



CELLECTA
USER MANUAL

CellTracker 50M Lentiviral Barcode Library

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Cellecta, Inc.

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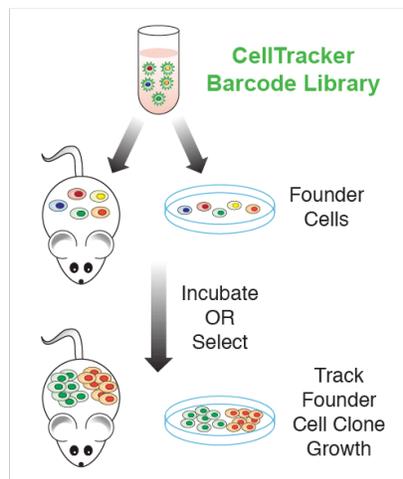
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1. CellTracker™ 50M Lentiviral Barcode Library

The CellTracker™ 50M Lentiviral Barcode Library (formerly known as the 13K × 13K Barcode Library) enables the tracking of individual clones derived from a population of cells. The pooled library contains more than 50 million lentiviral constructs with different barcode sequences. When a cell population is transduced with this barcode library, the barcodes integrate into the genomic DNA of the cells. The result is a select starter founder population where almost every cell has a different DNA-sequenceable barcode integrated into its DNA.

Since the barcode is stably integrated into the genome, it is passed on to any cell progeny when genomic DNA is replicated. This feature allows researchers to track cell proliferation over the course of an experiment. For instance, it is possible to:

- Track the fate of cell clones over a course of drug treatment.
- Study the effect of differentiation on hematopoietic cells.
- Assess the effect of treatment on implanted tumor cell populations.



Cellecta's CellTracker Barcode Library allows the tracking of founder cell clone growth.

The CellTracker 50M Barcode Library is constructed in a Cellecta third-generation lentiviral vector that expresses both TagRFP (Evrogen) and a Puromycin resistance gene under a human Ubiquitin C promoter. The specially-designed, optimized barcodes facilitate Next-Generation Sequencing (NGS) data analysis and barcode identification. Using an Illumina NextSeq or HiSeq NGS instrument, barcode sequences are identified and converted to lists with enumerated data.

The protocols included provide guidelines for packaging the CellTracker Barcode Library into VSV-G pseudotyped lentiviral particles, transduction of target cells, and the preparation of barcodes derived from transduced cells for NGS, enumeration, and analysis.

References and Product Citations for all Cellecta products can be found on the Cellecta website:

<https://www.cellecta.com/resources/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.

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.



[Download the PDF Version of the CellTracker 50M Barcode Library User Manual here](#)

2. Required Materials

The following sections detail the materials provided with Cellecta's 50M Barcode Lentiviral Library, and the additional materials required to carry out the protocols associated with using this library.

2.1. Materials Provided

Cellecta's CellTracker 50M Lentiviral Barcode Library is provided as plasmid DNA or as packaged VSV-G pseudotyped viral particles.

| Product | Cat. # | Amount | Storage |
|--|-----------|------------------------|---------|
| CellTracker 50M Lentiviral Barcode Library (plasmid) | BC13X13-P | 200 µg | -20°C |
| CellTracker 50M Lentiviral Barcode Library (virus) | BC13X13-V | 1 × 10 ⁸ TU | -80°C |

For information on the specific CellTracker Barcode Library that you have purchased, please review the Product Analysis Certificate (PAC) you received by email upon delivery. The PAC contains information such as the viral titer and volume (for libraries provided as viral particles), NGS data, complexity, mutation rate, and Lot Number.

PACs for pre-made CellTracker Barcode Libraries may be also downloaded from the Cellecta [Product Manuals and Certificates web page \(Page opens in new tab\)](#) and are organized by Lot Number.

Additional information can be found on the [CellTracker Lentiviral Barcode Library web page.](#)

2.2. 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 and 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)
- MgCl₂, 1M solution
- 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.

2.3. Additional Materials for Transduction of Cells

- Polybrene® (hexadimethrine bromide) (Sigma-Aldrich, Cat.# 107689)
- Puromycin (Sigma-Aldrich, Cat.# P9620-10ML)

NOTE: If starting from pre-packaged viral particles obtained from Cellecta, you will need the following materials included in the previous **Production of Lentivirus** list:

- 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)
- MgCl₂, 1M solution
- 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.

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

2.5. Additional Materials for NGS of sgRNA or Barcodes

- PCR and NGS primers for gRNA/barcode amplification and sequencing (IDT): See NGS Cassette Diagram provided as a link or attachment

For your convenience, we also offer [Library NGS Prep Kits](#) which provide primers and reagents required for amplification and multiplexing of up to 12 samples, as well as custom sequencing primers.

