



**CELLECTA**  
USER MANUAL

# **CRISPR Pooled Lentiviral sgRNA Libraries**

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Cellecta, Inc.

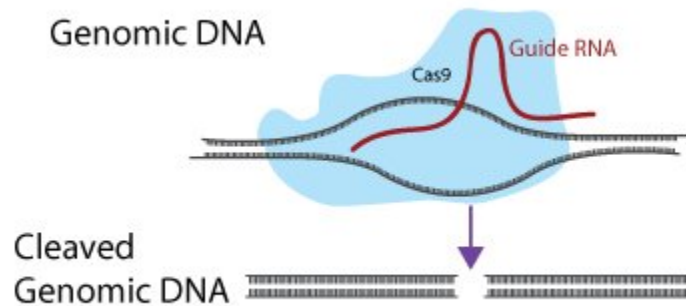
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# 1. CRISPR Pooled Lentiviral sgRNA Libraries

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The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) genes denoted as CRISPR/Cas9 system is a targeted gene-editing tool adapted from *Streptococcus pyogenes* that enables the permanent knockout of target genes. Single Guide RNAs (sgRNA or gRNA) direct the Cas9 nuclease to a specific genomic region, upon which the Cas9 cleaves the target gene and permanently knocks it out (see figure below).



*Guide RNA (sgRNA or gRNA) directs the Cas9 nuclease to a specific genomic region, upon which the Cas9 cleaves the target gene and permanently knocks it out.*

Variations of the CRISPR system have also been developed using engineered versions of the deactivated Cas9 (dCas9) protein to either interfere (i.e., repress) or activate gene expression without modifying genomic DNA. These gene-regulating variations of CRISPR use libraries with the same basic sgRNA design but target gene promoter regions rather than the coding sequence. The promoter targeting sgRNA, then, recruits a modified dCas9 protein which either activates or represses gene expression—depending on the dCas9 modification. Selective activation of targeted genes occurs when CRISPRa sgRNA expression constructs are used in cells expressing dCas9-VPH (or other dCas9-activator variations compatible with spCas9 sgRNA designs) and selective expression inhibition of targeted genes occurs when CRISPRi sgRNA constructs are used in cells expressing dCas9-KRAB (or other deactivated Cas9 repressors compatible with spCas9 sgRNA designs). Pooled screens can be run with both the CRISPR-activation (CRISPRa) and CRISPR-interference (CRISPRi) systems very similarly to standard knockout CRISPR screens using appropriately designed libraries.

Cellecta has employed a Two-Vector CRISPR/Cas9 lentiviral system for nearly all pooled CRISPR sgRNA libraries. With the Two-Vector CRISPR system, Cas9 is transduced into target cells, which are then selected for a high level of Cas9 expression. Generally, the higher the expression of Cas9, the more efficient is the knockout of the target gene. After a population of high-expressing Cas9 cells are obtained, they are then transduced with the sgRNA library such that most cells receive only one copy of a single sgRNA, thus knocking out only one target gene per cell. Upon selecting the transduced cells for a specific phenotype (e.g., cell death, cell proliferation, etc.), Next-Generation Sequencing (NGS) of the sgRNA sequences present in the genomic DNA of the remaining cells quantifies the fractional representation of each sgRNA sequence remaining in the population.

The protocols included provide the instructions on how to package the plasmid form of a pooled sgRNA library into viral particles, perform a CRISPR-based screen, and prepare experimental samples for NGS and deconvolution of raw sequencing data sets.

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.

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**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.

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*Last modified: 2019/08/21*

## 2. Materials Required for sgRNA Screening

The following sections detail the materials provided with Cellecta's pre-made or custom pooled lentiviral CRISPR sgRNA Libraries, and the additional materials required to carry out the protocols associated with screening these libraries.

*Last modified: 2018/07/03*

### 2.1. Materials Provided

Cellecta's pre-made and custom CRISPR Pooled Lentiviral sgRNA Libraries are provided either as plasmid DNA or as packaged VSV-G pseudotyped lentiviral particles. The pre-made human genome-wide pooled CRISPR sgRNA Knockout Libraries are available in two formats—as a single module or as three modules. Both library options cover all human protein-coding genes.

All pre-made human and mouse genome-wide CRISPR, CRISPRa, and CRISPRi libraries are all available in a single-module format.

#### Single-Module Genome-Wide 80K Knockout Libraries

Genome-wide coverage in single-module, pooled human and mouse libraries of nearly 80,000 constructs that can be conveniently used for a wide variety of screening approaches.

Human Libraries	# sgRNA	Catalog #	Amount	Storage
CRISPR Human Genome 80K Knockout Library (plasmid)	77,736	KOHGW-80K-P	200 µg	-20°C
CRISPR Human Genome 80K Knockout Library (virus)	77,736	KOHGW-80K-V8	2×10 <sup>8</sup> TU	-80°C
CRISPR Human Genome 80K Knockout Library (virus)	77,736	KOHGW-80K-V9	1×10 <sup>9</sup> TU	-80°C
CRISPR Human Genome 80K Knockout Library in EF1-Puro Vector (plasmid)	77,736	KOHGW-EP-80K-P	200 µg	-20°C
CRISPR Human Genome 80K Knockout Library in EF1-Puro Vector (virus)	77,736	KOHGW-EP-80K-V8	2×10 <sup>8</sup> TU	-80°C
CRISPR Human Genome 80K Knockout Library in EF1-Puro Vector (virus)	77,736	KOHGW-EP-80K-V9	1×10 <sup>9</sup> TU	-80°C

