



CELLECTA



# **Cellecta Pooled Bar-Coded Lentiviral shRNA Libraries**

*HT RNAi Genetic Screens  
with DECIPHER™ or Custom Pooled shRNA Libraries*

## **User Manual**

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## A. Background

The protocols below provide the instructions on how to package, titer, and transduce target cells with DECIPHER™ or custom pooled lentiviral shRNA libraries. Also provided are examples for both positive and negative selection screens. Additional protocols provide guidelines for the preparation of bar-coded probes for high-throughput (HT) sequencing and analysis of raw sequencing data sets. Please read the entire user manual before proceeding with your experiment.

The protocols and methods apply specifically to the following DECIPHER Modules, although the protocols are scalable and can be used for smaller libraries.

Library	Target Genes	# mRNA	# shRNA	Catalog #
Human Module 1	Signaling Pathways	5,043	27,500	DHPAC-M1-P
Human Module 2	Disease-Associated	5,412	27,500	DHDAC-M2-P
Human Module 3	Cell Surface, Extracellular, DNA Binding	4,922	27,500	DHCSC-M3-P
Mouse Module 1	Signaling Pathways	4,625	27,500	DMPAC-M1-P
Mouse Module 2	Disease-Associated	4,520	27,500	DMDAC-M2-P

**IMPORTANT: DO NOT mix modules.** In our experience, the maximum number of shRNAs per library should be no more than 30K in order to avoid working with an unmanageable number of cells. Representation and data may be severely affected in larger libraries if not enough cells are used.

**NOTE:** The module names are used solely for convenience to describe the major groups of genes targeted in the module. Many genes targeted in a module do not fall within the description, all modules target a variety of genes throughout the genome, and not all genes generally considered to fall under a specific description will be found in the module with the specific gene description. Please refer to the gene lists and complete gene annotations (available on the DECIPHER website at <http://www.decipherproject.net/support/>) associated with each module for detailed information regarding which genes are present in each specific module. Also, each module targets an orthogonal set of mRNA transcripts so there is no overlap in the targets between modules.

### Designing and Performing HT RNAi Genetic Screens

Specific screening protocols will vary depending on the particular biological mechanism to be studied. For general information and examples of successful genetic screening experiments, we recommend that you refer to the **References** section. Although the specific protocol and controls may be different depending on the cell type, functional assay, and selection protocol (e.g., FACS, apoptosis induction, toxic chemical survival, etc.), it is critical to carefully design your experiment in order to generate statistically significant data. With this in mind, consider the following suggestions when setting up your experiment:

#### *Model Phenotype Selection with Positive Control shRNA Construct(s).*

Before performing a large-scale genetic screen with a pooled lentiviral shRNA library, we suggest making several shRNA constructs designed against one or more particular target genes whose inactivation is known to elicit the desirable phenotypic changes in the target cells. Then, by packaging and transducing these positive control shRNA constructs into target cells, you can optimize the protocol for enrichment/depletion of cells with induced phenotypic changes for your experiment with the shRNA library. As negative control cells, which should not be enriched/depleted during the selection steps, you can use cells infected by non-targeting shRNA constructs (e.g. against Luciferase, or a scrambled shRNA control).

#### *Optimize the enrichment/depletion protocol.*

The quality of genetic screen data will depend significantly on the design and conditions used for the phenotype-specific selection step. A high enrichment/depletion level of target cells (optimally 50-100-fold) with a specific trait will help to identify shRNA constructs that are significantly enriched/depleted above the inevitable background level of non-enriched/depleted shRNA inserts. In most cases, transduced cells can be used to start a phenotypic screen at approximately 2-3 days after infection. However, this is based on anecdotal observations and the time it usually takes the lentiviral cassette to integrate and the shRNAs to express in most cell types. For certain genes and selections, the knockdown effect may happen quickly (1 day) or take significantly longer (4-7 days) to manifest.

When performing a genetic screen experiment, make an effort to minimize the time necessary for functional selection. Extended growth of phenotypically-selected cells reduces the reproducibility of identification of functional shRNAs in triplicate cell samples due to heterogeneity of cellular pools, differences in clonal cell growth, spontaneous apoptosis, etc. (*i.e.* "genetic drift"). Based on our experience, the maximum number of cell duplications for negative selection (viability) screens should be no more than 8-10 divisions. In the case of positive selection screens with high levels of enrichment (50-100-fold) of phenotype-specific cells, grow selected cell pools up to approximately  $1 \times 10^6$  cells and use all cells for purification of genomic DNA and bar-code amplification. For positive selection screens with low levels of enrichment (3-10-fold), consider designing an experiment with two sequential rounds of enrichment and using the entire pool of second-round enriched cells for genomic DNA isolation and bar-code amplification steps.

Use Reference Control Cells.

As a control for the genetic screen, it is important to use cells infected with the shRNA library but not selected for a specific phenotype or induced (treated) by a phenotype-inducing agent, etc. There are many options that can be considered for the selection of appropriate reference control cells, depending on your biological system. This control is necessary to use as a standard to measure the relative levels of each shRNA insert species in the transduced cell population without selection. Without this control, it is difficult to determine which shRNA species are enriched/depleted in the transduced cells after the selection step.

Design the experiment with at least triplicate samples.

Due to non-optimized conditions and variations in cell number, cultivation/treatment conditions, transduction efficiency, performance of DNA purification, bar-code amplification, high-throughput sequencing, etc., you may expect some variation in shRNA-specific bar-code HT sequencing reads between each experimental sample. In order to achieve statistically significant identification of genes involved in phenotypic responses, it is necessary to design the experiment with at least triplicate samples for each population of phenotype-selected and reference control cells.

## B. Components:

### **DECIPHER shRNA Libraries:**

The following components are included with each DECIPHER shRNA Library order:

- 120 µg of each plasmid library ordered, in pRSI9-U6-(sh)-UbiC-TagRFP-2A-Puro vector; enough to generate lentivirus for approximately 50-100 screens
- 10 µg empty library vector, as a packaging and transduction control; or, after linearization by BpiI restriction digest, for cloning individual constructs used to validate hits from your screen

The target gene lists, Product Analysis Certificate (PAC), and vector map and sequence can be downloaded from the DECIPHER Project website at <http://www.decipherproject.net/support/>. The complete shRNA and bar-code sequences are available upon request.

The following DECIPHER products and services are available from Collecta at additional cost. For more information, visit [www.cellecta.com](http://www.cellecta.com), email us at [sales@cellecta.com](mailto:sales@cellecta.com), or call 877-938-3910.

<b>DECIPHER Products and Services</b>	<b>Catalog #</b>
DECIPHER Module Packaging	CLVP-27K
DECIPHER Custom Lentiviral Constructs (positive and negative controls)	many
Cloning DECIPHER Module into Standard Library Vector (custom library)	DCLN-M-P
Linearized DECIPHER Vector, 50 reactions	DVU6CLIB-L
Ready-to-Use Packaging Plasmid Mix (pC-Pack2), 250 µg	CPCP-K2A

Note on The DECIPHER Project:

Plasmid DECIPHER shRNA Libraries and vector are distributed free of charge to academic and non-profit users under a Material Transfer Agreement (MTA). The MTA and Order Form can be downloaded from <http://www.decipherproject.net/support/>.

