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Cellecta CellTracker™
Lentiviral Barcode Library

User Manual

V3, 6/6/15

Table of Contents

A. Background

The protocols below provide guidelines for packaging the vector library into VSV-g pseudotyped lentiviral particles, transduction of target cells, and the preparation of barcodes derived from transduced cells for high-throughput (HT) sequencing and analysis.

Ensure that you have the latest version of this user manual. Please check the Cellecta website at http://www.cellecta.com/resources/literature/.

B. Required Materials:

The CellTracker Barcode Library is provided as plasmid DNA or as packaged VSV-g pseudotyped viral particles:

CellTracker Barcode Library, plasmid (Cat.# BC13X13-30M-P)	200 µg
CellTracker Barcode Library, packaged (Cat.# BC13X13-30M-V)	>1 × 10^8 TU

Additional Materials Required to Package the Plasmid Library:

- Ready-to-use Lentiviral Packaging Plasmid Mix (Cat.# CPCP-K2A). Libraries can be packaged into lentiviral particles with nearly any 2nd or 3rd generation HIV-based lentiviral packaging mix. Cellecta's lentiviral packaging mix contains two plasmids: psPAX2 and pMD2.G, pre-mixed in an appropriate ratio.
- 293T/17 Cell Line (ATCC, Cat.# CRL-11268™)
- Dulbecco's Modified Eagle Medium (D-MEM) (1X) (Mediatech CellGro, Cat.# 15-013-CV)
  - See General Note in Packaging Protocol
- Glutamine (L-Alanyl-L-Glutamine, Dipeptide L-glutamine) (Mediatech, Cat.# 25-015-CI)
- Fetal Bovine Serum (recommended: Mediatech, Cat.# MT 35-010-CV)
- D-PBS
- Trypsin-EDTA
- Tissue Culture Plates and Related Tissue Culture Supplies
- Lipofectamine™ Reagent (Invitrogen, Cat.# 18324-020)
- Plus™ Reagent (Invitrogen, Cat.# 11514-015)
- 500 ml, 0.2 µm filter units (Fisher Scientific Cat.# 09-741-05 or Thermo Scientific Cat.# 569-0020)

Additional Materials Required to Transduce Cells with Packaged Lentiviral Particles:

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

Additional Materials Required to Amplify and Sequence Barcodes:

- 15-ml BD FALCON screw-cap centrifuge tubes (12,000 RCF rated, PP, P:$CHCl_3$-resistant, BD Biosciences, Cat.# 352196)
- Buffer P1 (50mM Tris-HCl pH 8.0, 10mM EDTA) (QIAGEN, Cat.# 19051)
- RNase A (QIAGEN, Cat.# 19101)
- Sonicator for Genomic DNA Shearing
- Phenol:Chloroform pH 8.0 (Sigma-Aldrich, Cat.# P3803)
- DNase I, RNase-free (Epicentre, Cat.# D9905K)
- Titanium Taq DNA polymerase with PCR buffer (Clontech-Takara, Cat.# 639242)
- dNTP Mix (10 mM each)
- QIAquick PCR purification kit (QIAGEN, Cat.# 28106)

- QIAquick Gel Extraction Kit (QIAGEN, Cat.# 28706)
- Primer for sequencing barcodes in barcode constructs (IDT)*: See **Appendix, Section I.3**
- PCR primers for barcode amplification from genomic DNA (IDT)*: See **Appendix, Section I.3**
- HT sequencing primers (IDT)*: See **Appendix, Section I.3**
- HT Sequencing Kits (Illumina):

Platform	Kit Type	Illumina Cat.#	Description
GAIIx	Sequencing	FC-104-5001	TruSeq SBS Kit v5 – GA (36-cycle)
	Cluster Generation	GD-203-5001	TruSeq SR Cluster Kit v5 – CS – GA
HiSeq	Sequencing	FC-401-3002	TruSeq SBS Kit v3 – HS (50 cycle)
	Cluster Generation	GD-401-3001	TruSeq SR Cluster Kit v3-cbot-HS

Related Products and Services from Cellecta

- HT Barcode Sequencing of Transduced Cell Pellets from Genetic Screen, Cat.# CANA-SQ

C. Packaging Protocol

The following protocol describes the generation of packaged lentiviral particles from the plasmid CellTracker Barcode Library. The yield of recombinant lentiviral particles typically produced with this procedure is 1-10 × 10^6 TU/ml. The protocol is written for packaging with 10 x 15-cm plates to produce at least 3 × 10^8 TU of total lentiviral particles which can then be optionally concentrated. It is recommended to package at least this much lentivirus at a time to maintain barcode diversity in the library above 10 million.

General Note on Packaging

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

2-3 Days Prior to Starting Packaging

1. Start growing 293T cells in D-MEM medium plus glutamine (see **Required Materials**), supplemented with 10% FBS without antibiotics.

Day 0 – Plate Cells

2. Twenty four (24) hours prior to transfection, plate 12.5 × 10^6 293T cells in each of ten (10) 15-cm plates (or 150 cm^2 flasks). Use 30 ml of media per plate. Disperse the cells and ensure even distribution. At the moment of transfection, the cells should have reached ~80% confluency. Increase or decrease the number of 293T cells seeded if optimal confluency is not achieved in 24 hours. Incubate at 37°C in a CO_2 incubator for 24 hours.

Day 1 – Transfection (Ten 15-cm plates*)

3. In sterile 50-ml polypropylene tube, mix 600 µl (300 µg) of the Ready-to-use Packaging plasmid mix (see **Required Materials** for formulation) with 60 µg** of the plasmid library and add the plasmid mixture to 12 ml D-MEM medium without serum or antibiotics. Add 600 µl of Plus Reagent, mix, and incubate at room temperature for 15 min.