Mouse Libraries	# sgRNA	Catalog #	Amount	Storage
CRISPR Mouse Genome 80K Knockout Library (plasmid)	79,693	KOMGW-80K-P	200 µg	-20°C
CRISPR Mouse Genome 80K Knockout Library (virus)	79,693	KOMGW-80K-V8	2×10 <sup>8</sup> TU	-80°C

CRISPR Mouse Genome 80K Knockout Library (virus)	79,693	KOMGW-80K-V9	1×10 <sup>9</sup> TU	-80°C
CRISPR Mouse Genome 80K Knockout Library in EF1-Thy1.2 Vector (plasmid)	79,693	KOMGW-ET1.2-80K-P	200 µg	-20°C
CRISPR Mouse Genome 80K Knockout Library in EF1-Thy1.2 Vector (virus)	79,693	KOMGW-ET1.2-80K-V8	2×10 <sup>8</sup> TU	-80°C
CRISPR Mouse Genome 80K Knockout Library in EF1-Thy1.2 Vector (virus)	79,693	KOMGW-ET1.2-80K-V9	1×10 <sup>9</sup> TU	-80°C

### Three-Module Human Genome-Wide 150K Knockout Library

Deep coverage of 8 sgRNA per target gene divided into 3 modules that may be combined or used separately, depending on the type of screen desired.

Description	# sgRNA	Catalog #	Amount	Storage
CRISPR Human Genome Knockout Library, Module 1 (plasmid)	52,549	KOHGW-M1-P	200 µg	-20°C
CRISPR Human Genome Knockout Library, Module 2 (plasmid)	49,461	KOHGW-M2-P	200 µg	-20°C
CRISPR Human Genome Knockout Library, Module 3 (plasmid)	48,578	KOHGW-M3-P	200 µg	-20°C
CRISPR Human Genome Knockout Library, Modules 1, 2, & 3 (plasmid)	150,588 Total	KOHGW-M123-P	200 µg each	-20°C
CRISPR Human Genome Knockout Library, Module 1 (virus)	52,549	KOHGW-M1-V8	2×10 <sup>8</sup> TU	-80°C
CRISPR Human Genome Knockout Library, Module 2 (virus)	49,461	KOHGW-M2-V8	2×10 <sup>8</sup> TU	-80°C
CRISPR Human Genome Knockout Library, Module 3 (virus)	48,578	KOHGW-M3-V8	2×10 <sup>8</sup> TU	-80°C
CRISPR Human Genome Knockout Library, Module 1 (virus)	52,549	KOHGW-M1-V9	1×10 <sup>9</sup> TU	-80°C
CRISPR Human Genome Knockout Library, Module 2 (virus)	49,461	KOHGW-M2-V9	1×10 <sup>9</sup> TU	-80°C
CRISPR Human Genome Knockout Library, Module 3 (virus)	48,578	KOHGW-M3-V9	1×10 <sup>9</sup> TU	-80°C



**NOTE: Cas9 Expression Vectors are required but must be ordered separately. See [Related Products from Cellecta](#) for ordering information.**

### Single-Module Genome-Wide CRISPRa and CRISPRi Libraries

Human Libraries	# sgRNA	Catalog #	Amount	Storage
Human Genome-Wide CRISPRa sgRNA Library (plasmid)	106,400	KAHGW-106-P	200 µg	-20°C
Human Genome-Wide CRISPRa sgRNA Library (virus)	106,400	KAHGW-106-V8	2×10 <sup>8</sup> TU	-80°C
Human Genome-Wide CRISPRa sgRNA Library (virus)	106,400	KAHGW-106-V9	1×10 <sup>9</sup> TU	-80°C

Human Genome-Wide CRISPRi sgRNA Library (plasmid)	106,386	KIHGW-106-P	200 µg	-20°C
Human Genome-Wide CRISPRi sgRNA Library (virus)	106,386	KIHGW-106-V8	2×10 <sup>8</sup> TU	-80°C
Human Genome-Wide CRISPRi sgRNA Library (virus)	106,386	KIHGW-106-V9	1×10 <sup>9</sup> TU	-80°C

Mouse Libraries	# sgRNA	Catalog #	Amount	Storage
Mouse Genome-Wide CRISPRa sgRNA Library (plasmid)	109,186	KAMGW-109-P	200 µg	-20°C
Mouse Genome-Wide CRISPRa sgRNA Library (virus)	109,186	KAMGW-109-V8	2×10 <sup>8</sup> TU	-80°C
Mouse Genome-Wide CRISPRa sgRNA Library (virus)	109,186	KAMGW-109-V9	1×10 <sup>9</sup> TU	-80°C
Mouse Genome-Wide CRISPRi sgRNA Library (plasmid)	109,505	KIMGW-109-P	200 µg	-20°C
Mouse Genome-Wide CRISPRi sgRNA Library (virus)	109,505	KIMGW-109-V8	2×10 <sup>8</sup> TU	-80°C
Mouse Genome-Wide CRISPRi sgRNA Library (virus)	109,505	KIMGW-109-V9	1×10 <sup>9</sup> TU	-80°C

## Single-Module Genome-Wide CRISPRa and CRISPRi Dual-sgRNA Libraries

Human Dual-sgRNA Libraries	# sgRNA pairs	Catalog #	Amount	Storage
Human Genome-Wide CRISPRa Dual-sgRNA Library (plasmid)	105,130	KADHGW-105K-P	200 µg	-20°C
Human Genome-Wide CRISPRa Dual-sgRNA Library (virus)	105,130	KADHGW-105K-V8	2×10 <sup>8</sup> TU	-80°C
Human Genome-Wide CRISPRa Dual-sgRNA Library (virus)	105,130	KADHGW-105K-V9	1×10 <sup>9</sup> TU	-80°C
Human Genome-Wide CRISPRi Dual-sgRNA Library (plasmid)	105,135	KIDHGW-105K-P	200 µg	-20°C
Human Genome-Wide CRISPRi Dual-sgRNA Library (virus)	105,135	KIDHGW-105K-V8	2×10 <sup>8</sup> TU	-80°C
Human Genome-Wide CRISPRi Dual-sgRNA Library (virus)	105,135	KIDHGW-105K-V9	1×10 <sup>9</sup> TU	-80°C

