CELLECTA

USER MANUAL

CloneTracker™ 50M Lentiviral Barcode Library

v6a — Last update: 2019/12/04

Cellecta, Inc.

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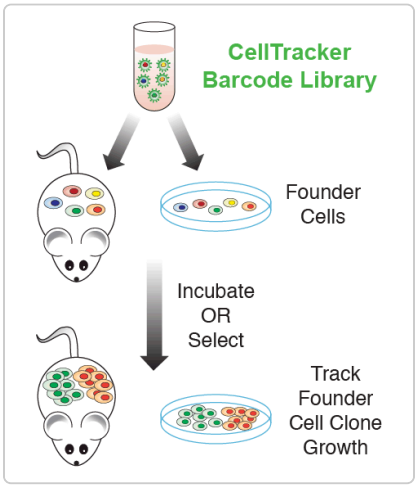
1. CloneTracker™ 50M Lentiviral Barcode Library

The CloneTracker™ 50M Lentiviral Barcode Library (formerly known as the CellTracker™ 50M Lentiviral Barcode Library and 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 CloneTracker Barcode Library allows the tracking of founder cell clone growth.

The CloneTracker 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 CloneTracker 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.

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

Last modified: 2019/05/15

2. Required Materials

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

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2.1. Materials Provided

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

Product	Cat. #	Amount	Storage
CloneTracker 50M Lentiviral Barcode Library (plasmid)	BC13X13-P	200 µg	-20°C
CloneTracker 50M Lentiviral Barcode Library (virus)	BC13X13-V	1×10^8 TU	-80°C
CloneTracker 13K Lentiviral Barcode Library (plasmid)	BC13-P	200 µg	-20°C
CloneTracker 13K Lentiviral Barcode Library (virus)	BC13-V	1×10^8 TU	-80°C

The barcode library sequences, vector information, Product Certificates, and additional resources are available on the CloneTracker Barcode Library web page.

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

Product Certificates for pre-made CloneTracker Barcode Libraries may also be downloaded from the Cellecta Product Certificates web page (Page opens in new tab) and are organized by Lot Number.

Additional information can be found on the CloneTracker Barcode Library web page.

Product Information

For information on the specific CloneTracker Barcode Library that you have purchased, please review the product information you received by email upon delivery (either by link or attachment):

Product Analysis Certificate (PAC)
The PAC contains important information such as the viral titer and volume (for libraries provided as viral particles), complexity, clone sequencing Q.C. data, NGS QC histogram, and Lot Number. PACs for pre-made CloneTracker Libraries may be also downloaded from the CloneTracker™ Barcode Library web page under the Resources tab.":https://www.cellecta.com/products-3/clonetracker-barcode-products/clonetracker-barcode-library/#tabs and are organized by Lot Number.

Barcode Sequence File
Provided as a Microsoft Excel file upon purchase, the sequence file contains the barcode sequences. If you have not received it, please contact us at tech@cellecta.com.

NGS Cassette Diagram

Available in SnapGene PDF format, the NGS cassette diagram shows the barcode sequence structure, design of the barcode expression cassette including location of PCR and NGS primers, and primer sequences. The NGS cassette of Cellecta's standard CloneTracker vector is available on the CloneTracker™ Barcode Library web page under the Resources tab.":https://www.cellecta.com/products-3/clonetracker-barcode-products/clonetracker-barcode-library/#tabs

Vector Map and Sequence

The CloneTracker vector sequence is available in SnapGene, GenBank/VectorNTI (.gb), and FASTA formats. The sequence files are available on the CloneTracker™ Barcode Library web page under the Resources tab.":https://www.cellecta.com/products-3/clonetracker-barcode-products/clonetracker-barcode-library/#tabs

Additional information on Cellecta's pre-made libraries can be found on the following web pages:

CloneTracker™ Barcode Library web page.

If interested in libraries expressing barcodes on RNA transcripts for use with RNA-Seq, please see the following products:

- CloneTracker XP™ Lentiviral Expressed Barcode Libraries
- CloneTracker™ XP-rLuc Lentiviral Expressed Barcode Libraries

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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
  - 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
- 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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2.3. 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, 1000x (Cat.# LTDR1)
- Puromycin (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

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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2.4. 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.

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2.5. 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

> 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® 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 HiSeq 2500 Rapid Run kits. Currently, 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.