10X 15-cm plates	**Component**
600 µl	Ready-to-use Packaging Plasmid Mix (0.5 µg/µl) *
60 µl	Plasmid CellTracker Barcode Library (1 µg/µl) **
12,000 µl	D-MEM, no FBS, no antibiotics

600 µl	Plus Reagent
13,260 µl	Total volume

**** IMPORTANT: DO NOT use less than 60 µg (ten 15-cm plates) to package a batch of CellTracker Barcode library. Some barcodes may be lost if the yield is less than 1 × 10^8 TU.***

4. Add 900 µl of Lipofectamine Reagent to 12 ml of D-MEM medium without serum or antibiotics in order to make a convenient master mix. Mix gently.

10X plates	**Component**
12,000 µl	D-MEM, no FBS, no antibiotics
900 µl	Lipofectamine
12,900 µl	Total volume

5. Add the diluted Lipofectamine Reagent (from step 4) to the DNA / Plus Reagent complex (from step 3), mix gently by flicking the tube or vortexing and incubate at room temperature for 15 min.

6. Add 2.5 ml of the DNA / Plus Reagent / Lipofectamine Reagent complex (from step 5) to each 15-cm plate from step 2, and mix complexes with medium by gentle rotation. Take care not to dislodge cells from the plate. Incubate at 37°C in the CO_2 incubator for 24 hours.

Day 2 – DNase I Treatment

7. At 24 hours post-transfection, replace the medium containing complexes with fresh 30 ml D-MEM medium supplemented with 10% FBS, DNase I (1 U/ml), $MgCl_2$ (5 mM), 20mM HEPES pH7.4. Continue incubation in the CO_2 incubator at 37°C overnight. Overnight DNase I treatment before harvesting virus does not negatively affect lentiviral titer or infectivity and helps prevent undesirable carryover of plasmid library into the virus prep.

   NOTE: Failure to change the media the day after transfection results in large carryover of plasmid (free and/or Lipofectamine-bound) in your lentiviral prep. This may cause problems with most downstream molecular biology applications, especially whenever there is a PCR step involved.

Day 3 – Collect Lentiviral Supernatant

8. At 48 hours post-transfection, collect all 30 ml of the virus-containing medium from each plate and filter the supernatant (300 ml) 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 of fresh D-MEM medium supplemented with 10% FBS, 20mM HEPES pH7.4 and continue incubation in the CO_2 incubator at 37°C for 24 hours.

   CAUTION: You are working with infectious lentiviral particles at this stage. Please follow the recommended guidelines for working with BSL-2 safety class materials (see **Safety Guidelines**).

9. Aliquot and store the supernatant at –80°C. Freezing and thawing usually results in ~20% loss of lentiviral titer with each cycle.

Cellecta offers lentiviral packaging services. Please contact us at sales@cellecta.com or visit our website at http://www.cellecta.com for more information.

D. Transduction Protocols and Lentiviral Titer Estimation

Transduction

Lentiviral transductions are performed by mixing cells and virus in culture media supplemented with Polybrene®. For both adherent and suspension cells, transductions are initiated in suspension and carried out overnight. Adherent cells are allowed to adhere to substrate during transduction and are transduced at a cell density that allows for 2-3 population doublings before reaching confluence. Suspension cells are typically transduced at higher density than standard growth density, and then they are diluted to standard growth density 18-24 hours after transduction.

Check Toxicity of Polybrene
Polybrene is a polycation that neutralizes charge interactions to increase binding between the lentiviral envelope and the plasma membrane. The optimal concentration of Polybrene depends on cell type and may need to be empirically determined. Excessive exposure to Polybrene can be toxic to some cells.

Before conducting the titer estimation experiment, we recommended performing a Polybrene toxicity titration in target cells. Grow cells in complete culture medium with a range of Polybrene concentrations (0 µg/ml, 1 µg/ml, 2 µg/ml, 3 µg/ml, 4 µg/ml, 5 µg/ml) for 24 hours, and then replace old medium with Polybrene-free complete culture medium. Grow cells for an additional 72 hours, and then check toxicity by counting viable cells. For your experiments, use the highest concentration of Polybrene that results in less than 10% cell toxicity compared to no Polybrene (typically, 5 µg/ml is recommended). For some cell types, you cannot use Polybrene.

Protocol For Titering lentiviral stock (RFP assay)

The CellTracker Barcode Library vector expresses the fluorescent protein TagRFP (excitation ~560nm emission ~590nm), allowing lentiviral titer estimation by flow cytometry (RFP assay) or by a combined flow cytometry/puromycin resistance assay (RFP/Puro^R assay). To check lentiviral titer, we recommend always using the same cells you will use in the screen. Most of the commonly used mammalian cell lines can be effectively transduced by lentiviral constructs. Relative titers can vary up to 50-fold depending on the chosen cell line.

Transduction (HEK293 cells):
The following protocol has been optimized for HEK293 cells. For other adherent cell types, parameters such as media, growth surface, time of detection, etc. will have to be adjusted.

Day 1

1. Quickly thaw the lentiviral particles in a water bath at 37°C. Transfer the thawed particles to a laminar flow hood, gently mix by rotation, inversion, or gentle vortexing, and keep on ice.
   CAUTION: Only open the tube containing the lentiviral particles in the laminar flow hood.
   NOTE: Unused lentiviral stock may be refrozen at –80°C, but it will typically result in a loss of about ~20% in titer.