Mouse Dual-sgRNA Libraries	# sgRNA pairs	Catalog #	Amount	Storage
Mouse Genome-Wide CRISPRa Dual-sgRNA Library (plasmid)	108,645	KADMGW-109K-P	200 µg	-20°C
Mouse Genome-Wide CRISPRa Dual-sgRNA Library (virus)	108,645	KADMGW-109K-V8	2×10 <sup>8</sup> TU	-80°C
Mouse Genome-Wide CRISPRa Dual-sgRNA Library (virus)	108,645	KADMGW-109K-V9	1×10 <sup>9</sup> TU	-80°C
Mouse Genome-Wide CRISPRi Dual-sgRNA Library (plasmid)	108,955	KIDMGW-109K-P	200 µg	-20°C
Mouse Genome-Wide CRISPRi Dual-sgRNA Library (virus)	108,955	KIDMGW-109K-V8	2×10 <sup>8</sup> TU	-80°C
Mouse Genome-Wide CRISPRi Dual-sgRNA Library (virus)	108,955	KIDMGW-109K-V9	1×10 <sup>9</sup> TU	-80°C



**NOTE: dCas9 Expression Vectors are required but must be ordered separately.** See [Related Products and Services from Cellecta](#) for ordering information.

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## Product Information

For information on the specific CRISPR sgRNA 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, mutation rate, clone sequencing Q.C. data, and Lot Number. PACs for pre-made CRISPR sgRNA Libraries may be also downloaded from the Cellecta [Product Manuals and Certificates web page](#) and are organized by Lot Number.

### sgRNA Sequence Files

Provided as a Microsoft Excel file upon purchase, the sequence file contains gene target names, sgRNA sequences, and NGS QC data including reads, distribution, complexity, and mutation rate. If you have not received it, please contact us at [tech@cellecta.com](mailto:tech@cellecta.com).

### NGS Cassette Diagram

Available in PDF format, the NGS cassette diagram shows the sgRNA sequence structure, design of sgRNA expression cassette including location of PCR and NGS primers, and primer sequences. The NGS cassettes of Cellecta's standard sgRNA vectors are available on the [Cloning and Expression Vector web page](#).

### Vector Map and Sequence

sgRNA library vector sequences are available in [SnapGene](#), GenBank/VectorNTI (.gb), and FASTA formats. The sequences of Cellecta's standard sgRNA vectors are available on the [Cloning and Expression Vector web page](#).

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

- [CRISPR Human Genome-Wide Knockout Libraries](#)
- [CRISPR Mouse Genome-Wide Knockout Library](#)
- [CRISPRa and CRISPRi Human Genome-Wide Libraries](#)
- [CRISPRa and CRISPRi Mouse Genome-Wide 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<sub>2</sub>, 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

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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<sub>2</sub>, 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:CHCl<sub>3</sub>-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 <b>Paired End (PE)</b>	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 <b>Single Read (SR)</b>	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 <b>Paired End (PE)</b>	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.

*Last modified: 2019/05/15*

## 2.6. Related Products and Services from Cellecta

### Related Products

Description	Cat.#	Quantity
<a href="#">NGS Prep Kit for sgRNA Libraries in pRSG16/17 (KOHGW)</a>	LNGS-120	Kit for 6-48 samples (48 preps of 50 µg each)
<a href="#">Supplementary Primer Set for LNGS-120</a> (12 Additional Index Primers)	LNGS-120-SP	48 Reactions (12 Index Primers x 4 reactions each)
<a href="#">Ready-to-Use Lentiviral Packaging Plasmid Mix</a>	CPCP-K2A	250 µg (25 transfections in 10-cm plates)
<a href="#">LentiFuge™ Viral Concentration Reagent</a>	LFVC1	1 ml of 1000X solution
<a href="#">LentiTrans™ Transduction Reagent</a> (Polybrene for Transduction)	LTDR1	1 ml of 1000x
<a href="#">LentiPrep™ Lentiviral Reagent Set with GFP Control for Packaging and Transduction</a>	LTSET-G	CPCP-K2A x 1 LFVC1 x 1 LTDR1 x 1 GFP Ctrl
<a href="#">LentiPrep™ Lentiviral Reagent Set with RFP Control for Packaging and Transduction</a>	LTSET-R	CPCP-K2A x 1 LFVC1 x 1 LTDR1 x 1 RFP Ctrl
<a href="#">CRISPR-Test™ Functional Cas9/dCas9 Activity Kits</a>	CRUTEST, CRITEST, CRATEST, CRTEST	Varies
<a href="#">CRISPR Cas9-Only and dCas9-Only Vectors</a>	Various	25 µg plasmid or 1×10 <sup>6</sup> TU virus

## Related Services

- [Lentiviral Library Packaging Services](#)
- [NGS of DNA from Genetic Screen](#)
- [Custom Pooled CRISPR sgRNA Libraries](#)
- [Custom Lentiviral CRISPR Constructs](#)

*Last modified: 2019/05/03*

## 3. Recommended Pilot Experiments

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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](mailto:sales@cellecta.com). Cellecta's pre-made libraries and constructs can be re-cloned in almost any of Cellecta's standard or custom vectors.