- 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 (Illumina):

| Platform | Kit Type | Illumina Cat.# | Description |
|----------------------------|-------------|----------------|---|
| NextSeq® | Sequencing | FC-404-2005 | NextSeq 500 v2 Kit (75 cycles, High-Output) |
| HiSeq® SR (Single-Read) | Sequencing | GD-401-3001 | TruSeq SBS Kit v3 – HS (50 cycles) |
| | Cluster Gen | FC-401-3002 | TruSeq SR Cluster Kit v3-cBot-HS |
| HiSeq® PE (Paired-End) | Sequencing | GD-401-3001 | TruSeq SBS Kit v3 – HS (50 cycles) |
| | Cluster Gen | PE-401-3001 | TruSeq PE Cluster Kit v3-cBot-HS |

NOTE: See Illumina website for information on HiSeq 2500 Rapid Run kits. Unfortunately, we **DO NOT** support NGS on the 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.

2.6. Related Products and Services from Cellecta

Related Products

| Description | Cat. # | Amount |
|--|-----------|--|
| NGS Prep Kit for Barcode Libraries in pRS16/17 (CellTracker 50M) | LNGS-200 | Kit for 6-48 samples (48 preps of 50 µg each, 12 multiplex) |
| Ready-to-Use Lentiviral Packaging Plasmid Mix | CPCP-K2A | 250 µg |
| LentiFuge™ Viral Concentration Reagent | LFVC1 | 1 ml of 1000X solution |
| CellTracker™ 13K Lentiviral Barcode Library (plasmid) | BC13K-P | 200 µg |
| CellTracker™ 13K Lentiviral Barcode Library (virus) | BC13K-V | 1×10^8 TU |
| CellTracker™ 4-Barcode-plus Cell Labeling Kit —Unique by Size, Sequence, and Individual Amplification | BC4P-V | 10^7 TU /construct (4) |
| CellTracker™ 5-Barcode Cell Labeling Kit —Unique Sequence and RFP Marker | BCRPP5-V | 10^7 TU /construct (5) |
| CellTracker™ 10-Barcode Cell Labeling Kit —Unique Sequence and RFP Marker | BCRPP10-V | 10^7 TU /construct (10) |

Related Services

- [Lentiviral Library Packaging Services](#)
- [Custom Pooled Barcode Libraries](#)
- [Custom Lentiviral Barcode Constructs](#)
- [NGS of DNA from Genetic Screen](#) (screening done with Cellecta libraries)

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 Polybrene.

Visit the [Cellecta Vector Information web page](#) for a list of our most common lentiviral vectors. If interested in using a vector not listed, please contact us at sales@cellecta.com. Cellecta's pre-made libraries and constructs can be re-cloned in almost any of Cellecta's standard or custom vectors.

3.1. 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 **X_b** .
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 **X_e** .

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:

$$\text{Doubling Time} = [T \times (\ln 2)] / [\ln (X_e / X_b)]$$

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.

$$Xb = 2 \times 10^6$$

$$T = 3 \text{ days}$$

$$Xe = 16 \times 10^6$$

$$\begin{aligned} \text{Doubling Time} &= [3 \times (\ln 2)] / [\ln(16,000,000 / 2,000,000)] \\ &= [3 \times (0.69)] / [\ln(8)] \\ &= 2.08 / 2.08 = \mathbf{1 \text{ day}} \end{aligned}$$

3.2. Calculating a Kill Curve

Most of the lentiviral vectors used in making Cellecta's pre-made libraries and constructs express a puromycin resistance gene. For other pre-made products as well as customized libraries and constructs, other selection markers such as blasticidin (Blast^R), hygromycin (Hygro^R), neomycin (Neo^R), or bleomycin (Bleo^R) may be substituted.

Regardless of the selection marker that the plasmids express, you need to know the concentration of antibiotic that kills untransduced cells within a given amount of time in order to successfully select transduced cells. We recommend the following methods for obtaining a "Kill Curve".

Puromycin Kill Curve

To create a Puromycin Kill Curve for your target cells, follow the protocol below.

1. Aliquot cells in a 12-well plate, at a density such that they are at 72 hours from confluency.
2. Add puromycin at concentrations of 0, 0.5, 1, 2, 5, and 10 µg/ml in six different wells.
3. Mix and place the cells at 37°C in a CO₂ incubator.
4. Grow cells under standard conditions for 72 hours.
5. Count viable cells, and determine the lowest concentration of drug that kills at least 90% of cells in 72 hours.

Use this concentration at the puromycin selection step during your experiment.

Blasticidin Kill Curve

To calculate a Blasticidin Kill Curve, follow the same protocol above and use the same concentrations as for the Puromycin Kill Curve. Determine the lowest concentration of drug that kills 99% of cells in 3-5 days.

Hygromycin Kill Curve

If using hygromycin, follow the same steps above but use 0, 50, 100, 200, 400, and 800 µg/ml hygromycin in six different wells.