### **Custom shRNA Libraries:**

The following deliverables are included with each Custom shRNA Library order:

- >200 µg library plasmid DNA
- Product Analysis Certificate, which includes HT Sequencing Q.C. distribution histogram and mutation rate
- List of shRNA, target, and bar-code sequences
- Library cloning vector map and sequence

Additional deliverables available:

- Pseudoviral packaged library

### **C. Required Materials:**

#### *Packaging of Library*

DECIPHER or Custom shRNA Libraries can be packaged into lentiviral particles with nearly any 2<sup>nd</sup> or 3<sup>rd</sup> generation HIV-based lentiviral packaging mix, or with Collecta's Ready-to-Use Packaging Plasmid Mix (pC-Pack2), Cat.# CPCP-K2A. Collecta can also provide libraries in ready-to-use packaged format.

#### *Materials Available Separately from Collecta or other vendors:*

- Positive control (targeting) lentiviral shRNA constructs (Custom, from Collecta; or generated by Customer)
- Negative control (non-targeting) lentiviral shRNA constructs (Custom, from Collecta; or generated by Customer)  
Linearized shRNA expression vector, for cloning individual constructs used to validate hits from your screen
- 293T/17 Cell Line (ATCC, Cat.# CRL-11268™)
- Dulbecco's Modified Eagle Medium (D-MEM) (1X) (Mediatech 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 GlutaMAX™ (Invitrogen) is used, addition of glutamine is not necessary.
- Glutamine (L-Alanyl-L-Glutamine, Dipeptide L-glutamine) (Mediatech, Cat.# 25-015-CI)
- Fetal Bovine Serum (recommended: Mediatech, Cat.# MT 35-010-CV)
- Puromycin
- D-PBS
- Trypsin-EDTA
- Polybrene® (hexadimethrine bromide) (Sigma-Aldrich, Cat.# 107689)
- 0.2-0.45 µm PES sterile syringe filters (Nalgene, Cat. # 194-2520)
- Tissue Culture Plates and Related Tissue Culture Supplies
- Lipofectamine™ Reagent (Invitrogen, Cat. # 18324-111)
- Plus™ Reagent (Invitrogen, Cat. # 11514-015)
- 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 shRNA inserts in control shRNA constructs (IDT)\*: See **Appendix**
- PCR primers for bar-code amplification from genomic DNA (IDT)\*: See **Appendix**
- HT sequencing primers (IDT)\*: See **Appendix**
- HT Sequencing Kits (Illumina):

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

**Related Services from Collecta**

- Custom Pooled shRNA Library Construction, Cat.# (many)
- RNAi Functional Genetic Screens with Pooled shRNA Libraries, Cat.# CRGS-X
- HT Bar-Code Sequencing of Cell Pellets from Genetic Screen (screening done with Collecta Library), Cat.# CANA-SQ
- DECIPHER Custom Constructs, Cat.# (many)
- Design and Cloning of 3 shRNA using Validated Algorithm, Cat.# CVSHC-FVP, -FVV
- Library Control Constructs and Vectors, Cat.# (many)
- Custom Peptide Libraries, Cat.# (many)

## D. Packaging Protocol for DECIPHER Pooled Lentiviral shRNA Libraries

The following protocol describes the generation of a packaged DECIPHER pooled lentiviral 27K shRNA library (27K shRNA complexity) using Invitrogen's Lipofectamine™ and Plus™ Reagent (see **Required Materials**). Other transfection reagents may be used, but the protocol should be adjusted to fit the manufacturer's protocol. The yield of recombinant lentiviral particles typically produced under these optimized conditions is  $1-10 \times 10^6$  TU/ml. In this protocol, using ten (10) 15-cm plates, at least  $3 \times 10^8$  TU of total pseudoviral particles can be made and then concentrated to up to 100-fold using several described methods.

1. Start growing 293T cells in D-MEM medium plus glutamine (see **Required Materials**), supplemented with 10% FBS without antibiotics, 2 to 3 days prior to transfection.

### Day 1 – Plate Cells

2. Twenty four (24) hours prior to transfection, plate  $12.5 \times 10^6$  293T cells in each of ten (10) untreated 15-cm plates (or 150 cm<sup>2</sup> 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 ~70%-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<sub>2</sub> incubator for 24 hours.

### Day 2 – Transfection

3. For each of the 10 plates, in sterile 14-ml polypropylene tubes, mix 60 µl (30 µg) of Packaging plasmid mix with 6 µg\* of the plasmid library and add the plasmid mixture to 1,200 µl D-MEM medium without serum or antibiotics. Add 60 µl of Plus Reagent, mix, and incubate at room temperature for 15 min.

per plate	Component
60 µl	Packaging plasmid mix (0.5 µg/µl)
6 µl	Plasmid shRNA Library (1 µg/µl) *
1,200 µl	D-MEM, no FBS, no antibiotics
60 µl	Plus Reagent
1,326 µl	Total volume

**\* IMPORTANT: DO NOT use less than 60 µg (ten 15-cm plates) to package a batch of DECIPHER library. A smaller amount may cause shRNA insert representation to be adversely affected.**

4. Add 990 µl of Lipofectamine Reagent to 13.2 ml of D-MEM medium without serum or antibiotics in order to make a convenient master mix. Mix gently.

1 plate	10 plates (11X*)	Component
1,200 µl	13,200 µl	D-MEM, no FBS, no antibiotics
90 µl	990 µl	Lipofectamine
1,290 µl	14,190 µl	Total volume

\* NOTE: Always add 1 or 2 extra reactions to avoid running out of master mix.

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 2.616 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<sub>2</sub> incubator for 24 hours.

### Day 3 – DNase Treatment (Optional)

7. At 24 hours post-transfection, replace the medium containing complexes with fresh D-MEM medium supplemented with 10% FBS, DNase I (1 U/ml), and MgCl<sub>2</sub> (4 mM). Continue incubation in the CO<sub>2</sub> incubator at 37°C for 24 hours. Overnight DNase I treatment before harvesting pseudovirus does not negatively affect viral titer or infectivity and helps prevent undesirable carryover of plasmid library into the pseudovirus prep.

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

#### Day 4 – Collect Pseudoviral Supernatant

8. At 48 hours post-transfection, collect all 30 ml of the pseudovirus-containing medium from each plate and filter the supernatant (300 ml) through a Nalgene 0.2 – 0.45  $\mu\text{m}$  PES filter (a low protein binding filter) to remove debris and floating packaging cells. Failure to filter supernatant could result in carry-over of cells into your pseudoviral prep.

**Note:** Usually, the peak of pseudovirus production is achieved at 48 hours post-transfection. We recommend collecting the supernatant only once, at 48 hours post-transfection, in order to achieve higher titers. Supernatant can also be collected at 72 hours post-transfection—replace the collected 48-hour supernatant with 30 ml of fresh D-MEM medium supplemented with 10% FBS and continue incubation in the  $\text{CO}_2$  incubator at  $37^\circ\text{C}$  for 24 hours.