*Last modified: 2018/12/04*

### 3.1. Cell Doubling Time

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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<sub>b</sub>**.
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<sub>e</sub>**.

**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  $2 \times 10^6$  cells. Three (3) days later, you count  $16 \times 10^6$  cells.

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

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

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

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

*Last modified: 2018/06/20*

## 3.2. Calculating a Kill Curve

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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<sup>R</sup>), hygromycin (Hygro<sup>R</sup>), neomycin (Neo<sup>R</sup>), or bleomycin (Bleo<sup>R</sup>) 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<sub>2</sub> 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.

*Last modified: 2018/10/10*

## 3.3. Check Toxicity of LentiTrans Transduction Reagent

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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. Cas9 Cell Preparation

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For CRISPR screens, cells must express some variation of (e.g., Cas9 nuclease, Cas9-KRAB, or Cas9-VPH) in conjunction with library sgRNA. Most CRISPR sgRNA libraries provided by Cellecta express only sgRNA so, before starting a screen, it is necessary to engineer target cells that express the appropriate Cas9 variant for the screen. After packaging the appropriate lentiviral Cas9 construct into VSV-G pseudotyped lentiviral particles, cells are transduced with the Cas9 packaged particles to generate stable cell lines to make the Cas9 cell lines. Please keep in mind the following points with regard to Cas9 construct transduction:

- Generally, the higher the expression of Cas9 (or dCas9), the greater the effect on the target gene will be (up to a point where too high expression and/or viral load become toxic for the host cell). For the standard CRISPR system, knockouts will occur more quickly and at higher frequency. For the CRISPRi and CRISPRa systems, gene repression or induction will typically be more pronounced. As a result, it is desirable to ensure strong expression of the Cas9 gene. If you have not used lentiviral vectors in your target cells before, we suggest checking which promoters will work best for the selection and marker genes. The efficacy of pol II promoters, such as CMV, UbiC, PGK, that drive expression of cDNA genes vary significantly across cell types. Cellecta sells pre-packaged control viruses expressing different marker genes from various promoters that can be used to determine which promoter will work the best in your cells.
- Cas9 is a large protein and lentiviral constructs with this gene are close to the limit for lentiviral packaging so Cas9 constructs package poorly. The yields are more than an order of magnitude lower than usually obtained with smaller sized lentiviral constructs. Packaging reactions should be set up at a scale to ensure you produce enough packaged viral particles for your experiments.



Cellecta offers Custom Cas9 Cell Line services. Please contact us at [sales@cellecta.com](mailto:sales@cellecta.com) or visit Cellecta's [Custom CRISPR Cas9 Cells web page](#) for more information.

### Protocol for Making Cas9-Expressing Cells for CRISPR Screens

This protocol assumes that you are using a Cas9 vector with the blasticidin resistance marker and have determined the virus dilution that gives 50% blasticidin-resistant cells (BR-50).

1. To generate the best Cas9 cell line for your screen or other experiments, follow the Individual Lentiviral Construct Packaging and Transduction of Packaged Lentiviral Viral Particles Protocols to transduce the parental cell line with increasing amounts of virus. For packaged Cas9 constructs purchased from Cellecta, use MOI below based on the transduction units of the viral lot:
  - MOI = 0 (negative control, no virus)
  - MOI = 1
  - MOI = 3
  - MOI = 10
2. 72h after transduction, start appropriate antibiotic selection (based on the selection marker of the Cas9 virus) and



grow cells under selection for 2 weeks. Be aggressive: use an antibiotic concentration that kills 100% control cells within 3-5 days.

3. Assay the MOI 1, 3, 10 Cas9 lines for CRISPR activity using the appropriate CRISPRtest Assay:
  - CRISPRuTest for Cas9/CRISPR-KO (Cat. # CRUTEST)
  - CRISPRaTest for dCas9-VPH/dCas9-VPR/CRISPRa (Cat. # CRATEST)
  - CRISPRuTest for dCas9-KRAB/CRISPRi (Cat. # CRITEST)

Based on CRISPRtest results, choose the best MOI Cas9 line for downstream applications.

4. Split the selected Cas9 cell line ("8x BR-50" in this case) into 3 samples, and continue selection with 1x, 2x, and 4x increases in blasticidin concentration.
  - Sample 1: Same blasticidin concentration used to calculate BR-50
  - Sample 2: 2x blasticidin concentration used to calculate BR-50
  - Sample 3: 4x blasticidin concentration used to calculate BR-50
5. After 1 week, select the Cas9 cells that survived the highest blasticidin concentration. Expand the selected Cas9 cell sample. This is the Cas9 cell line you will use in your screen or experiment.

