 (from the chart), and
>
> The **TITER** is:
> 100,000 × 0.3 / 0.01 = 3,000,000 TU/ml

Last modified: 29 December 2023

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5.5. Antibiotic Selection of Library Transduced Cells

Antibiotic selection of transduced cells must be carried out if depletion of non-transduced cells is needed in your experiment. However, it is important to avoid using too high an antibiotic concentration for two reasons: (1) you want to avoid the killing of a sizable fraction of transduced cells, and (2) you do not want to enrich for cells with multiple viral integrants. The general procedure for the selection of the appropriate antibiotic concentration is described in “section 3.2 Calculating a Kill Curve”. If the lentivector carries a fluorescent marker as well (e.g. RFP), the procedure below is recommended:

Determine the optimal concentration of antibiotic by flow cytometry.

1. Based on the titer efficiency of your cells (see Assessing Transduction Efficiency by Flow Cytometry or Antibiotic Selection section), set up transduction cell pools using the amount of viral particles that resulted in 20%-50% transduced cells.

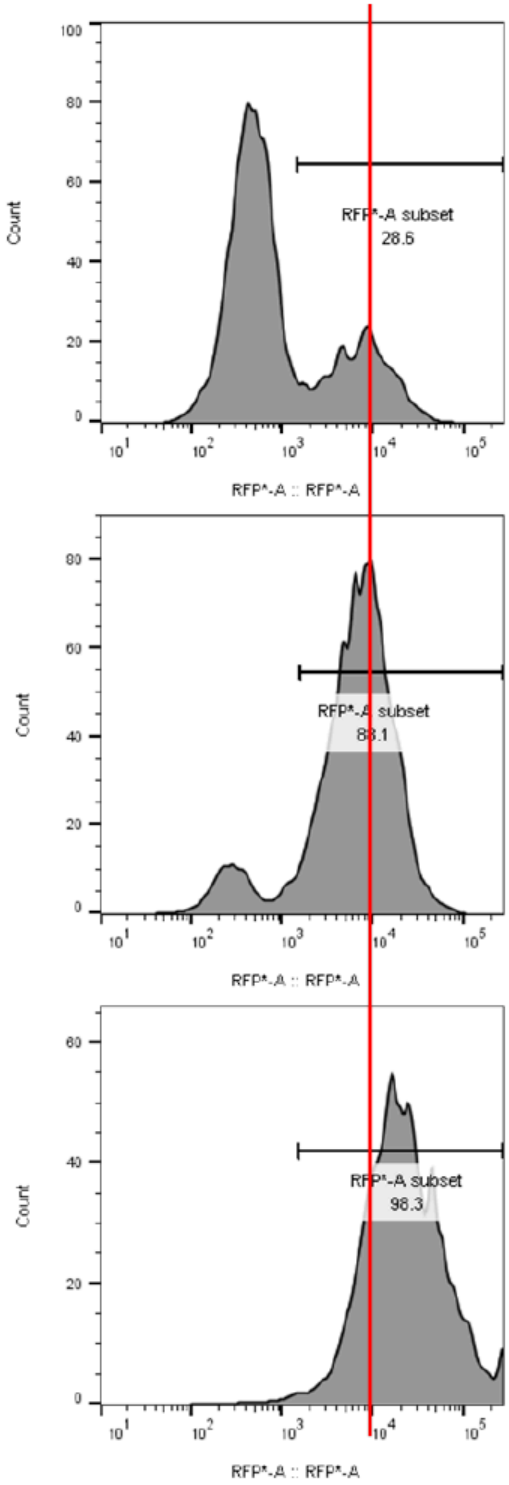
2. Aliquot same amount of cells in six 12-well wells (~50,000 cells /well).

3. Add increasing amount of antibiotic to each well (for puromycin selection, you would typically use a concentration range between 0.1 ug/ml – 10 ug/ml in 2-fold increments). Include a no-antibiotic control well.

4. Select the lowest antibiotic concentration that resulted in >90% transduced cells selection, while maintaining the fluorescence intensity of selected and unselected transduced unchanged.

In the example below, the top histogram is the cells transduced at MOI ~0.3, unselected; the middle histogram is the transduced cells selected with an antibiotic concentration which resulted in the enrichment of transduced cells to ~90%, without affecting RFP intensity; the bottom histogram is the transduced cells selected with an antibiotic concentration which resulted in the enrichment of transduced cells to ~99%, but also increased RFP intensity (at the expense of library representation).