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

9. Proceed to concentration step, or aliquot and store the non-concentrated supernatant at  $-80^\circ\text{C}$ . Freezing and thawing may result in 10 – 20% loss of pseudoviral titer with each cycle.

#### Concentrating Pseudovirus (Optional)

Although concentrating pseudovirus is optional, it is recommended if (1) very high titer pseudovirus stock may be needed to achieve desired MOI in hard-to-infect target cells or (2) pseudovirus should be suspended in another media (besides the standard PBS/10%FBS) which is optimal for sensitive target cells. However, because of the additional manipulation of samples, there is the added risk of contamination and loss of pseudovirus.

The following protocol was optimized to concentrate pseudovirus with high recovery. The protocol assumes that pseudoviral supernatant was harvested 48 hours after transfection and filtered as in step 8 above.

1. Aliquot pseudoviral supernatant in clear centrifuge tubes.
2. Add Polybrene to a final concentration of 5  $\mu\text{g}/\text{ml}$ , and incubate for 1 hour at  $+4^\circ\text{C}$ .
3. Centrifuge at 10,000 rpm for 1 hour at  $+4^\circ\text{C}$  in a Beckman JA-14 (or JA-10) or equivalent rotor. 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 tube on ice, resuspend the (in)visible pellet in PBS/10%FBS, make aliquots, and freeze at  $-80^\circ\text{C}$ .

Alternatively, you may concentrate pseudovirus by the any of the methods below. However, the yield of pseudovirus is superior ( $\sim 80\%$  recovery) using Collecta's protocol above.

- Ultracentrifugation at 50,000 x g for 90 min at  $+4^\circ\text{C}$
- Sucrose cushion ultracentrifugation
- PEG precipitation, followed by low speed centrifugation

## E. Pseudoviral Titer Estimation

DECIPHER lentiviral shRNA vectors all express both TagRFP and Puromycin Resistance markers. Titters are calculated based on the percentage of either RFP-positive or Puromycin-resistant cells.

We recommend that you estimate the titer of the pseudovirus-containing supernatant before proceeding with transduction experiments for the following reasons:

- To ensure that pseudoviral stock is viable
- To determine the amount of pseudoviral particles (transduction units (TU)) necessary to achieve the desirable percentage of transduced target cells (multiplicity of infection (MOI))
- To control the number of copies of integrated pseudoviral constructs per target cell

To check pseudoviral titer, we recommend choosing a cell line appropriate for your experimental system. 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.

### Check Toxicity of Polybrene®

Polybrene is a polycation that neutralizes charge interactions to increase binding between the pseudoviral envelope and the cellular 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, 2.5 µg/ml, 5 µg/ml) for 24 hours, and then replace old medium with Polybrene-free complete culture medium. Grow cells for an additional 48 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.

### Transduction Protocol For Titering in HEK293 cells.

Please read the entire protocol before beginning your experiment. For other plate formats, the volumes should be adjusted depending on the growth area of the well or plate.

**NOTE:** We highly recommend also titering the library directly in your target cells prior to beginning your experiment. This ensures the greatest accuracy of MOI and reproducibility of transduction. Use of positive transduction controls (as is the common practice in the protocols of other vendors) is not recommended, because of batch-to-batch variation due to transduction conditions and quality of cells at that time.

#### Day 1.

1. Quickly thaw the pseudoviral 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 pseudoviral particles in the laminar flow hood.

**Note:** Unused pseudoviral stock may be refrozen at -80°C, but it will result in a loss of about 10 – 20% in titer.

2. Trypsinize and resuspend cells to a density of  $1 \times 10^5$  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, 1 µl, 3.3 µl, 10 µl, 33 µl, and 100 µl pseudoviral stock, prepared by serial dilution, to six different wells. Mix and return cells to CO<sub>2</sub> incubator. Grow cells under standard conditions for 24 hours.

**\*Note:** Be sure to record the **original** # of cells at Time of Infection, as this is critical in titer calculation.

#### 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<sub>2</sub> incubator, and grow under standard conditions for 24 hours.

### Day 3. (48 hours after transduction)

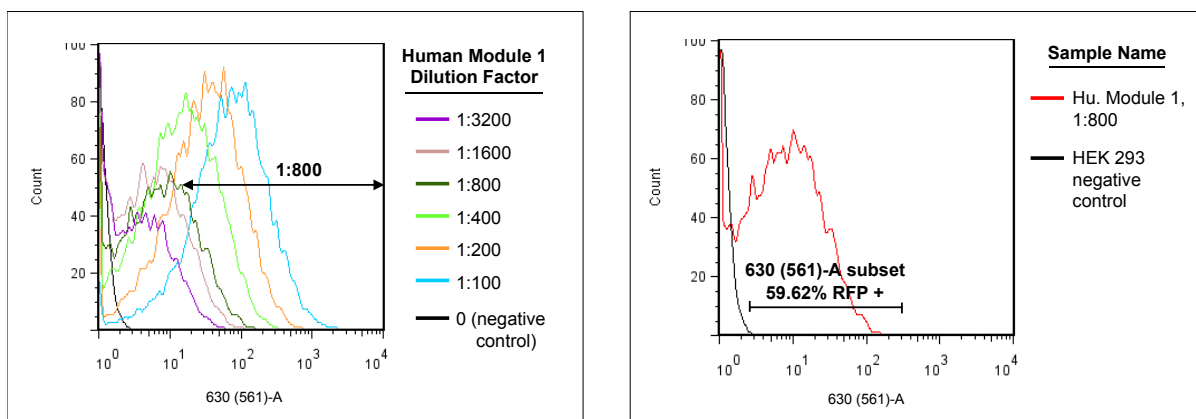
#### Fluorescence-based titering:

Transduced cells express RFP reporter protein from an internal UbiC promoter. By calculating the percentage of RFP-positive cells using flow cytometry, you can determine the pseudoviral titer. Detach cells from the plate by trypsin treatment, centrifuge, resuspend in 1X D-PBS at approximately  $10 \times 10^6$  cells/ml, and determine the percentage of transduced (RFP-positive) cells by flow cytometry. In order to set up a gate for counting RFP-positive cells, first analyze the background (RFP) level of control non-transduced cells (0  $\mu$ l of pseudoviral stock). Based on the percentage of transduced cells and the volume of pseudoviral stock used, calculate the original concentration of infective pseudoviral particles in the pseudoviral stock (transduction units) per ml (TU/ml).

**Note:** Depending on cell type, you may need to wait 72 hours after transduction before estimating titer by RFP fluorescence. For FACS Analysis of TagRFP, set excitation to 530 or 561 nm and emission between 580-600 nm.

#### Example of Titer Calculation:

FACS Analysis was performed on HEK 293 cells transduced with several dilutions of the DECIPHER Human Module 1 pseudoviral stock. Titer was calculated from a dilution that resulted in approximately 60% infected cells.



FACS Analysis of TagRFP for cells infected with varying dilutions of packaged DECIPHER library.