Other Antibiotic Kill Curves

Please contact [Cellecta Technical Support](#) if you need assistance.

3.3. Check Toxicity of Polybrene

Polybrene is a transduction enhancement reagent used during transduction of lentivirus into target cells. It is a polycation that neutralizes charge interactions to increase binding between the lentiviral envelope and the cell's plasma membrane. The optimal concentration of Polybrene depends on cell type and may need to be empirically determined. Excessive exposure to Polybrene can be toxic to some cells.

We recommend performing a Polybrene toxicity titration before transducing your target cells. Follow the protocol below.

1. In six wells of a 12-well plate, grow cells in complete culture medium for 24 hours with a range of Polybrene concentrations — 0, 1, 2, 3, 4, and 5 $\mu\text{g/ml}$.
2. Replace old medium with Polybrene-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 Polybrene that results in less than 10% cell toxicity compared to no Polybrene (typically, 5 $\mu\text{g/ml}$ is recommended). For some cell types, you cannot use Polybrene.

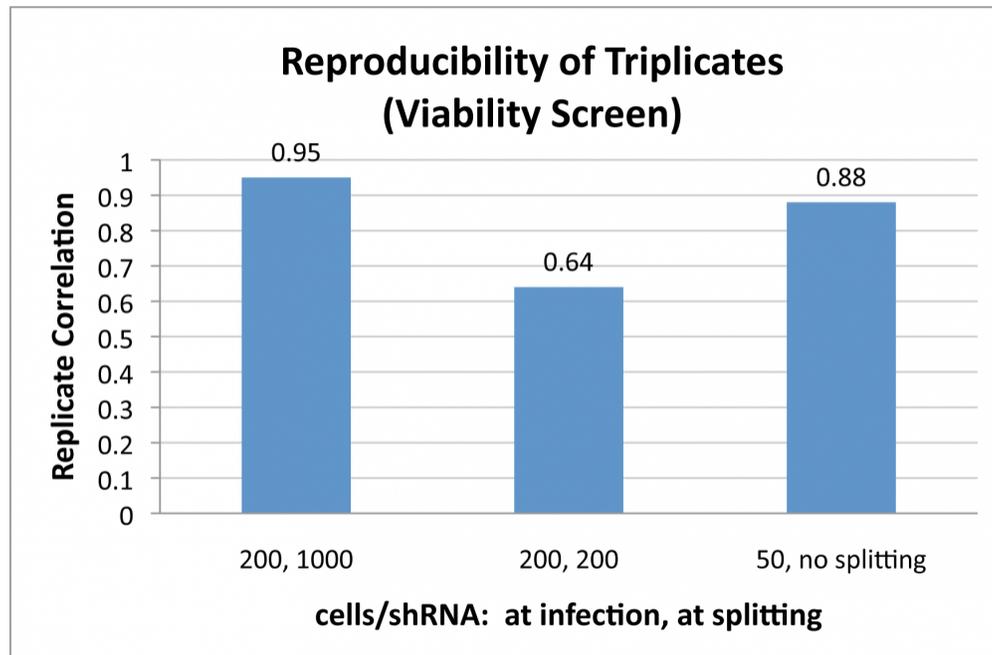
4. Library Packaging and Transduction of Target Cells

To package your lentiviral sgRNA, shRNA, or barcode library as VSV-G pseudotyped lentiviral particles, follow the standard lentiviral packaging protocol outlined until Core Protocols. 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 ($500 \times$ library complexity) are needed to start a screen with libraries of 50,000 sgRNAs.



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 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 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 CRISPR pooled sgRNA library and you know the MOI and number of starting cells you need to maintain representation, transduce your target Cas9-positive cells using the appropriate number and size of plates and the correct amount of virus.

4.1. Assess Fraction of Antibiotic-Sensitive Fluorescent-Positive Cells

Although the percent of transduced cells can be accurately determined by counting fluorescent cells by flow and the same promoter drives expression of both the fluorescent and antibiotic selection genes, some portion of these cells may not express the antibiotic selection gene (e.g., puromycin) at a threshold high enough to provide resistance. As a result, some fraction of the transduced fluorescent cells will not survive an antibiotic intended to kill off untransduced cells. Depending on how high the fraction of fluorescent-positive but antibiotic-sensitive cells, adjustments may be needed when transducing cells for screening. For this reason, we recommend determining the fraction of RFP- or GFP-positive cells (at a given MOI) that can survive antibiotic selection before using antibiotic in a screen.