2. Trypsinize and resuspend HEK293 cells to a density of 1 × 10^5 cells/ml in D-MEM supplemented with 10% FBS and 5 µg/ml Polybrene. Aliquot 1 ml/well in a 12-well plate and add 0 µl, 3 µl, 10 µl, 33 µl, and 100 µl of lentiviral stock (supernatant filtered to remove cells and cell debris, not concentrated) to six different wells. If concentrated virus is used, scale down virus volumes accordingly. Mix and return cells to CO_2 incubator. Grow cells under standard conditions for 24 hours.

   NOTE: It is important to accurately record the **original** # of cells at **Time of Transduction**, as this is critical in titer calculation. For adherent cells other than HEK293, choose a different # of cells at time of transduction, depending on cell size. As a rule of thumb, cells should be transduced at such a density such that they would become confluent in ~48 hours. For example, for HeLa cells, the suggested cell # is 50,000 cells/well in a 12-well plate.

Day 2

3. At 24 hours post-transduction, replace media with fresh D-MEM supplemented with 10% FBS and without Polybrene. Return cells to CO_2 incubator, and grow under standard conditions for additional 48 hours. Avoid confluence: trypsinize and re-plate cells if needed.

Day 4 (72 hours after transduction)

4. Detach cells from the plate by trypsin treatment, block trypsin with FBS/media, centrifuge, resuspend in 1X D-PBS, and determine the % of transduced (RFP-positive) cells by flow cytometry.

   NOTE: Attempting to determine the % of transduced cells by fluorescence microscopy is NOT RECOMMENDED.

   IMPORTANT: Flow cytometry settings to detect RFP-positive cells are the following: Excitation: 561nm (530nm laser is still acceptable), Emission: 600/20 band-pass filter, or similar (for TagRFP).

5. Proceed to **Lentiviral Titer estimation (RFP assay)**.

Alternative Transduction protocol (spinoculation) for hard to transduce cells

The following protocol has been optimized for K-562 cells. For other cell types, parameters such as media, growth surface, time of detection, etc. will have to be adjusted.

1. K-562 cells are transduced ("infected") using spinoculation. This is performed using multi-well tissue culture plates and a tabletop centrifuge capable of 1,200 × g and centrifugation of multi-well plates.

2. Grow K-562 cells and maintain them between 2×10^5 and 1×10^6 cells/ml. Do not let them become too dense or let the medium become yellow at any point.

3. For lentiviral library titration, K-562 cells are resuspended at 2×10^6 cells per ml in RPMI 10%FBS supplemented with 20mM HEPES pH7.4 and Polybrene 5 µg/ml. 0.5-ml aliquots are placed into each well in a 24-well plate (1×10^6 cells/well total). This cell density has proven effective for many suspension cell lines in-house at Cellecta. To each cell-containing well, add increasing amounts of lentiviral stock to be titered. For 100-fold concentrated lentiviral stock, for example, add 0 µl, 0.3 µl 1 µl, 3 µl, and 10 µl virus. Close the plate, mix by gentle agitation, wrap the perimeter with parafilm, and place the plate into centrifuge with an appropriate balance and centrifuge at 1,200 × g at +25°C for 2 hours.

4. Following centrifugation, remove plate(s) from centrifuge, carefully remove parafilm, and place in incubator. After 3 hours, "feed" cells with 0.5 ml additional complete medium per well (no Polybrene).

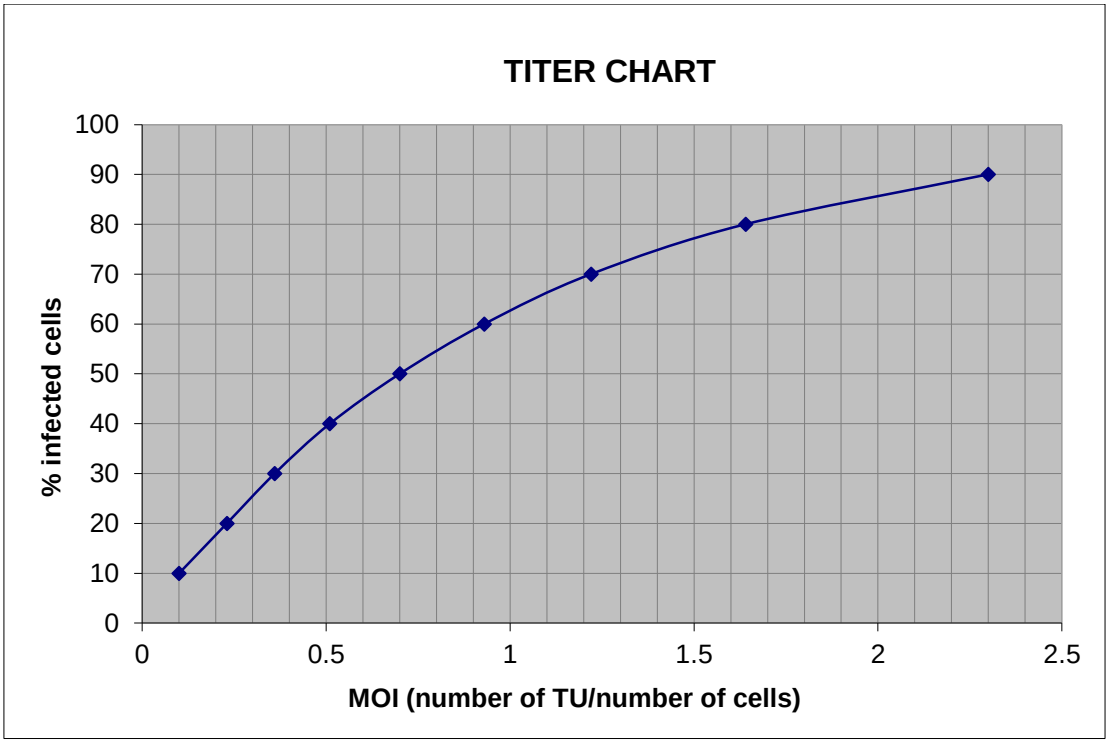
5. 24 hours after spinoculation, resuspend cells at 2×10^5 cells/ml in RPMI 10% FBS in the appropriate culture vessel and grow for additional 48 hours.