#### Formulas for determining titer and total TU:

$$(\text{Cell \# at Time of Infection}) \times (\text{Dilution factor}) \times (\% \text{ Positive cells}) / (\text{Volume of inoculum}) = \text{TU / Unit volume}$$

$$\text{TU / Unit volume} \times \text{Total Lentiviral Library volume} = \text{TOTAL TU}$$

#### Example Calculations:

$$(1.0 \times 10^5 \text{ cells}) \times (800) \times (.5962) / (0.3 \text{ ml}) = \mathbf{3.18 \times 10^8 \text{ TU/ml}}$$
 for HEK 293 target cells

$$3.18 \times 10^8 \text{ TU/ml} \times 1.5 \text{ ml Total Lentiviral Library volume} = \mathbf{4.77 \times 10^8 \text{ TU}}$$

#### Fluorescence Microscopy titering (NOT recommended):

You may also visualize the cells for RFP fluorescence by microscopy, but the results **will be inaccurate** due to lower detection sensitivity compared to flow cytometry.

#### Puromycin-based titering:

At 48 hours after infection, split cells into two samples. Grow one sample with antibiotic selection, and one sample without (**NOTE:** Before performing the following experiment, we recommend to first determine the optimal concentration of antibiotic using the **Puromycin Kill Curve** protocol below).

After 48 hours of growth in puromycin, count the number of viable cells in the selected and unselected samples. The ratio of selected/unselected viable cells gives the percentage of infected cells. Then, calculate relative pseudoviral titer as previously described. Please note that the titer determined by Puromycin selection may differ from the titer determined by counting RFP-positive cells using flow cytometry, and it also depends on cell type and selection conditions.

**Note:** Depending on cell type, you may need to wait 72 hours after transduction before estimating titer by Puromycin selection.

### **Puromycin Kill Curve**

In order to generate a purely transduced population of cells, it is important to determine the minimum amount of puromycin required to kill untransduced cells. This can be done empirically by generating a kill curve, as follows:

Trypsinize and resuspend cells to a density of  $1 \times 10^5$  cells/ml in growth media, aliquot 1 ml per well in a 12-well plate, and add puromycin at 0  $\mu\text{g/ml}$ , 0.5  $\mu\text{g/ml}$ , 1  $\mu\text{g/ml}$ , 2  $\mu\text{g/ml}$ , 5  $\mu\text{g/ml}$ , and 10  $\mu\text{g/ml}$  in six different wells. Mix and return cells to incubator. Grow cells under standard conditions for 48 hours.

For puromycin, the minimum antibiotic concentration to use is the lowest concentration that kills 100% of cells in 48-72 hours.

## F. Protocols for Genetic Screens with DECIPHER Pooled shRNA Libraries

To ensure reproducible and reliable results when using pooled shRNA libraries, it is critical that you infect enough cells to maintain sufficient representation of each shRNA construct present in the cellular library. The number of cells stably transduced with the shRNA library at the time of infection should exceed the complexity of the shRNA library by about 200-fold or optimally 1,000-fold. After infection, it would be best never to discard any cells at any time during the experiment (e.g. at splitting steps). If the number of cells is too high to grow and you are forced to discard a fraction of the cells, **the number of remaining cells should always exceed the complexity of the library by at least 1,000-fold (e.g. keep at least  $50 \times 10^6$  cells after splitting step).**

Additionally, when using pooled shRNA libraries, you should consider that the higher the percentage of transduced cells and MOI, the higher the percentage of infected cells that will bear two or more different shRNA constructs (see Tables 1 and 2 below). For most RNAi screens, we recommend optimizing transduction conditions and starting your experiment with 25-50% transduced cells.

**NOTE:** We highly recommend titrating the library directly in your target cells prior to beginning your experiment. This ensures the greatest accuracy of MOI and reproducibility of transduction. Use of positive transduction controls (as is the common practice in the protocols of other vendors) is not recommended because of batch-to-batch variation due to transduction conditions and quality of cells at that time.

% transduced cells:	10	20	30	40	50	60	70	80	90	>90*
MOI:	0.1	0.23	0.36	0.51	0.7	0.93	1.22	1.64	2.3	>2.5*

\* MOI cannot be reliably calculated if % of transduced cells is >90%.

**Table 1. Conversion of % transduced cells to MOI.**

MOI	Percentage of cells with 0, 1, 2, 3, or 4 integrants				
	0	1	2	3	4
0.1	90	9	0	0	0
0.2	82	16	2	0	0
0.3	74	22	3	0	0
0.4	67	27	5	1	0
0.5	61	30	8	1	0
0.6	55	33	10	2	0
0.7	50	35	12	3	0
0.8	45	36	14	4	1
0.9	41	37	16	5	1
1.0	37	37	18	6	2

**Table 2. Number of Integrations based on Poisson Distribution,** showing the expected number of shRNA integrants per cell at different MOIs. At a specific MOI, the number of cells having 0, 1, 2, 3, or 4 pseudoviral integrants per cell is listed. For example, at a MOI of 0.3, an estimated 12% (3 of 25) of the *infected* cells have more than one pseudoviral integrant. At an MOI of 0.8, about 34% (19 of 55) have more than one integrant.

## G. Examples of HT RNAi Screens

### **Positive Selection Screen:**

#### **Identification of shRNAs conferring resistance to TGF- $\beta$ mediated apoptosis in Human Hepatocellular Carcinoma Hep3B cells (treatment is TGF- $\beta$ ).**

A pooled lentiviral shRNA library of 27K complexity was used. Six independent transductions were performed. Each transduction consisted of  $1.5 \times 10^7$  cells infected at 35% efficiency ( $\sim 5.3 \times 10^6$  infected cells). Each transduction was treated as an independent sample. Each independent sample had an estimated average of  $\sim 200$  transduced clones per shRNA.

#### **Day 1.**

Cells were trypsinized and resuspended to a density of  $1 \times 10^5$  cells/ml in D-MEM supplemented with 10% FBS and 5  $\mu$ g/ml Polybrene. 25 ml of cells were aliquoted to each 15-cm plate (6 plates per replicate,  $1.5 \times 10^7$  cells per replicate), and enough pseudovirus was added to achieve  $\sim 9 \times 10^5$  infected cells per plate. Cells were returned to CO<sub>2</sub> incubator and grown under standard conditions for 24 hours.

#### **Day 2.**

At 18 hours post-transduction, media containing pseudovirus/Polybrene was replaced with fresh media (without Polybrene).

#### **Day 4.**

At 72 hours post-transduction, three (3) samples were harvested and stored as frozen cell pellets (untreated samples). Three cell samples were treated with DMEM media supplemented with TGF- $\beta$  (1 ng/ml) to induce apoptosis.

#### **Day 14.**

Cells that survived apoptosis were trypsinized, harvested, and centrifuged, and each sample was stored as a frozen cell pellet (TGF- $\beta$  treated samples).

Genomic DNA was then extracted and purified from the all 6 cell populations (both TGF- $\beta$  treated and untreated samples). shRNA-specific bar-codes were amplified from the entire amount of isolated genomic DNA (20-100  $\mu$ g) and enumerated by HT sequencing.