*Last modified: 2019/05/04*

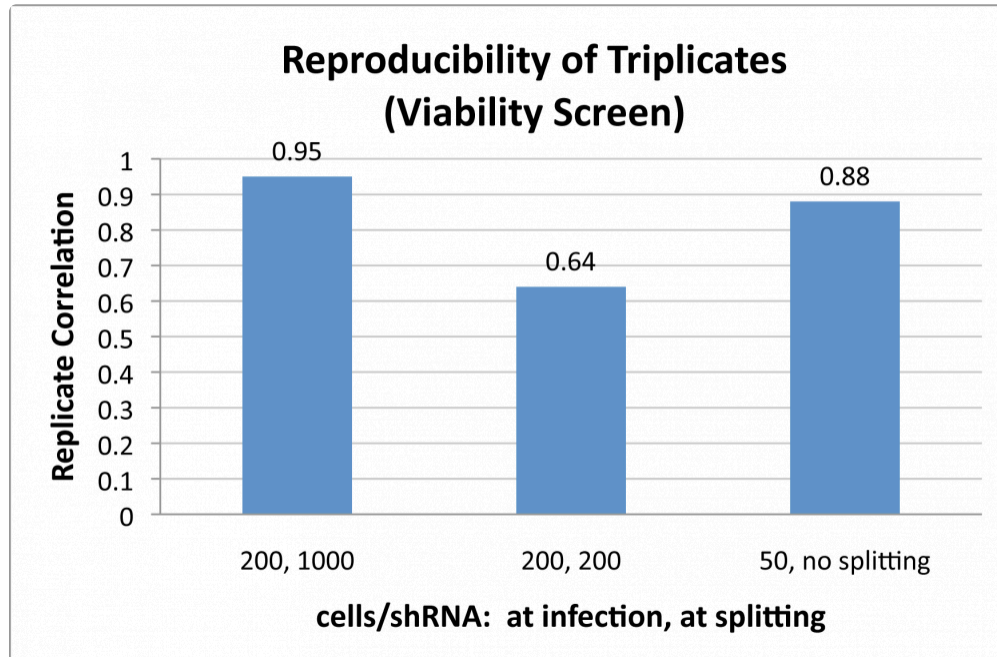
## 5. Library Packaging and Transduction of Target Cells

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

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

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

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

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

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

If a portion of propagated cells is removed during propagation (e.g., cells are split), the representation of the library can be skewed in the sample, which introduces significant random noise. This effect is readily seen in the middle bar in the above figure where the effect of starting with sufficient cells (i.e., 200-fold library complexity) is completely undercut by splitting cells

during propagation so that the final count of cells after 10 days is the same as the initial number of transduced cells (i.e., 200-fold library complexity). The correlation between triplicates falls dramatically when the cells are split to this degree.

Once you have calculated the titer of the packaged sgRNA or shRNA library and you know the MOI and starting number of cells you need to maintain representation, transduce your target cells (Cas9- or dCas9-positive cells if performing a CRISPR screen) using the appropriate number and size of plates and the correct amount of virus.

*Last modified: 2019/01/10*

## 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  $1 \times 10^6$  TU/ml and  $5 \times 10^6$  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 <sup>2</sup>	10 ml	$2 \times 10^7$ TU
15 cm	150 cm <sup>2</sup>	30 ml	$6 \times 10^7$ 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  $1 \times 10^5$  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](mailto:sales@cellecta.com) or visit Cellecta's [Lentiviral Packaging Services](#)

### 2-3 Days Prior to Packaging

- 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  $12.5 \times 10^6$  293T cells per **15-cm plate** (or 150 cm<sup>2</sup> flask) and use 30 ml of media per plate. If you are using **10-cm plates**, plate  $4 \times 10^6$  cells and use 10 ml of media per plate. Disperse the cells and ensure even distribution.
2. Incubate at 37°C in a CO<sub>2</sub> 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 <b>NOTE</b>
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
<b>1086 µl</b>	<b>1,332 µl</b>	<b>5,304 µl</b>	<b>10,608 µl</b>	<b>21,216 µl</b>	<b>31,824 µl</b>	<b>Total volume</b>

**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	1,200	4,800	9,600	19,200	28,800	D-MEM, no FBS, no antibiotics

μl	μl	μl	μl	μl	μl	
30 μl	90 μl	360 μl	720 μl	1,440 μl	2,160 μl	Lipofectamine Reagent
<b>1,030</b> μl	<b>1,290</b> μl	<b>5,160</b> μl	<b>10,320</b> μl	<b>20,640</b> μl	<b>30,960</b> μl	<b>Total volume</b>

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<sub>2</sub> 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<sub>2</sub> (5 mM), and 20mM HEPES, pH 7.4.
2. Continue incubation in the CO<sub>2</sub> 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<sub>2</sub> 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.

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## 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  $15,000 \times g$  for at least 1 hour at  $4^{\circ}\text{C}$ . 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  $-80^{\circ}\text{C}$ . 100-fold concentration is recommended (e.g., resuspend in 1 ml PBS if starting from 100 ml supernatant).

*Last modified: 2019/05/15*

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## 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  $1 \times 10^5$  cells/ml in D-MEM supplemented with 10% FBS and LentiTrans Reagent. For K-562 cells, we usually suspend at a density  $2 \times 10^6$  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<sub>2</sub> 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 LentiTrans Reagent).

## Day 1—Change Media

Between 16 to 24 hours post-transduction, remove media and replace with fresh complete media without LentiTrans Reagent. For suspension cells, spin down and resuspend cells in complete media at  $1-5 \times 10^5$  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  $\mu$ l 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	
<b>Excitation:</b>	561nm (530nm laser is still acceptable)
<b>Emission:</b>	600/20 band-pass filter, or similar
Flow Cytometry Settings for TagGFP	
<b>Excitation:</b>	488nm

<b>Emission:</b>	530/20nm band-pass filter, or similar
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- Use the formula below to calculate the percentage of transduced cells:

$(\text{Fluorescent cells} / \text{Total number of cells}) \times 100 = \%$  **infected cells** (use this for the titer calculation)

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

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

$([\text{antibiotic-selected cells} - \text{media-only}] / [\text{non-selected \{no antibiotic\} cells} - \text{media-only}]) \times 100 = \%$  **infected cells**  
(use for titer calculation)