6. 72 hours after spinoculation, perform titer as previously described.

NOTE: Use larger vessels for large-scale genetic screen transductions. Scale up all volumes accordingly.

Lentiviral Titer estimation (RFP assay)

Lentiviral titer is measured as Transduction Units/ml (TU/ml). One TU produces one integration event (i.e., one infection) in target cells. Infections (i.e., transductions) can be calculated from observed % of transduced cells according to the graph below.



The % of transduced cells is determined by flow cytometry (excitation=561nm emission=600/20 for TagRFP) by observing the % of RFP+ cells in the transduced cell sample. When the % of transduced cells is at or below 20%, the number of infections can be considered roughly equivalent to the number of transduced cells.

At higher transduction efficiencies, however, the fraction of transduced cells bearing multiple integrations increases, so that the number of infected cells after transduction is less than the number of TU. In other words, some cells are transduced 2 or 3 times. Use the graph to calculate the Multiplicity of Infection (i.e., the number of TU/cell) required to transduce a specific number of cells with MOIs above 0.2.

Titer is calculated according to the **TITER FORMULA** below:

TU/ml = (# of cells at Transduction) x MOI / (ml of Viral Stock used at Transduction)

Example:

IF: The original # of cells at Transduction was 100,000, and
The volume of virus stock used was 10 µl, and
The observed % of transduced (RFP+) cells is 25%,

THEN: The calculated MOI is 0.3, and
The **TITER** is: 100,000 × 0.3 / 0.01 = 3,000,000 TU/ml

Once titer is estimated, the amount of Lentiviral Stock necessary to transduce any given # of target cells at any transduction efficiency (range of 10-80% infected cells) can be backward calculated from the **TITER FORMULA** and **TITER CHART** above.

Example:

To transduce 20,000,000 cells at 50% transduction efficiency, with a Lentiviral Stock titer of 3,000,000 TU/ml, we calculated the required amount of Lentiviral Stock as follows:

1. We calculate the required MOI to achieve 50% transduction efficiency, using the TITER CHART:
   50% transduction efficiency = 0.7 MOI

2. We calculate the volume of Lentiviral Stock required using the TITER FORMULA:
   TU/ml = (# of cells at Transduction) x MOI / (ml of Viral Stock used at Transduction)
   3,000,000 = 20,000,000 × 0.7 / (ml Viral Stock)
   Viral Stock = 20,000,000 × 0.7 / 3,000,000 = 4.67 ml

Transduction of Founder Cells

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

Notes on Transducing Founder Cells

- Cell transduction is a random process following a statistical distribution. Therefore, if too high an MOI is used, many cells will take up more than one barcode. Cells with more than one barcode show up as more than one population in the final analysis. For example, if two barcodes integrate into one founder cell, and the cell produces 50 progeny, the data (after harvesting the cells and HT sequencing of the genomic DNA) will show two clonal populations with two different barcodes, each having the same number of cells. It will not be obvious that these two populations are from the same founder cell. For this reason, we typically recommend using low MOIs of <0.3 so that >90% of the transduced cells only contain one barcode (see graph above).
- Transducing larger populations of cells increases the frequency of having more than one founder cell with the same barcode. Since the CellTracker Barcode library has several million unique barcodes, a majority of cells will contain unique barcodes, even with library transductions of a million or more. However, with larger transductions, two or more founder cells can receive the same barcode. To minimize this, we recommend starting screens with less than a million cells if possible. For more details on the complexity and representation of barcodes in the library, and estimates of the number of barcodes repeats you should expect with transductions of different size founder cell populations, please refer to the QC information.
- Since the purpose of using this complete barcode library is to track the fate of cells from a founder population, we do not recommend splitting and discarding any cells during the course of an experiment:
  - Discarding any portion of cells will eliminate some of the barcodes unless the cell population has expanded to several orders of magnitude over the founder population size.
  - Even if the population has expanded many fold over, splitting or otherwise discarding cells may skew the clonal population sizes for different barcodes (i.e., clone that grow slowly versus cells that grow quickly may be differentially affected).
  - If you must discard cells, it is crucial to design the experiment to minimize the impact on the barcode representation in your samples.
- Following transduction, you may want to select cells with barcodes using Puromycin. For this, you should calculate a *Puromycin Kill Curve* using the following procedure:
  1. Aliquot cells in a 12-well plate, at such a density so they are at 72 hours from confluency.
  2. Add puromycin at 0 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 5 µg/ml, and 10 µg/ml in six different wells.
  3. Mix and return cells to incubator.
  4. Grow cells under standard conditions for 42-72 hours.

  Use the lowest concentration of Puromycin that kills >90% of cells in 42-72 hours.

E. Genomic DNA Extraction for Barcode Amplification and HT Sequencing

Identification of barcodes in the experimental samples requires amplification of the barcode portion of the integrated lentiviral constructs from sample genomic DNA. Subsequent high-throughput sequencing of barcodes by the Illumina GAIIx or HiSeq is done to quantify each barcode and, based on this, determine the relative size of each clonal population derived from a founder cell.