### **Negative Selection Screen:**

#### **Identification of genes essential for viability of AR-1 negative Human Prostate Cancer DU145 cells (treatment is time).**

The DECIPHER Human Module 1 pooled lentiviral shRNA library of 27K complexity was used. Six independent transductions were performed. Each transduction consisted of  $2 \times 10^7$  cells infected at 30% efficiency ( $\sim 6 \times 10^6$  infected cells). Each transduction was treated as an independent sample. Each independent sample had an estimated average of  $\sim 200$  clones per shRNA.

#### **Day 1.**

Cells were trypsinized and resuspended to a density of  $2 \times 10^5$  cells/ml in D-MEM supplemented with 10% FBS and 5  $\mu$ g/ml Polybrene. 25 ml of cells were aliquoted to each 15-cm plate (4 plates per replicate,  $2 \times 10^7$  cells per replicate), and enough pseudovirus was added to achieve  $\sim 1.5 \times 10^6$  infected cells per plate ( $\sim 6 \times 10^6$  cells per replicate). Cells were returned to CO<sub>2</sub> incubator and grown under standard conditions for 24 hours.

#### **Day 2.**

At 24 hours post-transduction, media containing pseudovirus/Polybrene was replaced with fresh media (without Polybrene).

#### **Day 3.**

At 48 hours post-transduction, three (3) samples were harvested and stored as frozen cell pellets (baseline samples). Puromycin was added to the three (3) remaining samples at a final concentration

of 1 µg/ml (or the concentration determined empirically by the Puromycin Kill Curve under **Pseudoviral Titer Estimation** above).

### Day 9.

Time-treated samples were harvested and stored as frozen cell pellets.

Genomic DNA was then extracted and purified from treated and untreated samples. shRNA insert bar-codes were amplified from genomic DNA and enumerated by HT sequencing.

## H. Genomic DNA Extraction for Bar-Code Amplification and HT Sequencing

Identification of shRNA bar-codes in the experimental samples requires amplification of the bar-code portion of the integrated lentiviral constructs from sample genomic DNA. Subsequent high-throughput sequencing of bar-codes by the Illumina GAIx or HiSeq is done to quantify each bar-code and generate digital expression data using Deconvolution software.

### Recommended Protocol – Using Sonicator or Ultrasonic Homogenizer

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

1. Resuspend cell pellet in 5 ml QIAGEN Buffer P1 supplemented with 100 µg/ml RNase A (see **Required Materials**), in a 15 ml screw-cap disposable **polypropylene** (phenol/chloroform resistant) centrifuge tube. If the number of cells in the sample is less than  $10^7$  cells, use only 1 ml of Buffer P1/RNase A buffer in 2 ml microfuge tubes.
2. Add 1/20 volume of 10% SDS, mix, and incubate 5 minutes at RT.
3. Using an ultrasonic homogenizer (see **Required Materials**), sonicate to shear DNA into ~10kb size fragments (approximately 10 seconds at high power). To prevent cross-contamination, thoroughly wash the ultrasound head with running water and dry-up with clean paper towel between samples. Alternatively, for smaller volumes, shear genomic DNA by passing cell lysate 5-10 times through a 22-gauge syringe needle.
4. Add 1 volume of phenol:chloroform pH 8.0 solution, vortex vigorously for 60 seconds, and centrifuge at 3,000 rpm for 30 minutes at RT (or 12,000 rpm for 5 minutes if using 2 ml microfuge tubes).

**NOTE:** You may skip steps 5 and 6 below (and go directly to step 7) if you centrifuge for 2 hours instead of 30 minutes at RT (or 20 minutes if using 2 ml tubes).

5. At this point, you may have a thin or very thick interphase. Do not worry about interphase contamination: take the whole upper phase + most of the interphase and transfer it into a new screw-cap disposable **polypropylene** (phenol/chloroform resistant) centrifuge tube. Any interphase contamination will be cleaned up in the next step.
6. Add 1 volume of chloroform, vortex vigorously for 60 seconds, and centrifuge again as above.
7. Now you should have a very thin and clean interphase. Transfer the upper phase to a new disposable centrifuge tube.
8. Add 0.1 volumes of 3M NaOAc, 0.8 volumes of isopropanol, and mix well. Centrifuge at 3,000 rpm for 30 minutes at RT (or 12,000 rpm for 10 minutes if using 2 ml microfuge tubes).
9. Wash pellet once with 70% ethanol. Centrifuge and air-dry pellet.
10. Dissolve DNA pellet in distilled water. If necessary, heat solution at 70°C to dissolve DNA precipitate. For the following amplification step, the optimal concentration of sonicated DNA is 5 µg/µl.

## I. Amplification of shRNA-specific Bar-Codes from Genomic DNA

The pooled bar-codes should be amplified from 200 µg of genomic DNA (isolated from cell samples in the previous step) by two rounds of PCR using Titanium Taq DNA polymerase mix (Clontech-Takara, see **Required Materials**). Use the entire amount of genomic DNA and a proportionally fewer number of 100-µl reactions per sample when amplifying bar-codes from samples generated by positive selection screens. The protocol was optimized using an ABI GeneAmp PCR System 9700. Use of other PCR enzymes and/or thermal cyclers may require additional optimization.

The lentiviral shRNA 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.

Please use 10 ng of plasmid shRNA library as an amplification control in the first round of PCR, and use the subsequent PCR products in all remaining steps.

### First Round of PCR

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

___ µl	Genomic DNA (50 µg)
3 µl	FwdHTS primer (10 µM)
3 µl	RevHTS primer (10 µM)
2 µl	50X dNTP (10 mM each)
10 µl	10X Titanium Taq Buffer
___ µl	Deionized water
1 µl	50X Titanium Taq
<hr/>	
100 µl	Total volume

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

### 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. Amplify each DNA sample with the FwdGex and RevGex primer set and perform HT sequencing on one sample per lane (in the flow cell) with the GexSeqN primer.

1. Combine together the 4 x 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	FwdGex primer (10 µM)
5 µl	RevGex primer (10 µM)
2 µl	50X dNTP (10 mM each)
10 µl	10X Titanium Taq Buffer
75 µl	Deionized water
1 µl	50X Titanium Taq
<hr/>	
100 µl	Total volume

94°C, 3 minutes	}	1 cycle
94°C, 30 seconds		<b>10, 12, or 14</b> cycles
65°C, 10 seconds		
72°C, 10 seconds		
68°C, 2 min		1 cycle

**NOTE:** Avoid overcycling of PCR reactions—this will usually result in the generation of a longer 130-150-bp band that corresponds to a fusion double bar-code product.

The amplified pooled bar-code cassettes are then analyzed on a 3.5% agarose-1XTAE gel. The results should reveal 106-bp amplified bar-code products. 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 106-bp bar-code band across all DNA samples from the genetic screen.