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2.6. Related Products and Services from Cellecta

Related Products

|_=. Description |_=. Cat.# |_=. Quantity | |<. NGS Prep Kit for Barcode Libraries in pRSI16/17 (CloneTracker™) |=. LNGS-200 |=. Kit for 6-48 samples
(48 preps of 50 µg each) | |<. Supplementary Primer Set for LNGS-200 (12 Additional Index Primers) |=. LNGS-200-SP |=. 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 | |<. CloneTracker™ 4-Barcode-plus Cell Labeling Kit

—Unique by Size, Sequence, and Individual Amplification |=. BC4P-V |=. 1 × 10^7 TU per construct (4) | |<. CloneTracker™ 5-Barcode Cell Labeling Kit

—Unique Sequence and RFP Marker |=. BCRPP5-V |=. 1 × 10^7 TU per construct (5) | |<. CloneTracker™ 10-Barcode Cell Labeling Kit

—Unique Sequence and RFP Marker |=. BCRPP10-V |=. 1 × 10^7 TU per construct (10) |

Related Services

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

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.

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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 ***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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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 CO_2 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 95% of cells in 3-5 days.

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.

Hygromycin Kill Curve

If using hygromycin, follow the same protocol as for the Puromycin Kill Curve but use 0, 50, 100, 200, 400, and 800 µg/ml hygromycin in six different wells.

Neomycin Kill Curve

If you are using a construct with a neomycin selection marker, you need to test for resistance to geneticin (G418). Use the same protocol as for the Puromycin Kill Curve, but use a range of concentrations between 400-800 µg/ml.

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3.3. 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: 2019/01/10

4. 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 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.

Last modified: 2019/01/10

4.1. 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.

Last modified: 2018/06/03

4.1.1. 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 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.

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4.2. 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 (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 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,	16-18 cycles*

68°C, 2 minutes	
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).

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**

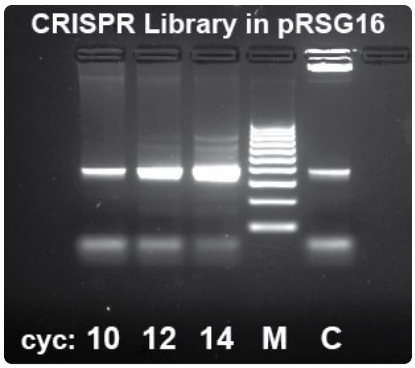
> * **Note:** Each sample to be sequenced together in a single Flow Cell or lane needs to be amplified with a different NRev Index Primer in the NGS Prep Kit. Be sure to use a different lettered NRev Index for each sample that will be loaded on the same Flow Cell or lane.

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	9 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 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.



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 of 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.

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

Last modified: 2019/11/27

4.3. 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 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 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 10nM concentration corresponds to 1.42 ng/µl based on A260 OD measurement. For an amplicon size of 750bp (dual-sgRNA product), a 10nM concentration corresponds to 5.3 ng/µl.

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

Last modified: 2019/09/06

4.4. 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 Illumina's NextSeq or HiSeq Flow Cells. In addition, if you are using the Cellecta NGS Prep Kits, 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 HiSeq instruments as well.

> **Note:** Please see the NGS Cassette Diagram for the specific Cellecta library or the Product Certificate for the NGS Prep 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 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 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 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 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 multiple samples in a Flow Cell or lane, 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). 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 (NGS Cassette Design or Product Certificate) 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), and specify 7 cycles to read 6-base Index sequence in the NGS Prep Kit.

Last modified: 2019/09/06

5. Transduction of Founder Cells

By transducing the CloneTracker or CloneTracker XP Barcode Library into a large pooled cell population, you can create a founder population in which each cell contains a unique integrated barcode. During transduction, the library of lentiviral constructs carrying each barcode enter the cells and stably integrate into the genomic DNA. Each lentiviral construct also has an RFP marker and puromycin selection to help maintain the barcode cassette.

Notes on Transducing Founder Cells

- Cell transduction is a random process following a statistical distribution. Therefore, if too high an MOI is used, many cells will take up more than one barcode. Cells with more than one barcode show up as more than one population in the final analysis. For example, if two barcodes integrate into one founder cell, and the cell produces 50 progeny, the data (after harvesting the cells and Next-Gen Sequencing of the genomic DNA) will show two clonal populations with two different barcodes, each having the same number of cells. It will not be obvious that these two populations are from the same founder cell. For this reason, we typically recommend using low MOIs of <0.2 so that >90% of the transduced cells only contain one barcode.

- Transducing larger populations of cells increases the frequency of having more than one founder cell with the same barcode. Since the CloneTracker Barcode Library has several million unique barcodes, a majority of cells will contain unique barcodes, even with library transductions of a million or more. However, with larger transductions, two or more founder cells can receive the same barcode. To minimize this, we recommend starting experiments with fewer than a million cells for libraries with 10M or more barcodes, and fewer than 300,000 cells for libraries with 1-5M barcodes, if possible. For more details on the complexity and representation of barcodes in the library, and estimates of the number of barcodes repeats you should expect with transductions of different size founder cell populations, please refer to the QC information available on the PAC or described in the Quality Control section.