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Distribution of RFP+ Cells

Last modified: 10 July 2025

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6. Extraction and Sequencing of sgRNA or Barcodes from Genomic DNA

Identification of sgRNA, shRNA, or barcode sequences in experimental cell or tissue samples from screens with pooled libraries requires (1) isolation and amplification of the target sgRNA or barcode inserts from the genomic DNA, and (2) Next-Generation Sequencing (NGS) of amplified sgRNA or barcode sequences using an Illumina NGS instrument. Following amplification, representation levels of each of the sgRNA or barcode sequences are then quantified to assess enrichment or depletion relative to the starting library or relevant control samples, depending on the type of screen.

Last modified: 21 September 2023

6.1. Genomic DNA Extraction

There are several protocols that can be used for DNA isolation. However, to accurately measure the relative fraction of each sgRNA or barcode present in a specific cell population, it is important to isolate the whole amount of genomic DNA from the cells derived from a genetic screen or barcoded sample. Purification of genomic DNA from just a fraction of cells at a particular time point or treatment condition in a screen, may not provide full representation the guides, hairpins, or barcodes present in the screened sample. As a result, the choice of DNA isolation protocol should be based on the number of cells in your samples.

We recommend the following protocols:

- For genomic DNA isolated from 10 million or more cells—which are typical from standard “dropout viability” CRISPR and RNAi screens with larger libraries—we highly recommend using the Large-Scale Genomic DNA Extraction Protocol. With this procedure, you usually obtain 50-100 µg of genomic DNA per 10 million cells. This amount of genomic DNA will overload most column-based DNA isolation kits and compromise yields. Conventional Genomic DNA Extraction is not column based and avoids DNA loss which can distort representation of the guides or barcodes in the population.

- For small and medium-sized populations of cells (from small libraries screens, positive selection screens where most of the cells are killed off, or FACS-based enrichment screens), we recommend using the following QIAGEN kits:
  - From 1 million to 10 million cells: Use the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Cat.# 69504)
  - For fewer than 1 million cells: Use the QIAGEN QIAamp DNA Micro Kit (QIAGEN, Cat.# 56304)

- After purification, you should resuspend your DNA at a concentration of ca. 1-2 µg/µl in water or Tris buffer (**Important:** Do not resuspend DNA in a buffer that contains EDTA. It will inhibit the PCR reaction.). DNA samples can be stored at +4°C for a few weeks or at -20°C for an extended period of time.

Last modified: 3 April 2025

6.1.1. Large-Scale Genomic DNA Extraction Protocol

> **NOTE:** Use of disposable tubes is highly recommended in order to avoid contamination.

1. Resuspend cell pellet in 5 ml QIAGEN Buffer P1 (with RNase A) in a 15 ml polypropylene (phenol:chloroform resistant), Falcon screw-cap centrifuge tube (12,000 RCF rated).

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2. Add 0.25 ml 10% SDS, mix, and incubate 5 minutes at room temperature.

3. Using an ultrasonic homogenizer, sonicate to shear DNA into 10-100 kb sized fragments. To prevent cross-contamination, thoroughly wash the ultrasound head with running water and dry with clean paper towels between samples.

4. Add 10 µl of Proteinase K, mix, and incubate 15 minutes at room temperature.

5. Add 5 ml Phenol:Chloroform:Isoamyl Alcohol solution, vortex hard, and spin down 60 min, 20°C at 8,000 rpm in JA-14 or equivalent rotor (Beckman).

6. At this point, there should be approximately 5 ml of clear upper phase. Transfer 4 ml of upper phase to a new 15 ml disposable screw cap tube (same as in Step 1).

7. Add 0.5 ml of 3M Sodium Acetate and 4 ml isopropanol, mix well, then spin down 30 min, 20°C at 8,000 rpm in a JA-14 or equivalent rotor.

> **NOTE:** To produce a more visible pellet that is compacted at the bottom of the tube, it is recommended to incubate overnight at room temperature before centrifugation.