*Last modified: 2019/05/04*

## 5.4. Lentiviral Titer Calculation

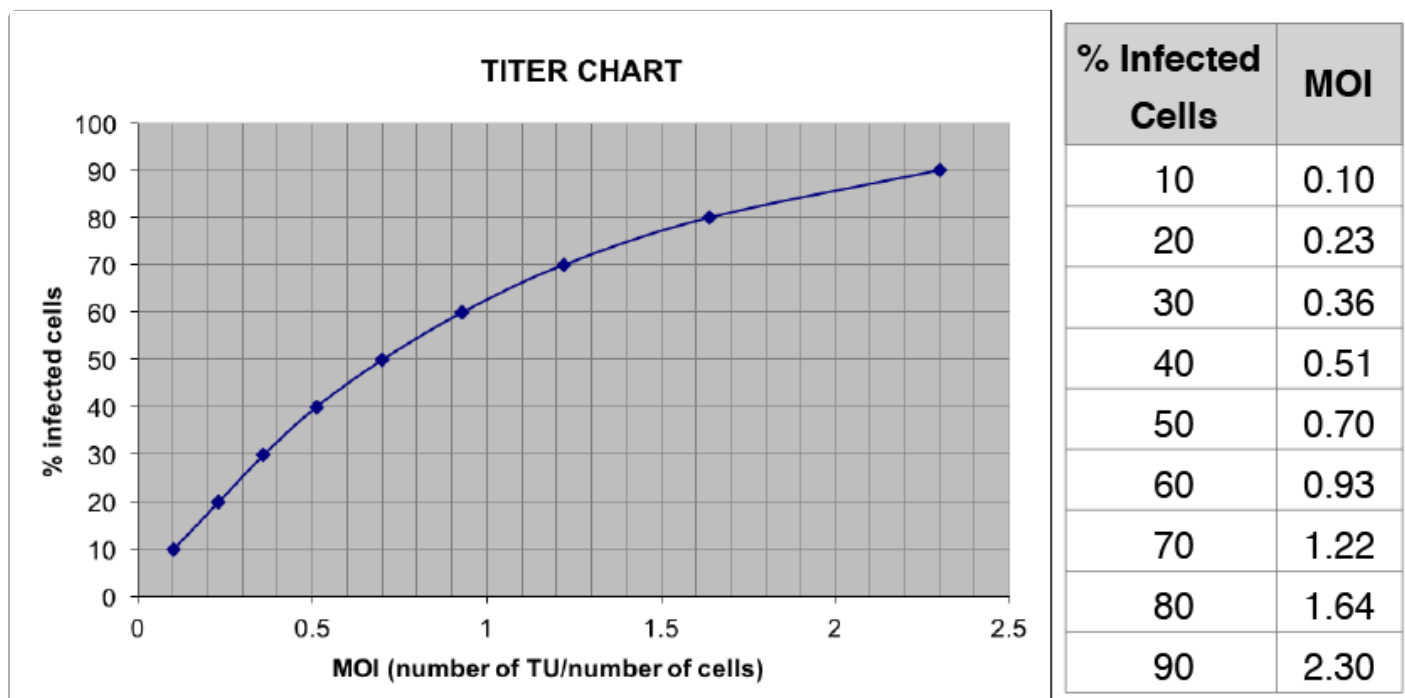
This section describes how to calculate the titer of packaged lentiviral particles. To calculate the number of transducible viral particles in the viral stock, you need to know the number of cells transduced at a specific dilution factor of the stock virus. This can be assessed by counting RFP-positive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



*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 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  $\mu$ l, 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:

$$100,000 \times 0.3 / 0.01 = 3,000,000 \text{ TU/ml}$$

*Last modified: 2019/01/10*

## 5.5. Antibiotic Selection of Library Transduced Cells

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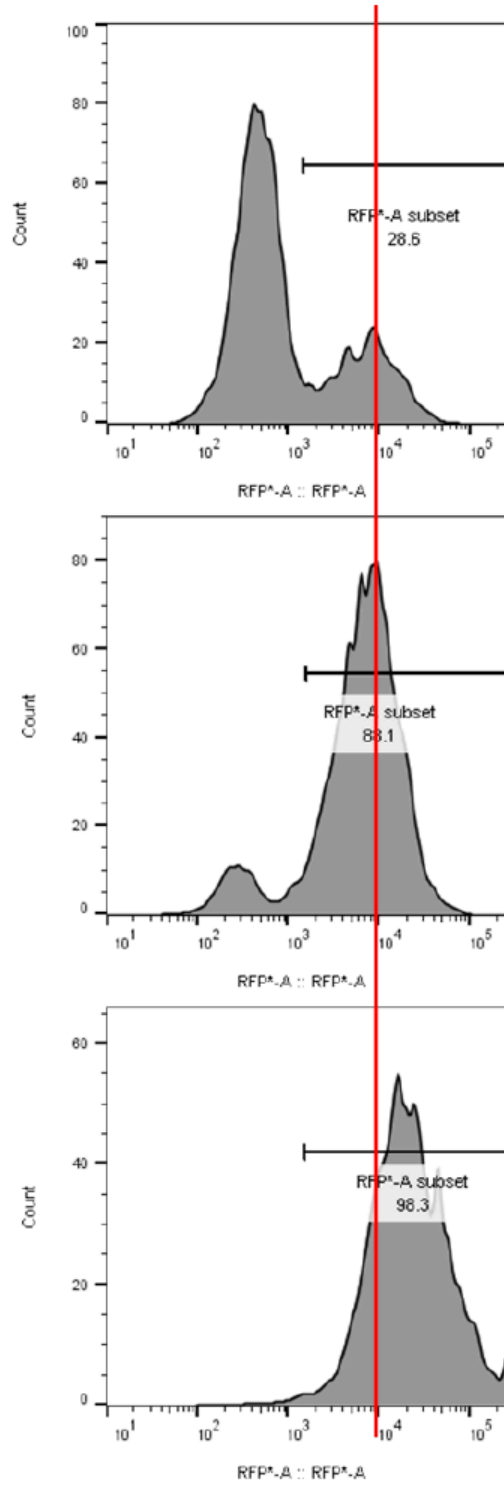
Determining the titer efficiency of your cells using the fluorescence marker is convenient. However, for most screening cases, it is practical to select using the antibiotic gene for transduced cells (based on the selection marker on the library vector). In this way, you get rid of non-transduced cells as soon as possible after library transduction.