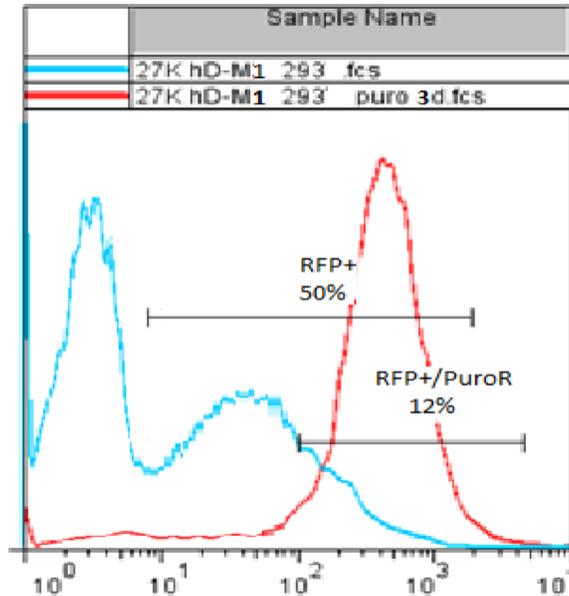
The protocol below is written with the assumption that the fluorescent marker is RFP and the antibiotic selection is puromycin. However, a similar approach can be used with fluorescence marker and antibiotic selection expressed from the same construct.

Assess the Fraction of RFP+ Cells that Survive Puro Selection

In order to calculate which fraction of RFP-positive cells are going to survive puromycin selection, it is first necessary to transduce your target cells and determine the viral titer of your stock by RFP using flow cytometry.

1. Based on the titer you assessed by RFP Assay, perform a small-scale transduction aiming at 50% infected cells.
2. Three (3) days after transduction, split cells into 2 samples and grow cells with or without puromycin for an additional 3 days.
3. Analyze both samples by flow cytometry.
4. By looking at the RFP intensity of puromycin-treated cells, calculate the percentage of cells that survived puromycin selection.

The figure below shows FACS analysis of transduced cells (**Blue: No puromycin selection; Red: Puromycin selection**). 50% of cells were RFP-positive, and 24% of the RFP-positive cells were *also* puromycin-resistant (12% of total).



Comparison of RFP+ cells vs. puromycin-resistant / RFP+ cells. Of the 50% RFP+ positive cells, 24% are also puro-resistant (12% of the total population).

IMPORTANT: The percentage of RFP-positive cells that are also puromycin-resistant is dependent on MOI, as it increases with the increase in percentage of RFP-positive cells bearing multiple integrations. In the example above, 24% of RFP-positive cells (12% of total cells) were puromycin-resistant when cells were infected at MOI 0.7 (50% RFP-positive cells). If the same cells were infected at an MOI of 0.5 (40% RFP-positive cells), less than 24% of RFP-positive cells would also be puromycin-resistant cells. Conversely, if cells were infected at MOI 2 (85% RFP-positive cells), a much higher percentage than 24% of RFP-positive cells would also be puromycin-resistant, due to a high percentage of RFP-positive cells bearing multiple integrants and therefore expressing high levels of the puromycin-resistance gene.

In the case described above, a 27K library genetic screen was started with at least 46×10^6 cells per replicate and transduction. Cells were infected at MOI 0.7 (50% transduction efficiency) to obtain 23×10^6 infected (RFP-positive) cells, of which about 5.5×10^6 (24%) will be puromycin-resistant (~200 puromycin-resistant cells per library construct).

5. Extraction and Sequencing of sgRNA or Barcodes for 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 NextSeq or HiSeq instrument. Following amplification, representation levels of each of the sgRNA or barcodes sequences are then quantified to assess enrichment or depletion relative to the starting library or relevant control samples, depending on the type of screen.

We currently do not support NGS of samples on the Illumina MiSeq.

5.1. Genomic DNA Extraction

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 genetic screen. 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 effector constructs. There are several protocols that can be used for DNA isolation. Depending on the number and type of cells, some approaches may work better than others.

- Genomic DNA isolated from more than 10 million cells will overload most column-based DNA isolation kits and compromise yields. To avoid this loss of genomic DNA, which can distort representation of the guides or barcodes in the population, we highly recommend using the conventional Genomic DNA Extraction Protocol. With this procedure, you usually obtain 50 µg-100 µg of genomic DNA from 10 million cells.

This protocol is typically recommended to purify DNA from a “dropout viability” screen which often requires growth of 25-100M cells per time point or treatment variation, depending on the size of the library.

- 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. DNA samples can be stored at +4°C for a few weeks or at -20°C for an extended period of time.

5.1.1. 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).

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 μ l 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 dH₂O to a concentration of ~2 mg/ml.
11. Incubate 30 minutes at 80°C before spectrophotometer reading.

NOTE: Expected yield is about 10 μ g genomic DNA per 1 million cells.

5.2. Amplification of sgRNA or Barcode Sequences from Genomic DNA

The protocol is based on two rounds of PCR to amplify the integrated sgRNA or shRNA barcode sequences 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 are 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 you to use the original shRNA/sgRNA/barcode plasmid library (10 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.

- Although it is typically not necessary, you may use the packaged effector library as a positive control. In order to use 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, the RevUbiC1 primer adjacent to the cloning side (see the Cassette Design information for your library) 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.