8. Discard supernatant, add 10 ml of 70% ethanol, and spin down 5 min, 20°C at 8,000 rpm in a JA-14 or equivalent rotor.

9. Discard supernatant and air-dry pellet.

10. Dissolve DNA pellet in an appropriate volume of dH_2O to a concentration of ~2 mg/ml.

11. Incubate 30 minutes at 80°C before spectrophotometer reading.

> **NOTE:** Expected yield is about 10 µg genomic DNA per 1 million cells.

Last modified: 29 December 2023

6.2. Amplification of sgRNA or Barcode Sequences from Genomic DNA

The protocol is based on two rounds of PCR to amplify the integrated sgRNA, shRNA barcode sequences, or clonal barcodes from the genomic DNA. Also, the second round of PCR includes primers that add the sample-specific indexes and sequences (P5 and P7) complementary to primers immobilized in Illumina flow cells. With the PCR reactions, the optimization of cycles is required to avoid over-cycling. Using an optimal cycle number is necessary to generate a sufficient amount of amplicons to sequence while maintaining the relative representation of each sgRNA or barcode in the cell population.

- As an amplification/sequencing positive control, we recommend that you use the original shRNA/sgRNA/barcode plasmid library (1 ng) used in the screen. This positive control can be used as a baseline in NGS data analysis to measure the enrichment or depletion of effector constructs in the experimental samples. If you do not have the plasmid library, we suggest either using any construct in the same vector to serve as a positive control for PCR or the packaged library to serve as an amplification/sequencing positive control as described in the following bullet.

- Although it is typically not necessary, you may use the packaged effector library as a positive control. In order to use

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the packaged library as a positive control, you can start with a volume containing sufficient viral particles equal to at least a few hundred-fold the complexity of the library. For example, for a 50,000-construct library, you should use a volume containing 10-25 million transduction units (TU). Also, before starting PCR, you will need to reverse transcribe the viral RNA sequence using a reverse primer that targets the sense (upper) strand of the insert site at a location where it will generate a full transcript of the target sgRNA/barcode region, including the PCR primer sites. For most Cellecta libraries containing the UbiC promoter, the RevUbiC1 primer (5'-AGGCAGCGCTCGCCGTGAGGA-3') adjacent to the cloning site can be synthesized and used for the reverse transcription step.

First Round PCR

The goal of the first PCR is to amplify shRNA barcodes, sgRNA inserts, or barcode library sequences from genomic DNA isolated from each experimental cell population. The amount of starting DNA can vary depending on the type of screen. For samples with up to 50 µg of genomic DNA, run a single 100 µl reaction for the each sample. For samples with more than 50 µg of DNA, we recommend dividing up the run into multiple 100 µl PCR reactions, each with a maximum of 50 µg DNA.

1. For each sample, prepare a PCR mix according to the table below, where ***x*** is the volume of genomic DNA (typically 50 µl at 1 µg/µl). Many samples will have more than 50 µg of DNA. For these samples, you will need to scale up the master reaction below as needed for each sample, then divide the reaction into multiple tubes with 100 µl in each. For example, if you have 180 µg of DNA in one sample, you should prepare a 400 µl master mix, then aliquot 100 µl in four PCR tubes for amplification (180 µg / 50 µg = 3.6, so round to 4 reactions).

Volume	Component
x µl	Genomic DNA (up to 50 µg)
3 µl	Forward 1st Round PCR Primer
3 µl	Reverse 1st Round PCR Primer
2 µl	dNTP Mix
10 µl	Taq Polymerase Buffer
80 – ***x*** µl	PCR-Grade Water
2 µl	Taq Polymerase
100 µl	**Total volume**

2. Mix gently, centrifuge briefly to collect droplets, divide samples with more than 50 µg of genomic DNA into 100 µl PCR reactions as necessary.

3. Perform PCR using the following cycling conditions.

95°C, 2 minutes	1 cycle
95°C, 30 seconds, 65°C, 30 seconds, 68°C, 2 minutes	16-18 cycles*
68°C, 2 minutes	1 cycle

> * **Note:** Depending on your library, the specific number of cycles may need some optimization. For barcode libraries (and shRNA libraries with barcodes), 16 cycles is typically sufficient. For sgRNA libraries and dual-sgRNA libraries, 18 cycles is recommended. If you are using less than 50 µg of genomic DNA in a 100 µl PCR reaction, increase the number of cycles (e.g., for 25 µg, use one extra cycle). However, please note that we do not recommend analyzing the First Round PCR product by gel or equivalent (Bioanalyzer, etc.). You may not see a band at all or just a light band at most. You should evaluate the PCR product only after running both First and Second Round PCR.