While this antibiotic is easily accomplished by antibiotic selection. However, during antibiotic selection, it is important to avoid using too high an antibiotic concentration for two reasons: (1) you want to avoid the killing of a sizeable fraction of transduced cells, and (2) you do not want to enrich for cells with multiple viral integrants.

### Determine the optimal concentration of antibiotic for library screens.

1. Based on the titer efficiency of your cells (see Determine Transduction Efficiency by Flow Cytometry or Antibiotic Selection section), set up transduction cell pools using the amount of viral particles that resulted in 20%-50% transduced cells.
2. Aliquot same amount of cells in six 12-well wells (~50,000 cells /well).
3. Add increasing amount of antibiotic to each well (for puromycin selection, you would typically use 0.5  $\mu$ g/ml, 1  $\mu$ g/ml, 2  $\mu$ g/ml 4  $\mu$ g/ml, 8  $\mu$ g/ml). Include a no-antibiotic control well.
4. Select the lowest antibiotic concentration that resulted in >90% transduced cells selection, while maintaining the fluorescence intensity of selected and unselected transduced unchanged.

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



*Distribution of RFP+ Cells*

*Last modified: 2019/05/06*

## 6. CRISPR Screening Recommendations

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### Recommendations for Positive Selection Viability Screens (a.k.a. Survival or Rescue Screens)

Rescue screens allow for identification of genes that are functionally required for cell sensitivity to a treatment or to understand underlying drug mechanisms. Positive screens are also known as enrichment screens. Many positive screens use FACS to look for modulators of signaling molecules such as NF- $\kappa$ B, p53, c-myc, HSF-1, and HIF-1 $\alpha$  using fluorescent reporter cell lines, or cells expressing specific antibody-detectable markers, such as specific receptors.

#### Length of the Screen

A positive screen involves a selection that eliminates most of the cells. With this sort of screen, the goal is to isolate a small population of cells with sgRNAs that enable the cells to pass through the selection step. The critical factor here is the nature of the selection, which ultimately determines the screen procedure.

**NOTE:** In most cases, it is advisable to wait 1-2 weeks after sgRNA library transduction before carrying out the selection step. The wait period is needed to allow knockout of the alleles of the target gene in most transduced cells, and the development of the resistant phenotype before applying selection. Cells should then be harvested as soon as positive selection is completed. Growing and expanding clones after positive selection is not advised.

#### MOI of Transduction

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

**Example:** The starting population should be at least 25 million infected cells to start a screen with a library with 50,000 sgRNAs.

#### Antibiotic Selection of Transduced Cells

In order to prevent loss of transduced cells and enrichment for multiple integrants, it is recommended that antibiotic selection of transduced cells based on library lentivector selection marker (usually puromycin) is started no sooner than 72 hours after library transduction. Also, it is recommended to use the appropriate antibiotic concentration that removes the non-transduced cells with minimal killing of transduced cells. To determine this concentration, please follow the procedure described in “Antibiotic Selection of Library Transduced Cells”.

## Maintenance of the Cells

A positive selection screen often involves the comparison of two types of samples: selected and unselected (control) samples. After transduction and before selection, it is best practice not to discard any cells. However, this is often not practical. If cells have to be discarded or split before beginning the selection, the number of remaining cells in each sample should always exceed the complexity of the library by at least 1,000-fold (e.g., keep at least  $5.0 \times 10^7$  cells after every splitting step, for a 50K library).



**IMPORTANT:** After the selection step, *all the cells in the selected samples* should be collected for genomic DNA purification and sgRNA PCR amplification. For the control samples, follow the aforementioned 1,000-fold rule—you should collect enough cells to equal 1,000-fold the complexity of the library.



Similarly, when amplifying sgRNAs from isolated DNA, you should always use all of the genomic DNA recovered from cell samples, up to or exceeding the amount corresponding to 1,000 cells  $\times$  the library size (e.g. 50,000). For diploid cells, 50 million cells contain  $\sim 400 \mu\text{g}$  of genomic DNA.

## Baseline Controls for Positive Selection Screens

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

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## Recommendations for Negative Selection Viability Screens (a.k.a. Dropout Viability Screens)

A standard dropout viability screen (negative selection screen) relies on the fact that some of the gRNAs in the screen are either cytotoxic or cytostatic (presumably by having knocked out an essential target gene). Cells with gRNAs that do not inhibit growth grow normally, populating the culture; cells with lethal gRNA do not propagate.

**The endpoint analysis involves looking for gRNA sequences that are underrepresented in the sample population relative to the original library.**

### Length of the Screen

For a CRISPR dropout viability screen to work, the cells need to be cultured long enough for knockouts to develop in all the alleles of each gRNA's target gene, and then for cells with unaffected growth to significantly increase their proportion relative to the affected cells.

**NOTE:** The length of any particular screen may need to be altered depending on the specifics (e.g., cell growth rates,

types of targets of interest, use of additional compounds, etc.).

- Typically, we find allowing for 3 weeks after transduction to be a good starting point.

If the screen is not run long enough, all the gRNA counts will be in a narrow range and it will be difficult to identify significantly depleted gRNA sequences from background variability. If the screen is run too long, the range of representation of gRNA sequences may become broader due to the natural growth variance in different cells in the population. This phenomenon, often referred to as genetic drift, can increase the background variance of the screen and make it difficult to identify significantly depleted gRNAs from background variability.