Notes on Genomic DNA Extraction and Amplification

- If the experiment is intended to track the fate all the cells derived from each clone in a founder cell population (e.g., you want to determine the growth rates of all the founder clones):
  - The best approach is to isolate genomic DNA and amplify pooled barcodes from the whole population of progeny cells (e.g. the whole tumor in a xenograft model).
  - If it is not possible to use all the progeny cells, you should start isolation with a minimum of 200-fold the number cells as were in the initial founder population
- If the experiment is intended to just identify which fraction of cells from a founder cell population that are still present in a population (e.g., survive some sort of selection that eliminates a portion of the initial founder population), you should start isolation with a minimum of 10-fold the number cells as were in the initial founder population.
- Pooled barcodes should be amplified by two rounds of PCR using Titanium Taq DNA polymerase mix (Clontech-Takara, see **Required Materials**).
- The protocol was optimized using an ABI GeneAmp PCR System 9700. Use of other PCR enzymes and/or thermal cyclers may require additional optimization.

Recommended Protocol

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

1. Suspend cell pellet in 5 ml QIAGEN buffer P1 (with RNaseA) in 15 ml POLYPROPYLENE (phenol/chloroform resistant), BD FALCON screw-cap centrifuge tube (12,000 RCF rated, BD Biosciences Cat.# 352196).

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

3. Using an ultrasonic homogenizer (see **Required Materials**), sonicate to shear DNA into 10-100 kb sized fragments. To prevent cross-contamination, thoroughly wash the ultrasound head with running water and dry-up with clean paper towel between samples.
4. Add 5 ml phenol/chloroform pH8.0 solution, vortex hard and spin down 60 min, +20°C at 8,000 rpm in JA-14 or equivalent rotor (Beckman).

5. You should have about 5 ml of clear upper phase. Transfer 4 ml of upper phase to new 15 ml DISPOSABLE screw cap tube (same as in Step 1).

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

7. In order to have a more visible pellet, compacted at the bottom of the tube, it is recommended to incubate overnight at RT before centrifugation.
   IMPORTANT: If starting material is less than 5 million cells, add carrier before centrifugation (linear polyacrylamide, 25 µg/ml final) and spin down for a longer time (60 min).

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

9. Discard supernatant and air-dry pellet.

10. Dissolve DNA pellet in appropriate volume of dH_2O to a concentration of approximately 2 mg/ml. Expected yield is about 10 µg per 1 million cells.

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

F. Amplification of Barcodes from Genomic DNA

The lentiviral library and PCR primer designs include sequences complementary to the sequences of the immobilized primers necessary for generating amplification clusters in Illumina's GAIIx or HiSeq Flow Cells. ***Our library design is only compatible with Single-Read Flow Cells (in the Single-Read Cluster Generation Kit)***, because our primers are not complementary to the sequences immobilized on Paired-End flow cells (in the Paired-End Cluster Generation Kit). See **Required Materials** for the appropriate Illumina catalog numbers.

Use 10 ng of plasmid library as an amplification control in the first round of PCR, and use the subsequent PCR products in all remaining steps. The protocol below is for 200 ug of genomic DNA. For whole amount of genomic DNA use proportionally more tubes.

First Round of PCR

1. For each sample, prepare 4 × 100 µl reactions containing 200 µg of genomic DNA:

___	µl	Genomic DNA (50 µg)
3	µl	FwdHTS3 primer* (10 µM)
3	µl	R2 primer* (10 µM)
2	µl	50X dNTP Mix (10 mM each)
10	µl	10X Titanium Taq Buffer
___	µl	Deionized water
1	µl	50X Titanium Taq
100	µl	Total volume

94°C, 3 minutes	1 cycle
94°C, 30 seconds 65°C, 10 seconds 72°C, 20 seconds	16 cycles
68°C, 2 min	1 cycle

** Please see Appendix for primer sequences.*

Second Round of PCR

The second round of PCR—nested PCR—is required in order to significantly reduce genomic DNA carryover into the samples used for HT sequencing.

1. Combine together the 4 × 100 µl First Round PCR reactions and use a 2 µl aliquot in the second round of analytical PCR with nested primers in each 100 µl reaction:

2	µl	First Round PCR Product
5	µl	Gex1-Bpi primer* (10 µM)
5	µl	NR2 primer* (10 µM)
2	µl	50X dNTP Mix (10 mM each)
10	µl	10X Titanium Taq Buffer
75	µl	Deionized water
1	µl	50X Titanium Taq
100	µl	Total volume

94°C, 3 minutes	1 cycle

94°C, 30 seconds 65°C, 10 seconds 72°C, 10 seconds	)	**10, 12, or 14** cycles
68°C, 2 min		1 cycle

** Please see Appendix for primer sequences.*

NOTE: Avoid overcycling of PCR reactions—this will usually result in the generation of a longer fragment that corresponds to a fusion double barcode product.

The amplified pooled barcode cassettes are then analyzed on a 3.5% agarose-1XTAE gel (load 5 µl/lane). The results should reveal a bright band at 267 bp for the Gex1-Bpi/NR2 amplicon. The goal of this analytical PCR step is to optimize the starting amount of First Round PCR product and the number of cycles (if necessary) in order to achieve equal intensities of a single 267-bp barcode band across all DNA samples from the genetic screen. Select optimal amount of starting amount and cycle number which produce bright single band without overcycling.