- 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). Also include a positive reference control, in one 100 µl reaction by amplifying 10 ng of original plasmid effector library used in the screen.

| 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 |

- Mix gently, centrifuge briefly to collect droplets, divide samples with more than 50 µg of genomic DNA into 100 µl PCR reactions as necessary.
- Perform PCR using the following cycling conditions.

| | |
|--|---------------|
| 94°C, 2 minutes | 1 cycle |
| 94°C, 45 seconds, 65°C, 15 seconds, 72°C, 30 seconds | 16-20 cycles* |
| 72°C, 2 minutes | 1 cycle |

- Depending on your library, the specific number of cycles may need some optimization. For barcodes (and shRNA libraries with barcodes), typically 16 cycles is sufficient, and for sgRNA libraries, 18-20 cycles may be necessary.

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.

- The 12 NRev Index Primers 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 lane for sequencing (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 sequencing lane of a Flow Cell.

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

- If you are starting from more than 50 μ g of genomic DNA, it is important to combine all individual reactions for each sample together into a single tube.
- Perform the Second Round of nested PCR using a 5 μ l aliquot of the First Round PCR product for each sample:

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

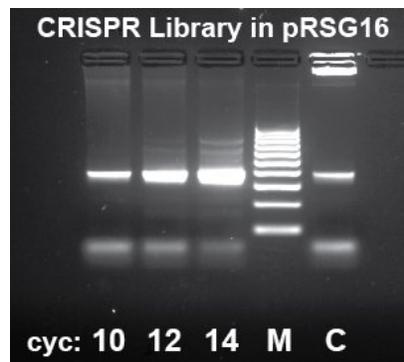
Note: Each sample to be sequenced together in a single Flow Cell or lane needs to be amplified with a different NRev Index Primer. Be sure to use a different lettered NRev Index for each sample that will be loaded on the same Flow Cell.

- Perform PCR under the following cycling conditions.

| | |
|--|-------------|
| 94°C, 2 minutes | 1 cycle |
| 94°C, 45 seconds, 65°C, 15 seconds, 72°C, 30 seconds | 12 cycles** |
| 72°C, 2 minutes | 1 cycle |

** Additional cycles may be needed for samples as described in Step 5 below.

4. Analyze the amplified products from Step 3. 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 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 shRNA bar-code or sgRNA products is typically between 125 to 300 bp and provided in the effector Cassette Design information specific for your library.



CRISPR Human Genome 80K Knockout Library NGS PCR product analysis by gel electrophoresis



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 (\pm 2-fold) no additional 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 weak or missing bands back into the PCR instrument for additional cycles. For example, if 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.
 - 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.

- Overcycling often generates additional higher molecular 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 the Second PCR for that sample using another 5 μ l of DNA from the First PCR and a fewer number of cycles.

DNA samples can be stored at +4°C at this stage.

5.3. 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. Purify and concentrate the PCR products from each sample using QIAquick PCR Purification Kit following the manufacturer's protocol. Samples should be eluted in a volume of approximately 30 μ l.
2. After purifying and concentrating, run each purified PCR product with 10X loading buffer on a 3.5% agarose-1XTAE gel with size of wells which accommodate at least 50 μ l.
3. Using a transilluminator and scalpel, excise the narrow band that corresponds to the correct target sgRNA or barcode amplicon size.
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 each PCR product from the gel fragment using a QIAquick Gel Extraction kit following the manufacturer's protocol. Elute the purified PCR product in 20 μ l 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 each 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 10nM concentration corresponds to 1.42 ng/ μ l based on A260 OD measurement.

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

5.4. Sequencing sgRNA Inserts or Barcodes

The second round PCR primers include sequences (P5 and P7) complementary to the sequences of the immobilized primers necessary for generating amplification clusters in Illumina's NextSeq or HiSeq Flow Cells. In addition, nested reverse (NRev) primers during the Second Round of PCR added sample-specific index sequences which allow to combine and multiplex up to 12 samples for NGS. The primers were designed using the NextSeq instrument but are compatible with HiSeq instruments as well.

Note: Please see the Product Insert for the specific kit you purchased for information on compatibility with HiSeq Single-Read (SR) and Paired-End (PE) Reagent Kits.

The number of sequencing cycles (read length) required depends on the gRNA or barcode length. Please refer to your library information (for Cellecta Libraries, see the corresponding Library Cassette Diagram).

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 pools needs to have a unique index sequence.

Note: The number of samples which can be sequenced in one lane or flow cell is based on the complexity of effector library and expected total number of reads generated in NextSeq or HiSeq 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” for the NextSeq or HiSeq System depending on which instrument you are using:
 - NextSeq: <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 the provided custom **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 multiplex samples in a Flow Cell, also add the **Index NGS Primer** directly into the appropriate well (i.e., spike in either with the Illumina index primer or the reverse sequencing primer, see Note). 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.