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Second Round PCR

The Second Round of PCR is required in order to significantly reduce genomic DNA carryover into the NGS step. Additionally, the Second Round PCR primers add the P5 and P7 sequences that are complementary to the immobilized primers in the NGS Illumina Flow Cells.

- For Cellecta NGS Prep Kits and Supplementary Index Primer Sets, the NRev Index Primers (up to 24) each contain a different index sequence which will be used to deconvolute the sequencing results for each sample mixed together in the same Flow Cell or sequencing lane (i.e., multiplex sequencing). It is important, therefore, to use a different NRev Index Primer (each signified by a different letter) to amplify each sample that will be run together in the same Flow Cell or lane.

For the Second Round of PCR, start with an aliquot of 5 µl of the First-Round PCR reaction for each sample.

1. If you are starting from more than 50 µg of genomic DNA, it is important to combine all individual reactions for each sample together into a single tube.

2. Perform the Second Round of nested PCR using a 5 µl aliquot of the First Round PCR product for each sample:

Volume	Component
5 µl	First Round PCR Product
5 µl	NFwd Primer
5 µl	NRev (Index) Primer*
2 µl	dNTP Mix
10 µl	Taq Polymerase Buffer
71 µl	PCR-Grade Water
2 µl	Taq Polymerase
100 µl	**Total volume**

> * **Note:** Each sample to be sequenced together in a single Flow Cell or lane needs to be amplified with a different NRev Index Primer in the Cellecta NGS Prep Kit. Be sure to use a differently lettered NRev Index for each sample that will be loaded on the same Flow Cell or lane.

3. Perform PCR under the following cycling conditions.

95°C, 2 minutes	1 cycle
95°C, 30 seconds, 65°C, 30 seconds, 68°C, 2 minutes	12 -16 cycles**
68°C, 2 minutes	1 cycle

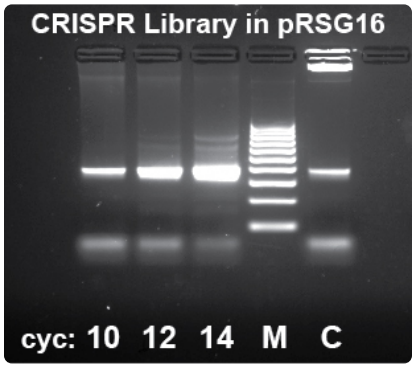
> ** **Note:** Additional cycles may be needed for samples as described below.

4. Analyze the amplified products from the Second Round of PCR. This analysis may be done on an Agilent® 2100 Bioanalyzer® instrument with the Agilent® High Sensitivity DNA Kit (Cat.# 5067-4626) or a Fragment Analyzer (Advanced Analytical) instrument using Standard Sensitivity NGS Analysis Kit (Cat.# DNF-473-1000) using the manufacturer's protocol, if you have access to this equipment. If not, then we recommend the following procedure using agarose gel electrophoresis to analyze the samples:
    - Run all samples including positive control on 3.5% agarose-1xTAE gel. Mix 5 µl of aliquot from the each

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sample with 2x loading buffer (e.g. 10% sucrose with Bromophenol Blue tracking dye).

- Analyze the intensity of the bands after electrophoresis (i.e., Bromophenol Blue tracking dye about 80% down the gel). The typical results of agarose gel electrophoresis analysis are shown in the figure below. The expected size of amplified barcode, shRNA barcode, and sgRNA products is typically between 125 to 400 bp or dual-sgRNA product around 750bp and is provided in the NGS Cassette Design information specific for your library or NGS Prep Kit Product Certificate. If you did not receive Cellecta's Order Information email with links to this information, please contact us at tech@cellecta.com.



CRISPR Human Genome 80K Knockout Library NGS PCR product analysis by gel electrophoresis. In this example, 10 cycles is the recommended cycle number for the Second Round PCR step.