Repeat second-round amplification of barcodes from each sample using the optimized volume of First Round PCR product, 2 × 100 µl of Second Round PCR product per sample, and 12-14 cycles of PCR. Set up 2 × 100 µl reactions for each sample containing an adjusted "equal" amount of First Round PCR product (2 µl or more):

2 µl	First Round PCR Product
5 µl	Gex1-Bpi primer* (10 µM)
5 µl	NR2 primer* (10 µM)
2 µl	50X dNTP Mix (10 mM each)
10 µl	10X Titanium Taq Buffer
75 µl	Deionized water
1 µl	50X Titanium Taq
100 µl	Total volume

94°C, 3 minutes		1 cycle
94°C, 30 seconds 65°C, 10 seconds 72°C, 10 seconds	)	**12 or 14** cycles
68°C, 2 min		1 cycle

** Please see Appendix for primer sequences.*

Analyze the PCR products by gel-electrophoresis on a 3.5% agarose-1XTAE gel in order to ensure equal yields of amplified barcodes for all samples. Combine amplified barcodes from the 2 × 100 µl Second Round PCR reactions and purify the samples as follows:

1. Purify the each specific PCR product with the single column from QIAquick PCR purification kit (QIAGEN) following the manufacturer's protocol,
2. Separate by electrophoresis in a preparative 3.5% agarose-1XTAE gel,
3. Cut out band and extract DNA from the gel using the QIAquick gel purification kit (QIAGEN), and
4. Quantitate using A260 nm measurement using NanoDrop spectrophotometer (or equivalent) and adjust concentration to 10nM (~0.75 ng/µl).

G. HT Sequencing of Barcodes

HT sequencing of pooled amplified barcodes can be performed on the Illumina GAIIx (~20-30 million reads per sample) or HiSeq (~80-100 million reads per sample) using the GexSeqS sequencing primer and following the manufacturer's protocol. The final concentration of GexSeqS primer in the reaction should be 500 nM. For the cluster generation step, use 20 fmoles (2 µl of 10 nM PCR product) of the gel-purified band from the 2nd round of PCR. The number of cycles (read length) required is 44.

The Barcode library and PCR primer designs include sequences complementary to the sequences of the immobilized primers necessary for generating amplification clusters in Illumina's GAIIx or HiSeq flow cells. ***Our design is only compatible with Single-Read Flow Cells (in the Single-Read Cluster Generation Kit)***, because our primers are not complementary to the sequences immobilized on Paired-End flow cells (in the Paired-End Cluster Generation Kit).

See **Required Materials** for a list of recommended Illumina kits for HT Sequencing of Barcode Library samples. HT sequencing of samples on the Illumina MiSeq is not supported.

Please contact us at sales@cellecta.com for information on our HT sequencing and data analysis services.

** Please see Appendix for HT sequencing primer sequences.*

H. Barcode Enumeration (Conversion of raw HT sequencing data to number of reads for each barcode)

For help with Barcode Enumeration, please contact Cellecta Technical Support at tech@cellecta.com.

I. Troubleshooting

Low Lentiviral Titer (<10^6 TU/ml in supernatant)

1. **Poor transfection efficiency (48 hour post-transfection, less than 80% of 293T cells are very brightly fluorescent)**

   Problem: 293T Cells have too high or too low density
   Solution: Plate fewer or more cells in order to have about 80% confluency at time of transfection.

   Problem: Plasmid DNA/Lipofectamine/Plus Reagent ratios are incorrect
   Solution: Optimize the ratios using the guidelines provided in the Lipofectamine protocol.

2. **Inefficient production of the virus**

   Problem: 293T Cells are of poor quality
   Solutions:
   - Optimize growth conditions, check growth medium, and don't grow 293T cells for more than 20 passages.
   - Check for mycoplasma contamination.
   - Do not overgrow the cells (do not allow the cells to reach more than 90% confluency in order to keep the culture continuously in logarithmic growth phase).

   Problem: Lentiviral supernatant harvested too early or too late
   Solution: Harvest supernatant 48 hours and 72 hours after transfection.

   Problem: 293T cell media is too acidic at time of virus harvesting
   Solution: Make sure to replace media 24 hours before harvesting, and make sure to supplement media with HEPES pH 7.4 20mM final.

3. **Inefficient transduction of titering cells**

   See below.

Inefficient Transduction of Packaged Library

1. **Poor transduction efficiency**

   Problem: Target cells have too high or too low density
   Solution: Plate fewer or more cells in order to have 20-50% confluency at transduction stage.

   Problem: Target cell line may be difficult to transduce
   Solutions:
   - Use a higher concentration of lentiviral particles.
   - Perform "Spinoculation" to improve transduction efficiency.
   - Check to see if Polybrene was added at 5 µg/ml.

   Problem: Wrong amount of Polybrene added during transduction stage
   Solution: If Polybrene is toxic to the target cells, optimize Polybrene concentration in the range of 0 – 5 µg/ml by performing a toxicity titration as described in **Transduction Protocols and Lentiviral Titer Estimation.**

   Problem: Loss of lentiviral titer during storage
   Solution: Ensure storage of aliquoted packaged library at –80°C. Each freeze-thaw cycle typically causes reduction of the titer by ~20%. Use a fresh stock for transduction.

   Problem: The RFP assay is performed too early
   Solution: Normally, the maximal expression of RFP from the integrated provirus is expected to develop by 72 hours after transduction. However, some cells exhibit delayed expression. Try the assay at a later time, such as 96 hours.

   Problem: The RFP assay is performed with the wrong flow cytometry settings.
   Solution: RFP+ cells are to be detected using a 561nm laser for excitation (530nm still acceptable) and 600/20 band-pass filters (or similar) for detection (for TagRFP). Using blue laser (488nm) for excitation leads to gross underestimation of viral titer.

   Problem: In the RFP assay, the % of transduced cells is determined by fluorescence microscopy instead of flow cytometry.
   Solution: Use flow cytometry.