- Since the purpose of using the CloneTracker Barcode Library is to track the fate of cells from a founder population, we do not recommend splitting and discarding any cells during the course of an experiment:
    - Discarding any portion of cells will eliminate some of the barcodes unless the cell population has expanded to several orders of magnitude over the founder population size.
    - Even if the population has expanded many fold over, splitting or otherwise discarding cells may skew the clonal population sizes for different barcodes (i.e., clones that grow slowly versus cells that grow quickly may be differentially affected).
    - If you must discard cells, it is crucial to design the experiment to minimize the impact on the barcode representation in your samples.

- Following transduction, you may want to select cells with barcodes using puromycin. To empirically determine the concentration of antibiotic that kills untransduced cells within a given amount of time, you should calculate a Puromycin Kill Curve as described in the **Recommended Pilot Experiments** section.

In this section of the manual, you will find step-by-step protocols for general packaging, transducing, and titering lentiviral plasmid libraries and constructs.

Last modified: 2019/07/11

5.1. Lentiviral Packaging

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

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 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
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 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 15-cm 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.

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.

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: 2019/05/15

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 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 $1\text{-}5 \times 10^5$ 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.

Last modified: 2019/02/15

5.3. Determine 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, 10 µl of concentrated stock to 5 different wells (or, for non-concentrated virus, scale up volumes ca. 100-fold). 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-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
Flow Cytometry Settings for TagGFP	
Excitation:	488nm
Emission:	530/20nm 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:

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

Last modified: 2019/05/04

5.4. Lentiviral Titer Calculation

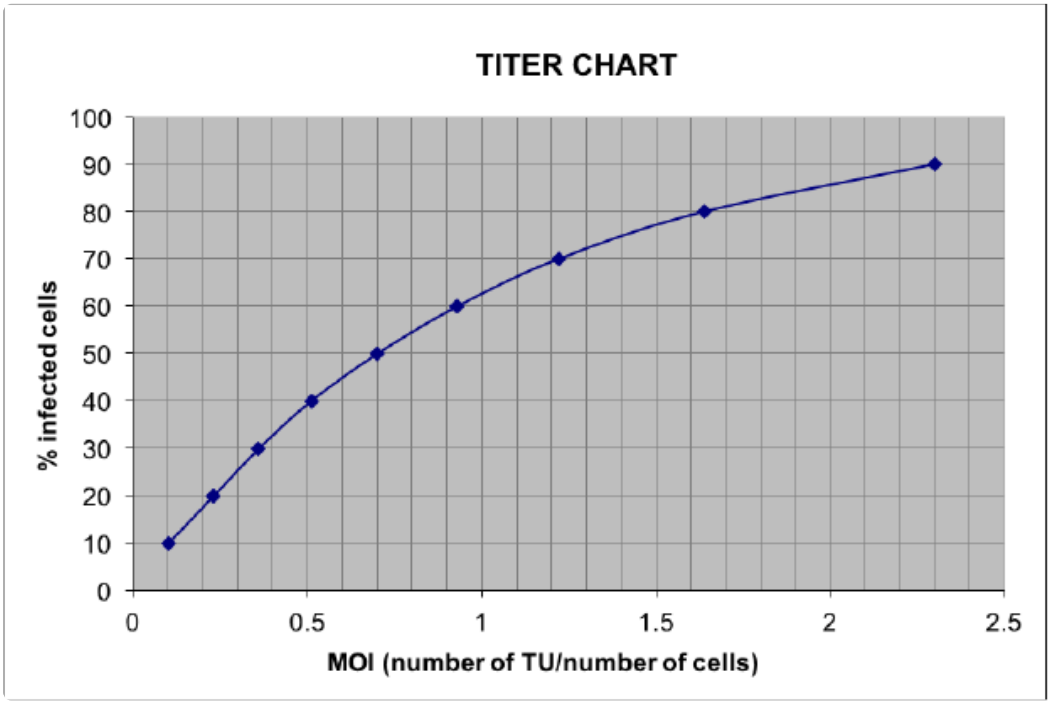
This section describes how to calculate the titer of packaged lentiviral particles. To calculate the number of transducible viral particles in the viral stock, you need to know the number of cells transduced at a specific dilution factor of the stock virus. This can be assessed by counting RFP-positive 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).



% 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: 2019/01/10

6. 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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7. 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/bmbl5/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.

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8. Contact Us

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