## Antibiotic Selection of Transduced Cells

In order to prevent loss of transduced cells and enrichment for multiple integrants, it is recommended that antibiotic selection of transduced cells based on library lentivector selection marker (usually puromycin) is started no sooner than 72 hours after library transduction. Also, it is recommended to use the appropriate antibiotic concentration that removes the non-transduced cells with minimal killing of transduced cells. To determine this concentration, please follow the procedure described in “Antibiotic Selection of Library Transduced Cells”.

## Maintaining Library Representation throughout Screen

As mentioned above, the number of cells stably transduced with the sgRNA library at the time of transduction should exceed the complexity of the sgRNA library by at least 500-fold. For a library with 50,000 sgRNAs, the starting population should be at least 25 million infected cells. The MOI of transduction should be kept at or below 0.5 to ensure that most transduced cells carry only one integrated provirus.

After transduction, the ideal is to never discard any cells at any time during the experiment (e.g., at treatment, harvesting, DNA purification, etc.). However, this is often not practical—especially for a negative screen where most of the cells propagate normally. If the number of cells becomes too large and you are forced to discard a fraction, the number of remaining cells should always exceed the complexity of the library by at least 1,000-fold (e.g., for a 50K library screen, keep at least 50 million cells after every splitting step). Similarly, when amplifying gRNAs from isolated DNA, you should always use all of the genomic DNA recovered from cell samples, up to a number of cells corresponding to 1000x the library size.

## Baseline Controls for Negative Screens

In a simple screen aimed at identifying gRNAs which are cytotoxic in a given cell line, we typically use the library itself as the baseline control since the sgRNA frequency distributions in both plasmid and packaged lentiviral libraries are virtually identical. The plasmid library has already been sequenced as part of the QC when we made the library, so it is not necessary to re-sequence the library at this point. If you would also like to use transduced cells as a baseline control, we typically recommend harvesting and sequencing genomic DNA from cells by about 18 hours post-transduction.



**IMPORTANT:** If using a 18-hour post-transduction as baseline control, it is highly recommended that you use LentiFuge-purified virus (See [Related Products from Cellecta](#)) for transduction in your experiment, to minimize issues with plasmid library contamination in your baseline.



In more complex experiments, aiming at identifying differential toxicity between isogenic cell lines or between compound-treated and non-treated cells, other baselines will be needed.

*Last modified: 2019/05/04*

## 7. Extraction and Sequencing of sgRNA or Barcodes from Genomic DNA

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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*

### 7.1. Genomic DNA Extraction

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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*

#### 7.1.1. Large-Scale Genomic DNA Extraction Protocol

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**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  $\mu$ 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 dH<sub>2</sub>O to a concentration of ~2 mg/ml.
11. Incubate 30 minutes at 80°C before spectrophotometer reading.

**NOTE:** Expected yield is about 10  $\mu$ g genomic DNA per 1 million cells.

*Last modified: 2017/03/10*

## 7.2. Amplification of sgRNA or Barcode Sequences from Genomic DNA

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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 ( $180 \mu\text{g} / 50 \mu\text{g} = 3.6$ , 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
<b>100 µl</b>	<b>Total volume</b>

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.

- 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.
- 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
<b>100 µl</b>	<b>Total volume</b>

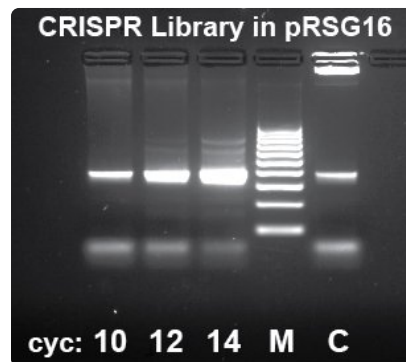
\* **Note:** Each sample to be sequenced together in a single Flow Cell or lane needs to be amplified with a different NRev Index Primer in the NGS Prep Kit. Be sure to use a different lettered NRev Index for each sample that will be loaded on the same Flow Cell or lane.

- 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 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 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  $\mu$ l 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*

## 7.3. NGS Sample Purification

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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/ $\mu$ l and 60 ng/ $\mu$ l, combine 6  $\mu$ l and 2  $\mu$ l 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  $\mu$ l.

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  $\mu$ 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 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  $\mu$ 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 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*

## 7.4. Sequencing sgRNA Inserts or Barcodes

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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*

## 8. Technical Support

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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?

### Email Addresses

Technical Support: [tech@cellecta.com](mailto:tech@cellecta.com)

General information: [info@cellecta.com](mailto:info@cellecta.com)

### Phone Numbers

Phone: +1 650 938-3910

Toll-free: (877) 938-3910

For the latest technical news and updates, visit Cellecta's blog at: <https://www.cellecta.com/blog-news/>

*Last modified: 2018/06/20*

## 9. Safety Guidelines

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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.

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## 10. Contact Us

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### Email Addresses

General information: [info@cellecta.com](mailto:info@cellecta.com)

Sales: [sales@cellecta.com](mailto:sales@cellecta.com)

Orders: [orders@cellecta.com](mailto:orders@cellecta.com)

Technical Support: [tech@cellecta.com](mailto:tech@cellecta.com)

### Mailing Address

Cellecta, Inc.  
320 Logue Ave.  
Mountain View, CA 94043  
USA

### Phone Numbers

Phone: +1 650 938-3910

Toll-free: (877) 938-3910

Fax: +1 650 938-3911

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<https://www.cellecta.com/blog-news/>



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