2. **Transduction affects target cell viability**

   Problem: Polybrene is toxic for target cells
   Solution: Optimize the concentration and exposure time to Polybrene during the transduction step. For some sensitive cells, Polybrene should not be used.

   Problem: Virus-containing conditioned media is toxic to target cells.
   Solution: Concentrate and resuspend the virus in target cell growth media, PBS 10% FBS, or PBS 1% BSA.

Difficulties with Probe Preparation and HT Sequencing

1. **No PCR Product**

   Problem: Incorrect primers or bad reagents used, or missing reagents.
   Solutions:
   - Include 10 ng of plasmid library DNA as a positive control. If it produces the correct amplification product, the problem lies with the genomic DNA or previous PCR prep. If not, confirm use of the correct primers and reagents.
   - Verify that primer sequences are correct. Please see **Appendix, Section I.3**.

2. **No barcodes present in HT Sequencing results**

Problem: Incorrect primer used in Illumina-Solexa Cluster Generation step.
Solution: Ensure that you or the HT Sequencing core facility uses the GexSeq Sequencing primer (see **Appendix, Section I.3**), NOT the Sequencing primer that comes with the Illumina Cluster Generation Kit.

Problem: Incorrect Cluster Generation kit used.
Solution: Ensure that you or the HT Sequencing core facility uses the proper **Single-Read** Cluster Generation Kit (see **Required Materials**).

J. Technical Support

For additional information or technical assistance, please contact us by phone or email:

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K. Safety Guidelines

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

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter upstream of 5'LTR in the lentivector allows efficient Tat-independent production of lentiviral RNA, reducing the number of genes from HIV-1 that are used in this system.
- Number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (gag, pol, rev). The corresponding proteins are expressed from different plasmids lacking packaging signals and share no significant homology to any of the expression lentivectors, pVSV-G expression vector, or any other vector to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (gag, pol, rev) will be present in the packaged lentiviral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.
- Lentiviral particles will carry only a copy of your expression construct.

Despite the above safety features, use of HIV-based vectors falls within NIH Biosafety Level 2 criteria due to the potential biohazard risk of possible recombination with endogenous lentiviral sequences to form self-replicating virus or the possibility of insertional mutagenesis. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at:

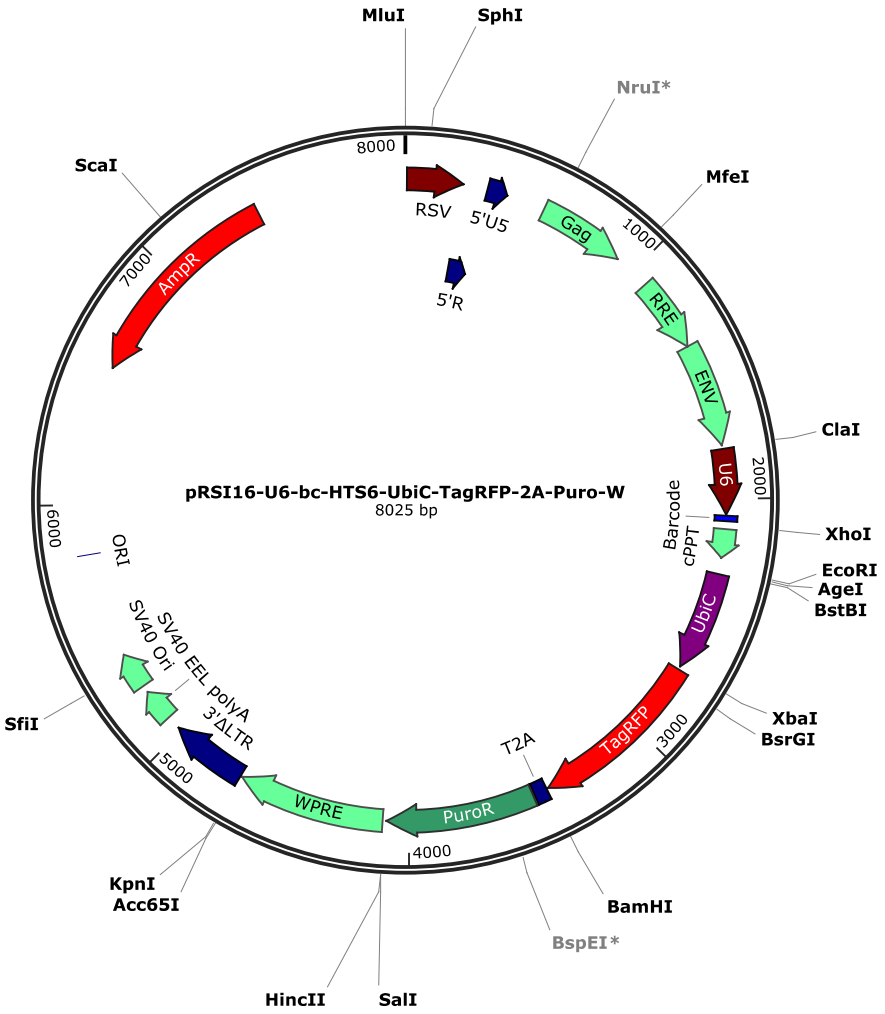
http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm

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.

L. Appendix

1. Map of CellTracker Lentiviral Barcode Library Vector



** All Cellecta lentiviral vectors are covered by a lentiviral expression system license owned by Life Technologies Corporation (LTC). See **Terms and Conditions.***

2. Barcode Library Vector Cassette

CellTracker Library in pRSI16 vector: - size of Gex1-Bpi/NR2 amplicon is 267 bp.