DECIPHER Library NGS PCR product analysis by gel electrophoresis

5. Quantify the amount of the amplified product for each sample using software tools provided with instrument or gel imager. If all samples show similar yield of amplified products (+/- 2-fold) no addition cycling is required and you should proceed to NGS Samples Purification. If there is a greater than 2-fold variance, you should put the samples with very weak or missing bands back into the PCR instrument for additional cycles. For example, if the amount of PCR product in the sample is 4, 8, or 16-fold less than in other samples, run that sample for an additional 2, 3, or 4 PCR cycles, respectively. For samples in the +/- 5-cycle range, don't run additional cycles.
    - If you run additional cycles on any samples, quantify the reaction products again by using one of the instruments mentioned above or gel electrophoresis to ensure that all samples have correctly amplified products.
    - Avoid overcycling! Try to use the minimum number of cycles which allows quantitation of the bands. Importantly, for dual-sgRNA libraries, overcycling induces a significant level of recombination between different dual-sgRNA molecules. Overcycling also often generates additional higher molecular weight bands. These bands do not normally interfere with NGS unless they are very strong. Also, overcycling has the potential to compress the representation of the targeted sequences if the reaction exceeds the exponential phase. Therefore, it is desirable to minimize the cycle number. If there are excessive higher molecular weight bands, you can rerun Second Round PCR for that sample using another 2 µl of DNA from the First Round PCR and a fewer number of cycles.

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DNA samples can be stored for several days at +4°C at this stage or move directly onto NGS Sample Purification

Last modified: 27 December 2024

6.3. NGS Sample Purification

To maximize sequencing of the targeted sgRNA or barcodes and minimize background sequences, we recommend purifying the amplified PCR products by preparative gel electrophoresis.

1. Based on quantitative analysis of PCR product yield for each of the samples, combine the PCR products from each sample at the same amount. For example, for samples with yields of correct PCR product of 20 ng/µl and 60 ng/µl, combine 6 µl and 2 µl of PCR reaction, respectively.

Purify and concentrate the PCR products from the combined sample using the QIAGEN QIAquick PCR Purification Kit, following the manufacturer's protocol. The combined sample should be eluted in a volume of approximately 30 µl.

2. After purifying and concentrating, run each purified combined PCR product with 10X loading buffer on a 3.5% agarose-1XTAE gel with well sizes that accommodate at least 50 µl.

3. Using a scalpel and a 365 nm (UV-A) lamp, excise the narrow band that corresponds to the correct target sgRNA or barcode amplicon size. DO NOT use a 302/312 nm (UV-B) lamp!

CAUTION! Be sure to use UV safety glasses to protect your eyes when viewing and excising the DNA from the gel on the transilluminator.

4. After excision, purify the combined PCR product from the gel fragment using a QIAquick Gel Extraction kit following the manufacturer's protocol. Elute the purified PCR product in 20 µl of elution buffer.

> **Note:** Be sure to centrifuge QIAquick columns at maximum speed for at least 3 minutes before eluting DNA to avoid ethanol contamination in the purified PCR product.

5. Quantify extracted DNA in the combined sample by A260nm OD measurement using a NanoDrop spectrophotometer (or equivalent), and then adjust the concentrations in all samples to 10 nM. For example, if the amplicon size is 200bp, the 10 nM concentration corresponds to 1.32 ng/µl based on A260 OD measurement. For an amplicon size of 750bp (dual-sgRNA product), a 10 nM concentration corresponds to 4.95 ng/µl.

DNA samples can be stored at -20°C at this stage.

Last modified: 3 April 2025

6.4. Sequencing sgRNA Inserts or Barcodes

The Second Round nested PCR primers contain sequences (P5 and P7) complementary to the sequences of the immobilized primers necessary for generating amplification clusters on any of Illumina's NGS Instruments. In addition, if you are using a Cellecta NGS Prep Kit, the NRev Index Primers add sample-specific index sequences which allow you to combine and multiplex up to 24 samples on a single flow cell or lane. The primers were designed with the NextSeq instrument in mind, but they are compatible with other Illumina instruments as well.

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IMPORTANT: For certain libraries, such as sgRNA and dual-sgRNA, the Cellecta 6-nt Index should be read using Read 2, **not** in the Illumina Index 1. Please check the Product Certificate for the appropriate LNGS Kit for more information.

The number of sequencing cycles (read length) required depends on the sgRNA or barcode length. Please refer to your library information (NGS Cassette Diagram for the specific Cellecta library or the Product Certificate for the NGS Prep Kit).