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 Insert) provided with the Primer components for the specifics about how to set up the NGS Primers for your kit.

- Specify the appropriate number of cycles to sequence the complete gRNA or barcode region (please refer to Product Insert), plus 7 cycles to read the 6-base Index sequence.

When sequencing is completed, demultiplex the run to into separate FASTQ (*.bcl files) files. This can be done using the [Illumina BaseSpace Hub](#) or, alternatively, demultiplexing and fastq file generation may be done faster using a UNIX server and the [Illumina bcl2fastq software](#). Using the bcl2fastq software also avoids the rather slow step of downloading Fastq files from BaseSpace. You can obtain the bcl2fastq program at [this link](#).

- The [Bioinformatics I/O Blog](#) on the University of Glasgow's Centre for Viral Research Site (for instance, see blog articles [How to generate a Sample Sheet from sample/index data in BaseSpace](#) and [How to demultiplex Illumina data and generate fastq files using bcl2fastq](#)).
- The [BaseSpace Sequence Hub Help Center](#) from Illumina. In particular, see [How to download data from Run](#).

After demultiplexing the data, the barcode or guide sequence reads should be aligned with the library reference file and counted using appropriate software.

6. Core Protocols for Lentiviral Packaging and Transduction

This section provides key protocols for packaging, transducing, and titering lentiviral plasmid libraries and constructs.

6.1. Lentiviral Packaging

The following protocol describes the generation of pseudoviral packaged lentiviral constructs using Invitrogen's Lipofectamine™ and Plus™ Reagent (see Required Materials). 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, CRISPR, cDNA, or promoter reporters, and sgRNA/shRNA libraries in lentiviral vectors.

- The yield of recombinant lentiviral particles typically produced under these optimized conditions is typically between 1×10^6 TU/ml and 5×10^6 TU/ml for individual lentiviral constructs less than 7kb from LTR to LTR. For larger lentiviral constructs with a packaging sequence greater than 7kb, for example construct containing the Cas9 gene, the yield will be much lower (approximately 1×10^5 TU/ml for Cas9 constructs).



IMPORTANT: For libraries in sgRNA-spCas9 vectors (Single-Vector CRISPR system), you should expect a significantly (10- to 50-fold) lower yield (approximately 1×10^5 TU/ml) because of the large vector size.

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(-). 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.

Day 0 – Plate Cells

1. Twenty four (24) hours prior to transfection, plate 12.5×10^6 293T cells in a 15-cm plate (or 150 cm² flask). Use 30 ml of media per plate. Disperse the cells and ensure even distribution.
2. Incubate at 37°C in a CO₂ 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.

| Plate Size | Estimated Virus Yield |
|------------|-----------------------|
| 10-cm | 1×10^6 TU |
| 15-cm | 3×10^6 TU |



Cellecta offers Lentiviral packaging services. Please contact us at sales@cellecta.com or visit Cellecta's [Lentiviral Packaging Services](#)

Day 1 – Transfection into 293T Cells

- 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 |
| 1086 µl | 1,332 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 plasmids DNA at other concentrations, adjust the volume accordingly (e.g., for 0.5 µg/µl use twice the indicated µl).

- 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 μ l | 1,290 μ l | 5,160 μ l | 10,320 μ l | 20,640 μ l | 30,960 μ l | 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 DNA / PLUS Reagent / Lipofectamine complex (from Step 3) to each 15-cm plate (from the previous **Day 0 – Plate Cells** step), 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 CO₂ incubator for 24 hours.

Day 2 – DNase I Treatment

1. At 24 hours post-transfection, replace the medium containing complexes with 30 ml fresh D-MEM medium supplemented with 10% FBS, DNase I (1 U/ml), MgCl₂ (5 mM), and 20mM HEPES, pH 7.4.
2. Continue incubation in the CO₂ 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.

NOTE: 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 plate) through a Nalgene 0.2 μ m 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 of fresh D-MEM medium supplemented with 10% FBS and 20mM HEPES pH 7.4, and continue incubation in the CO₂ 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.

Optional Virus Concentration Protocol

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 another media (besides D-MEM/10%FBS) that is optimal for sensitive target cells.
 - If packaging an shRNA or sgRNA library for screening because the concentration minimizes problems that might arise from genomic DNA carryover.
1. Aliquot lentiviral supernatant in clear, sterile centrifuge tubes.
 2. *(Recommended)* Add Cellecta's LentiFuge™ Viral Concentration Reagent (see [Materials for Production of Lentivirus](#)) according to the protocol described in the LentiFuge User Manual.
 3. Centrifuge at $15,000 \times g$ 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).

6.2. Transduction of Cells with Lentiviral Particles

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 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 (e.g., for K-562 cells we typically maintain 2×10^5 and 1×10^6 cells/ml).