ClaI-U6-FwdU6-1>FwdU6-2>EcoRI>FwdHTS3>Gex1-Bpi>BarCode18TTCG BarCode18<GexSeqS<XhoI<cPPT<Gex2-NR2<R2<RevUbiC1

```
                        FwdHTS3
                TCGGATTCAAGCAAAAGACGGCATA Gex1-Bpi
          Bpi           TCAAGCAGAAGACGGCATACGAAGACA          BC18                BC18                                  XhoI
U6-ACCGGAGTCTTCTTTTTTGAATTCAAGCAAAAGACGGCATACGAAGACAGTTCG-NNNNNNNNNNNNNNNNNTTCGNNNNNNNNNNNNNNNNN-TTCGGACTGTAGAACTCTGAACCTCGAGCAA
U6-TGGCCTCAGAAGAAAAAACTTAAGTTCGTTTTCTGCCGTATGCTTCTGTCAAGC-NNNNNNNNNNNNNNNNNAAGCNNNNNNNNNNNNNNNNN-AAGCCTGACATCTTGAGACTTGGAGCTCGTT
                                                                                                      AAGCCTGACATCTTGAGACTTGGAGA
                        cPPT                                                                                                  GexSeqS
TTTAAAAGAAAAGGGGGGATTGGGGGGTACAGTGCAGGGGAAAGAATAGTAGACATAATAGCAACAGACATACAAACTAAAGAATTACAAAAACAAATTACAAAAATTCAAAATT
AAATTTTCTTTTCCCCCCTAACCCCCCATGTCACGTCCCCTTTCTTATCATCTGTATTATCGTTGTCTGTATGTTTGATTTCTTAATGTTTTTGTTTAATGTTTTTAAGTTTTAA
                                                  EcoRI     AgeI    BstBI BspEI           UbiC promoter
TCTGCGTTGTTGTCGGTGCTCGTTCTCTGCTCTTCACGCTACTGAATTCATCACCGGTTCTTCGAAGGCCTCCGCGCCGGGTTTTGGCGCCTCCCGCGGGCGCCCCCCTCCTCACGGCG
AGACGCAACAACAGCCACGAGCAAGAGACGAGAAGTGCGATGACTTAAGTAGTGGCCAAGAAGCTTCCGGAGGCGCGGCCCAAAACCGCGGAGGGCGCCCGCGGGGGGAGGAGTGCCGC
AGACGCAACAACAGCCACGAGAGCCACCAGCGGCATAGTAA                                                                    AGGAGTGCCGCTCGCGACGGA
        Gex2-NR2        AAGAGACGAGAAGTGCGATGA                                                                          RevUbiC1
                                 R2
```

3. HT Sequencing Primers

Primer Name	Used for	Sequence (IDT preferred)
FwdHTS3	1st Round	5′-TCGGATTCAAGCAAAAGACGGCATA-3′
R2	1st Round	5′-AGTAGCGTGAAGAGCAGAGAA-3′
Gex1-Bpi	2nd Round	5′-TCAAGCAGAAGACGGCATACGAAGACA-3′
Gex2-NR2	2nd Round	5′-AATGATACGGCGACCACCGAGAGCACCGACAACAACGCAGA-3′
GexSeqS	HT Sequencing	5′-AGAGGTTCAGAGTTCTACAGTCCGAA-3′ (HPLC Purified)
FwdU6-1	Standard sequencing	5′-CAAGGCTGTTAGAGAGATAATTGGAA-3′
FwdU6-2	Standard sequencing	5′-CCTAGTACAAAATACGTGACGTAGAA-3′

4. Barcode Library Vector Features

Feature	Function	Source
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull et al., 1998).	Rous sarcoma virus
HIV-1 truncated 5′ LTR	Permits viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).	HIV-1
HIV-1 psi (ψ) packaging signal	Allows viral packaging (Luciw, 1996).	HIV-1
HIV-1 Rev response element (RRE)	Permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems et al., 1991; Malim et al., 1989).	HIV-1
U6 promoter	Human U6 promoter drives RNA Polymerase III transcription for generation of shRNA transcripts.	Human
cPPT	Central polypurine tract, cPPT, improves transduction efficiency by facilitating nuclear import of the vector's preintegration complex in the transduced cells.	HIV-1
UbiC promoter	Ubiquitin C promoter drives expression of TagRFP and PuroR.	Human
TagRFP	TagRFP fluorescent protein (Evrogen) serves as an indicator of successful transduction.	sea anemone *Entacmaea quadricolor*
2A (T2A)	*Thosea asigna* virus 2A translational cleavage site containing 18 amino acid residues. Cleavage occurs via a co-translational ribosome skipping mechanism between the C-terminal glycine and proline residues, leaving 17 residues attached to the end of TagRFP and 1 residue to the start of the puromycin resistance marker.	Thosea asigna virus
PuroR	Puromycin-resistant marker for selection of the transduced cells.	*Streptomyces alboniger*
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element—enhances the stability of viral transcripts.	Woodchuck hepatitis virus
ΔU3/HIV-1 truncated 3′ LTR	3' Self-inactivating long terminal repeat. Allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes (Dull et al., 1998). The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells. Required for viral reverse transcription; self-inactivating 3' LTR with deletion in U3 region prevents formation of replication-competent viral particles after integration into genomic DNA.	HIV-1
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.	SV40
SV40 Ori	Allows for episomal replication of plasmid in eukaryotic cells.	SV40
AmpR	Ampicillin resistance gene (β-lactamase) for selection of plasmid in bacterial cells.	bacterium *Salmonella paratyphi*
pUC ori	pUC bacterial origin of replication.	pUC

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