Repeat second-round amplification of bar-codes from each sample using the optimized volume of First Round PCR product, 3 x 100 µl of Second Round PCR product per sample, and 12-14 cycles of PCR. Set up 3 x 100 µl reactions for each sample containing an adjusted "equal" amount of First Round PCR product:

2 µl	First Round PCR Product
5 µl	FwdGex primer (10 µM)
5 µl	RevGex primer (10 µM)
2 µl	50X dNTP (10 mM each)
10 µl	10X Titanium Taq Buffer
75 µl	Deionized water
1 µl	50X Titanium Taq
<hr/>	
100 µl	Total volume

94°C, 3 minutes	}	1 cycle
94°C, 30 seconds		<b>12 or 14</b> cycles
65°C, 10 seconds		
72°C, 10 seconds		
68°C, 2 min	1 cycle	

Analyze the PCR products by gel-electrophoresis on a 3.5% agarose-1XTAE gel in order to ensure equal yields of amplified bar-codes for all samples. Combine amplified bar-codes from the 3 x 100 µl Second Round PCR reactions and purify the samples as follows:

1. Purify the PCR product with the 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).

## **J. HT sequencing of Pooled shRNA-specific Bar-codes on Illumina's GAIIX or HiSeq HT Sequencing Platform**

HT sequencing of pooled amplified bar-codes can be performed on the Illumina GAIIX (~20-30 x 10<sup>6</sup> reads per sample) or HiSeq (~80-100 x 10<sup>6</sup> reads per sample) using the GexSeqN sequencing primer and following the manufacturer's protocol. The final concentration of GexSeqN primer in the reaction should be 500 nM. For the cluster generation step, use 10 pmol of the purified library. The number of cycles (read length) required depends on the length of the bar-code and whether there is an additional sequence, e.g. the 2-nt DECIPHER "Module Identifier" (see **Appendix**). For the DECIPHER libraries, the minimum number of cycles required is 20 (18nt bar-code + 2nt identifier).

The shRNA 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 DECIPHER Library samples.

Please contact us at [sales@collecta.com](mailto:sales@collecta.com) for information on our HT sequencing and data analysis services.

## **K. Bar-code Enumeration (Conversion of raw sequencing data to number of reads for each bar-code)**

For DECIPHER shRNA Libraries, step-by-step protocols for bar-code deconvolution and enumeration are included with the downloadable software available at <http://www.decipherproject.net/software/>. For Custom shRNA Libraries, please contact Collecta Technical Support at [tech@collecta.com](mailto:tech@collecta.com).

## **L. Statistical Analysis of shRNA hits enriched/depleted in genetic screen**

Please contact Collecta Technical Support at [tech@collecta.com](mailto:tech@collecta.com) for assistance.

## M. Troubleshooting

### **Low Pseudoviral Titer (<10<sup>6</sup> TU/ml in supernatant)**

#### **1. Poor transfection efficiency**

*Problem:* 293T Cells have too high or too low density

*Solution:* Plate fewer or more cells in order to have about 70%-80% confluency at transfection stage.

*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 pseudovirus**

*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:* Pseudoviral supernatant harvested too early or too late

*Solution:* Harvest supernatant 48 hours after transfection.

### **Inefficient Transduction of Packaged shRNA Library**

#### **1. Poor infection efficiency**

*Problem:* Target cells have too high or too low density

*Solution:* Plate fewer or more cells in order to have about 50% confluency at infection stage.

*Problem:* Target cell line may be difficult to transduce

*Solutions:*

- Use a higher concentration of pseudoviral particles.
- Optimize the transduction protocol and use HEK293 cell line as positive control cells.
- Perform "Spinoculation" to improve transduction efficiency (email us at [tech@cellecta.com](mailto:tech@cellecta.com) for protocol).
- Check to see if Polybrene was added at 5 µg/ml.

*Problem:* Wrong amount of Polybrene added during infection stage

*Solution:* If Polybrene is toxic to the target cells, optimize Polybrene concentration in the range of 0 – 5 µg/ml.

*Problem:* Loss of pseudoviral titer during storage

*Solution:* Ensure storage of aliquoted packaged shRNA library at –80°C. Each freeze-thaw cycle causes reduction of the titer by 10 – 20%. Use a fresh stock for transduction.

*Problem:* The assay is performed too early

*Solution:* Normally, the maximal expression of integrated provirus is expected to develop by 48-72 hours after infection. However, some cells exhibit delayed expression. Try the assay at a later time, such as 96 hours.

#### **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:* Pseudovirus-containing conditioned media is toxic to target cells.

*Solution:* Concentrate and resuspend the pseudovirus in target cell growth media.

*Problem:* Pseudovirus itself is toxic to target cells (Polybrene and/or conditioned media is not toxic).

*Solutions:*

- Decrease the incubation time of pseudovirus with target cells.
- Perform two sequential transductions, with short incubation times.

### **3. No expression of RFP or Puro<sup>R</sup> (or shRNAs) in target cells**

*Problem:* The UbiC or U6 promoter is not functional in target cells.

*Solutions:*

- Change the target cells.
- Replace the ineffective promoter(s) with EF1, CMV, or PGK (for marker expression), and/or H1 (for shRNA expression).
- Contact Collecta at [tech@collecta.com](mailto:tech@collecta.com) to have the library cloned in another vector with different promoter(s).

## **Difficulties with Probe Preparation and HT Sequencing**

### **1. No PCR Product**

*Problem:* Incorrect primers or bad reagents used, or missing reagents.

*Solution:* 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.

### **2. No bar-codes 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 GexSeqN Sequencing primer (see **Required Materials**), 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**).

## N. Technical Support

### DECIPHER Project Users:

For help with using the DECIPHER Pooled Lentiviral shRNA Library, please email technical support at [support@decipherproject.net](mailto:support@decipherproject.net) with the answers to the questions below (if applicable).

#### Library Used:

1. Which library did you use, and which Module(s)?
2. What are the lot numbers?

#### Packaging the Library:

3. What was the pseudoviral titer, and what was the total number of TU packaged?
4. How was the pseudovirus concentrated? (*if applicable*)

#### Transducing Target Cells:

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

#### RNAi Screen:

10. Could you briefly explain your experiment?
11. How many infected cells were used?

#### Sample Preparation & HT Sequencing

12. What HT sequencing system and which Illumina HT Sequencing Kits did you use?
13. How much PCR product was used for HT Sequencing?
14. How many sequences were read per sample?
15. Would you be able to send us the raw data so that it may help us diagnose the issue?

### **All Users:**

For additional information or technical assistance, please refer to the questions above and contact us by phone or email:

Phone: +1 (650) 938-3910  
Toll-Free: +1 (877) 938-3910  
Fax: +1 (650) 938-3911

#### E-mail:

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

Blog: <http://www.cellecta.com/blog/>

Postal Mail: Collecta, Inc.  
320 Logue Ave.  
Mountain View, CA 94043

## O. 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 viral 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 pseudoviral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.
- Pseudoviral 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 viral 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/bmb14/bmb14s3.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 pseudoviral 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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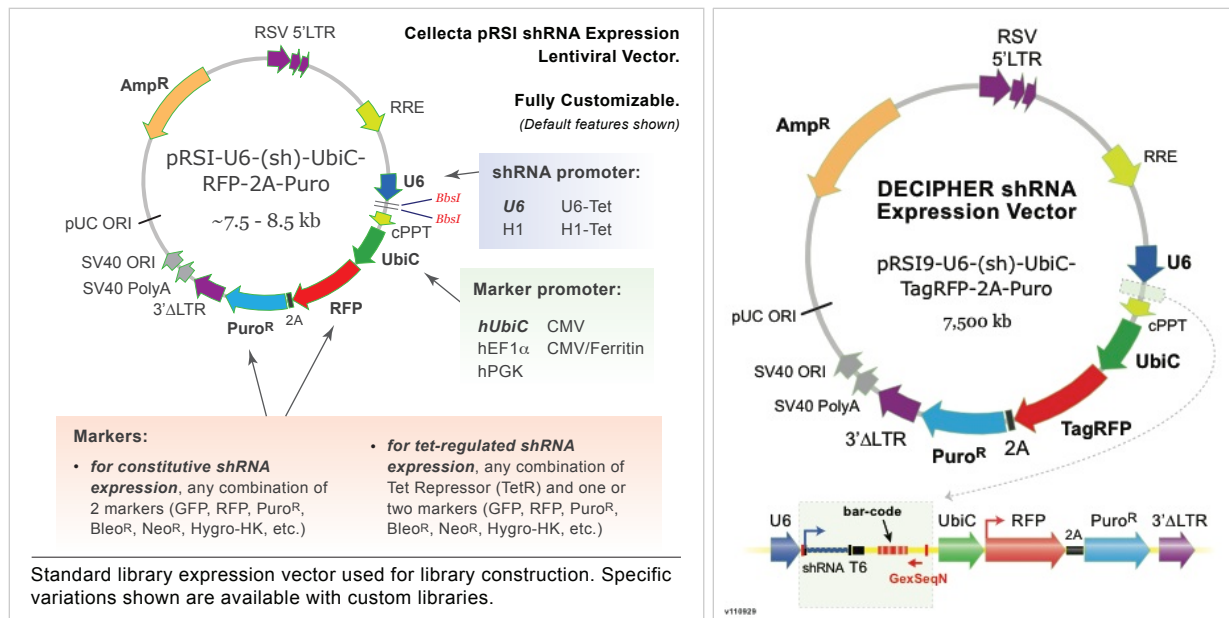
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#### **Lentiviral delivery vector reviews:**

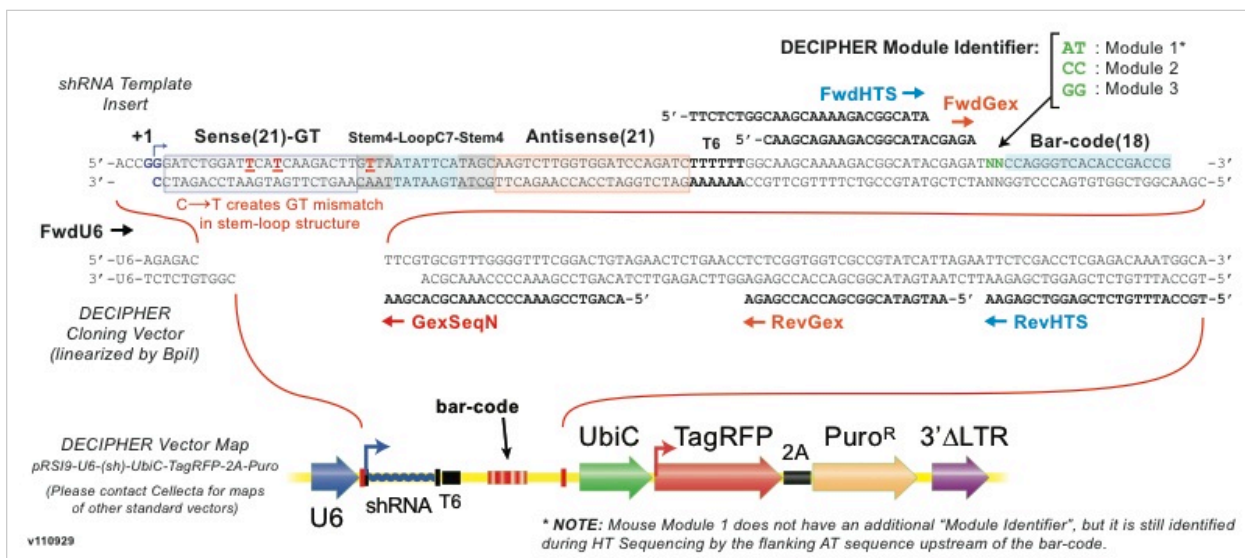
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## Q. Appendix

### 1. Lentiviral shRNA Expression Vector Maps\* (Standard, Customizable, and DECIPHER)



### 2. DECIPHER pRSI9 U6 shRNA Expression Cassette (may vary in other vectors – contact Collecta)



Primer Name	Use	Sequence (IDT preferred)
<b>FwdHTS</b> (was FwdHTS2)	1 <sup>st</sup> Round	5' -TCTCTGGCAAGCAAAGACGGCATA-3'
<b>RevHTS</b> (was RevcPPT-5)	1 <sup>st</sup> Round	5' -TGCCATTTGTCTCGAGGTTCGAGAA-3'
<b>FwdGex</b> (was Gex1MS)	2 <sup>nd</sup> Round	5' -CAAGCAGAAGACGGCATAACGAGA-3'
<b>RevGex</b> (was Gex2M)	2 <sup>nd</sup> Round	5' -AATGATACGGCGACCACCGAGA-3'
<b>GexSeqN</b>	HT Sequencing	5' -ACAGTCCGAAACCCCAAACGCACGAA-3' (HPLC Purified)
<b>FwdU6</b> (was Fwd-U6-1)	Standard sequencing	5' -CAAGCTGTTAGAGATAAATTGGAA-3'

\* All Collecta lentiviral vectors, including the DECIPHER vectors, are covered by a lentiviral expression system license owned by Life Technologies Corporation (LTC). See **Terms and Conditions**. The complete sequence of the DECIPHER Library vector can be downloaded at <http://www.decipherproject.net/support/>.