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

An important parameter for transduction is the multiplicity of infection (MOI). Depending on the purpose of transduction and objectives of the experiment, different MOIs may be appropriate. For individual constructs, typically, an MOI 1 based on the Cellecta viral titer as a reference (i.e., use about as many viral particles as the number of cells plated) is suitable or, for hard to transduce cells, you may want to increase the MOI to 2-4 fold. For library screens where you want most of the cells to only pick up a single construct, you should titer the packaged library in the specific cells you plan to screen, and use an MOI that transduces less than half the target population.



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 1—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 cells in appropriate complete media and 5 µg/ml Polybrene and distribute them into culture plate wells as described below. Be sure to set up enough cultures for all dilutions of the virus you want to run plus an uninfected control. For HEK293 cells, we usually suspend at a density of 1×10^5 cells/ml in D-MEM supplemented with 10% FBS and 5 µg/ml Polybrene. For K-562 cells, usually at 2×10^6 cells per ml in RPMI/10%FBS supplemented with 20mM HEPES, pH 7.4 and Polybrene, 5 µg/ml
3. Aliquot a portion of cells into each well of a microtiter plate:
 - For adherent cells, aliquot 1 ml/well of target cells into a 12-well plate at a density of $5-10 \times 10^5$ cells/well. The optimal number of cells to plate at the time of transduction will vary depending on cell size. As a rule of thumb, cells should be transduced at a density such that they would become confluent in ~48 hours. For example, for HeLa cells, the suggested cell number is 50,000 cells/well in a 12-well plate; for HEK293, the cell number should be 100,000 cells/ml.
 - For suspension cells, aliquot 0.5 ml/well into a 24-well plate or 25-ml aliquots wells of a 48-well plate ($5-10 \times 10^5$ cells per well total)
4. To each cell-containing well, add an appropriate amount of lentivirus. Be sure to include one well of cells only (no virus) as a negative control. If titering, add increasing amounts of virus, for example, 0, 1, 3.3, 10, and 33 µl of concentrated stock to 5 different wells. If non-concentrated virus is used, scale up virus volumes accordingly.

NOTE: To determine viral titers, it is important to accurately record the *original* number of cells at the **Time of Transduction**.

5. Close the plate and mix by gentle agitation:
 - For adherent cells, place the plate into the CO₂ 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 $1,200 \times g$ 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 Polybrene).
6. Quickly thaw the lentiviral 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.

Day 2—Change Media:

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

Day 3 or 4—Split Cells

As the culture approaches confluence, split the cells 1:4 to 1:8, depending on the type of cells, and incubate for an additional 48-72 hours in complete medium containing an antibiotic if appropriate at the concentration (we recommend determining a “kill curve” for each antibiotic you plan to use with your target cells)

For lentiviral constructs with either a fluorescent marker or antibiotic resistance marker, viral titers can be determined from the fraction of fluorescent or antibiotic resistant cells in the population.

To measure transduction efficiency by flow using a fluorescent protein marker:

1. Resuspend cells in plate resuspend in 1X D-PBS.
2. If trypsin is used, block it with FBS/media, centrifuge
3. Determine the percentage of transduced (RFP-positive) cells by flow cytometry. For detection of Cellecta’s RFP-positive cells use the following settings:

| Flow Cytometry Settings for TagRFP | |
|------------------------------------|---|
| Excitation: | 561nm (530nm laser is still acceptable) |
| Emission: | 600/20 band-pass filter, or similar |

IMPORTANT: Attempting to determine the percentage of transduced cells by fluorescence microscopy is NOT recommended. It is likely to lead to inaccurate titer estimation.

If you select a culture where the percentage of fluorescent cells is less than 25%, then the number of viral particles at infection (the MOI) approximately equals the number of fluorescent cells (i.e., one virus per transduced cells) so the titer calculation can be simplified as follows:

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

To measure transduction efficiency by puromycin, blasticidin, or other antibiotic selection:

1. Verify that your vector has an antibiotic resistance marker, then return cells to the CO₂ incubator and grow under standard conditions for an additional 24 hours.
2. Add an appropriate amount of your antibiotic to the culture, then incubate 2-3 additional days.
3. 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. Incubate 1 hour at 37°C. Read the intensity of the alamarBlue staining using a plate reader. Using the no-cell media-only control as background, calculate the percentage of alamarBlue staining as follows:

[(alamarBlue Selected – Media-only Background) / (Non-infected cells – Media-only Background)] x 100 = % **infected cells** (use this for the titer calculation)

Day 5

After determining transduction efficiency, change media to remove dead cells, and dilute culture as needed to keep cells from confluency, and proceed with incubation in complete medium with puromycin (or other antibiotic) for your experiment.

For Cellecta's tet-inducible guide or hairpin constructs, you may want to split culture into two separate populations and add doxycycline to a 0.1 µg/ml final concentration to one of the cultures to induce expression of the sgRNA or shRNA.