Guidelines for preparing samples for sequencing:

- Combine together equal amounts (each adjusted to 10 nM) of experimental and positive control samples to be sequenced together. Importantly, each sample in the combined pool needs to have a unique index sequence.

We recommend that you DO NOT combine Cellecta samples with samples of different origin on a single flow cell or lane. It may cause sequencing errors.

> **Note:** The number of samples which can be sequenced in one lane or flow cell is based on the complexity of the pooled library and expected total number of reads generated with the Illumina instrument. You will need to determine how many target reads you want per sample based on your experiment.

- Prepare the samples for NGS by following the Illumina “Denature and Dilute Libraries Guide” specific for your system.
  - NextSeq 500: https://support.illumina.com/downloads/nextseq-500-denaturing-diluting-libraries-15048776.html
  - HiSeq: https://support.illumina.com/downloads/hiseq-denature-dilute-libraries-guide-15050107.html

- Spike in 5-15% of the PhiX Control to the pooled indexed sample to increase the nucleotide diversity.

- Mix Cellecta’s **Seq NGS Primer** with the Illumina primer mix (for the NextSeq, spike in the primer at the appropriate cartridge position—usually at Read 1). For indexing multiple samples in a flow cell or lane using a Cellecta NGS Prep Kit, also add the **Index NGS Primer** directly to the appropriate well (i.e., spike in either with the Illumina index primer or the reverse sequencing primer; see Note below). Each primer should have a final concentration of 300 nM for the NextSeq, or 500 nM for the HiSeq. We recommend measuring the final volume of the primer mix in each well to ensure accurate spiked-in primer concentrations. For primer spike-in information on other Illumina instruments, please refer to this Illumina guide: https://knowledge.illumina.com/library-preparation/general/library-preparation-general-reference_material-list/000001542

> **Note:** Depending on the way the amplification and sequencing primers were designed, the position of the Seq NGS and Index NGS primers may vary. Refer to the supplementary information (Product Certificate or NGS Cassette Diagram file) provided for the specifics on how to set up the NGS Primers for your specific library.

- Specify the appropriate number of cycles to sequence the complete sgRNA or barcode region (please refer to Product Certificate or NGS Cassette Diagram file), and specify the indicated number of cycles to read the 6-base Index sequence if using a Cellecta NGS Prep Kit.

Last modified: 7 May 2024

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7. Technical Support

Please provide answers to the questions below (where applicable) and then contact us for assistance.

Library Used:

1. Which library did you use?
2. What is the lot number?

Packaging the Library:

1. What was the lentiviral titer, and what was the total number of TU packaged?
2. How was the virus concentrated? (if applicable)

Transducing Target Cells:

1. What MOI did you use to transduce your target cells?
2. What target cells did you use?
3. How many replicates did you use? (i.e., duplicate, triplicate, etc.)
4. Did you use puromycin after transduction, and at what concentration?
5. For how long did you use puromycin on the cells?

Library Screen:

1. Could you briefly explain your experiment?
2. How many infected cells were used?

Sample Preparation & NGS:

1. Describe the protocol you used to amplify the barcodes.
2. What NGS system and which Illumina Kit did you use?
3. How much PCR product was used for NGS?
4. How many sequences were read per sample?

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8. Safety Guidelines

The HIV-based lentivector system is designed to maximize its biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The tat-transactivator domain has been removed from the upstream 5'-LTR in the lentivector to disable transcription from the 5'-LTR.
- The three proteins (gag, pol, env) are necessary for replication and packaging of the lentiviral vector. When packaging lentiviral particles, the genes encoding these three packaging proteins are separately introduced and expressed in the packaging cells using separate ordinary plasmids that lack packaging signals and share no significant homology to the lentivector. As a result, none of the HIV-1 genes (gag, pol, env) will be present in the packaged lentiviral genome.
- None of the HIV-1 genes (gag, pol, env) will be present in the packaged lentiviral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.
- Lentiviral particles will carry only a copy of your expression construct.

Despite the above safety features, use of replication-incompetent lentiviral particles falls within NIH Biosafety Level 2 criteria. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at:

> https://www.cdc.gov/labs/bmbl/

It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and follow standard microbiological practices, which include:

- Wear gloves and lab coat at all times when conducting the procedure.
- Always work with lentiviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area are to be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.

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9. Contact Us

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