For 6-7 days is normally enough to observe shRNA knockdown and significant sgRNA knockout. Depending on the specific sgRNA or shRNA, the target gene, and the stability of the protein, longer cell incubations may be required to observe knockdown or knockout effect. In particular, it may take up to 10 days to see CRISPR sgRNA in a majority of the cells. Also, for inducible constructs, knockdown or knockout may require several days incubation after induction.

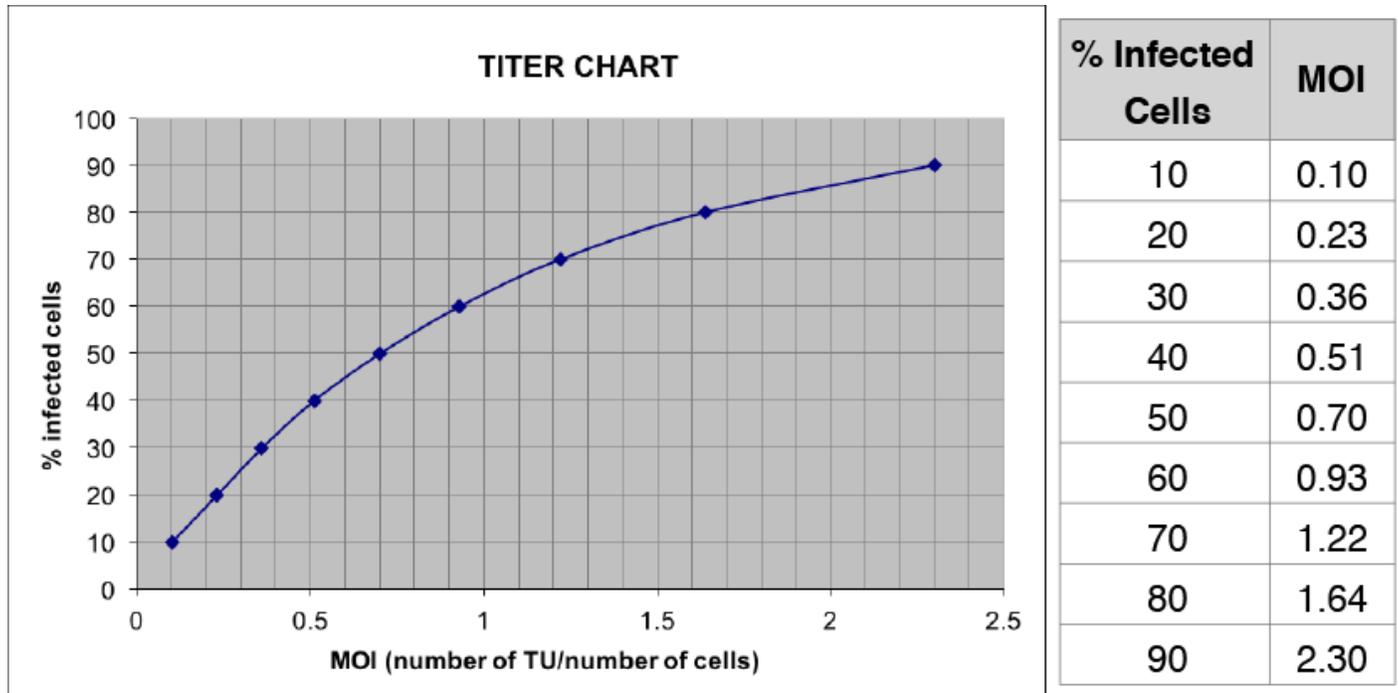
6.3. Lentiviral Titering

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 occurring. 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.
 - 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 80% transduced cells (i.e., MOIs in the range of ~0.2-1.5).



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:

$$\text{TU/ml} = (\# \text{ of cells at Transduction}) \times [\text{MOI} / (\text{ml of Lentiviral Stock used at Transduction})]$$

- # 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 number 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:

$$100,000 \times 0.25 / 0.01 = 2,500,000 \text{ TU/ml}$$

7. Technical Support

Please see Cellecta's General Troubleshooting Guide. If you don't find the solution to your problem in this Guide, 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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For the latest technical news and updates, visit Cellecta's blog at: <https://www.cellecta.com/blog-news/>

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 RSV promoter upstream of 5'LTR in the lentivector allows efficient Tat-independent production of lentiviral RNA, reducing the number of genes from HIV-1 that are used in this system.
- Number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (gag, pol, rev). The corresponding proteins are expressed from different plasmids lacking packaging signals and share no significant homology to any of the expression lentivectors, pVSV-G expression vector, or any other vector to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (gag, pol, rev) 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 HIV-based vectors 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/biosafety/publications/bmb15/index.htm>

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.

9. Contact Us

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For the latest technical news and updates, visit Cellecta's blog at:

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10. PDF Download Link



[Download the PDF Version of the CellTracker 50M Barcode Library User Manual here](#)