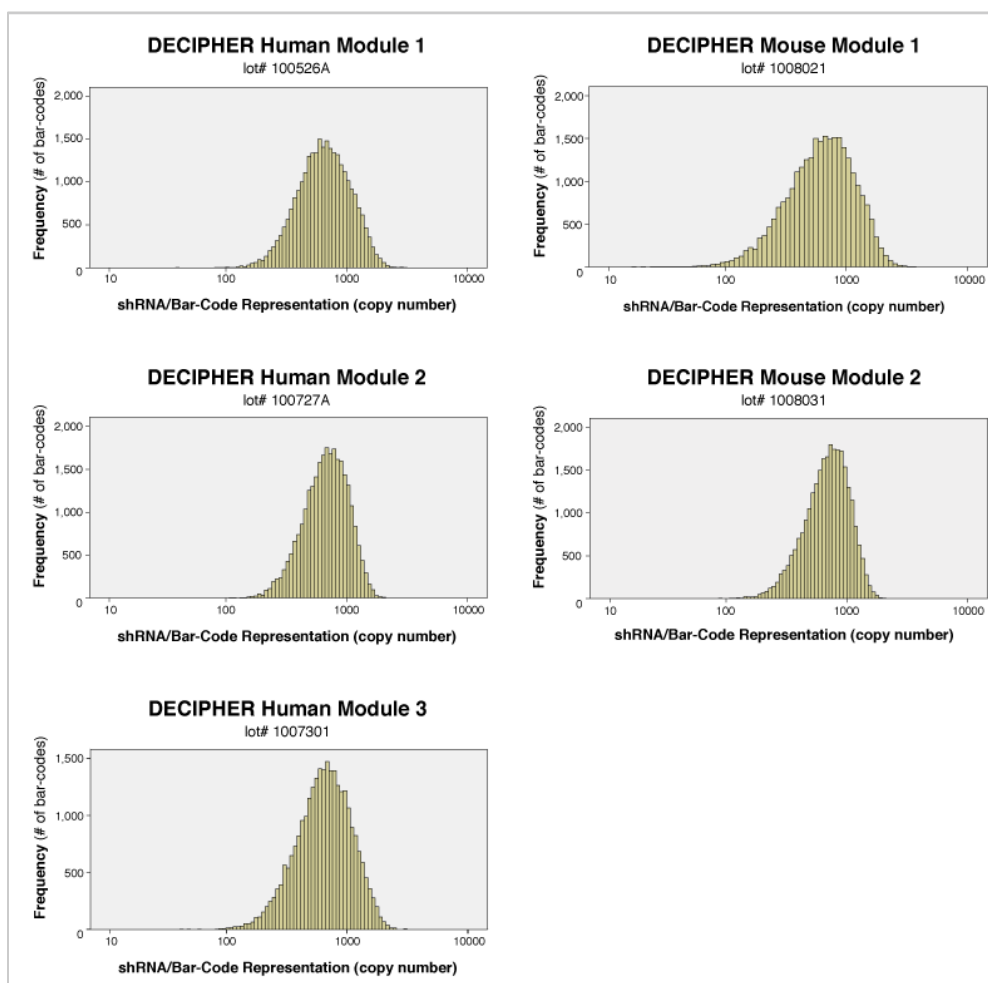
### 3. DECIPHER Vector Features

Feature	Location*	Function
Rous Sarcoma Virus (RSV) enhancer/promoter	7 - 233	Allows Tat-independent production of viral mRNA (Dull et al., 1998).
HIV-1 truncated 5' LTR	233 - 413	Permits viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).
HIV-1 psi ( $\psi$ ) packaging signal	569 - 922	Allows viral packaging (Luciw, 1996).
HIV-1 Rev response element (RRE)	1072 - 1370	Permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems et al., 1991; Malim et al., 1989).
U6	1803 - 2064	Human U6 promoter drives RNA Polymerase III transcription for generation of shRNA transcripts.
HIV-1 cPPT	2194 - 2311	Central polypurine tract, cPPT, improves transduction efficiency by facilitating nuclear import of the vector's preintegration complex in the transduced cells.
UbiC promoter	2360 - 2758	Human Ubiquitin C promoter drives expression of TagRFP and PuroR
TagRFP	2772 - 3482	TagRFP fluorescent protein (Evrogen) serves as an indicator of successful transduction (origin: sea anemone <i>Entacmaea quadricolor</i> )
T2A	3489 - 3542	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
PuroR	3549 - 4145	Puromycin-resistant marker for selection of the transduced cells (origin: <i>Streptomyces alboniger</i> )
$\Delta$ U3/HIV-1 truncated 3' LTR	4176 - 4489	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
SV40 polyadenylation signal	4546 - 4677	Allows transcription termination and polyadenylation of mRNA.
SV40 Ori	4707 - 4853	Allows for episomal replication of plasmid in eukaryotic cells
AmpR	6036 - 6896 (c)	Ampicillin resistance gene ( $\beta$ -lactamase) for selection of plasmid in bacterial cells (origin: <i>Salmonella paratyphi</i> )
pUC ori	5277 (c)	pUC bacterial origin of replication.

**Distance from 5'LTR to 3' $\Delta$ LTR: 4,483 bp**

\* (c): element on complementary strand

#### 4. DECIPHER Library HT Sequencing Q.C. Data



#### 5. DECIPHER Library Individual Clone Sequencing Q.C. Data

Approximately 200 random clones picked from the Plasmid DECIPHER pooled shRNA libraries were analyzed (40 per module x 5 modules). Of the 200 clones, 96 were sequenced.

The lots tested were:

Human Module 1, lot# 100526A  
Human Module 2, lot# 100727A  
Human Module 3, lot# 1007301  
Mouse Module 1, lot# 1008021  
Mouse Module 2, lot# 1008031

Insert Rate (from 200 clones): **>95%**

Total number of nucleotides sequenced: **5,800** (shRNA portion is approx. 60 nt)

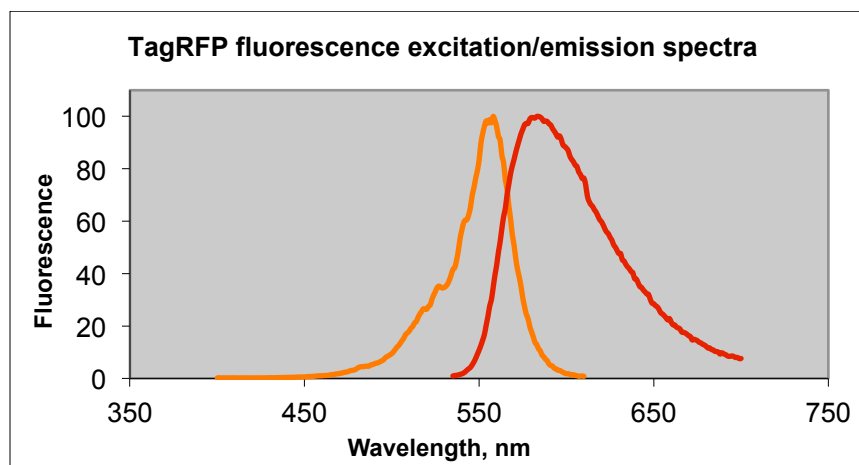
Number of clones with perfect sequence (no mutations, deletions, nor insertions in shRNA portion (sense-loop-antisense): **89 (91%)**

Number clones with at least one mutation, deletion, or insertion: **7** (1 insertion (A), 6 mutations (A>G, A>T, A>T, T>A, C>A, A>G))

Mutation/Insertion/Deletion rate: **0.12%** (i.e. appr. 1 Mut/Del/Ins in 800 nucleotides)

## 6. Properties of the TagRFP Fluorescent Protein

The pRSI9-U6-(sh)-UbiC-TagRFP-2A-Puro DECIPHER shRNA Expression Vector contains Evrogen's full-length wild-type TagRFP gene for the high level of expression of the fluorescent protein from the Ubiquitin C promoter in mammalian cells. TagRFP is a monomeric red (orange) fluorescent protein generated from the wild-type RFP from sea anemone *Entacmaea quadricolor* [Merzlyak et al., 2007]. It possesses bright fluorescence with excitation/emission maxima at 555 and 584 nm, respectively. TagRFP is about three times brighter than mCherry protein [Shaner et al., 2004], which makes it the brightest monomeric red fluorescent protein available so far. Due to its exceptional properties, TagRFP is an excellent fluorescent marker that can be used instead of mCherry for monitoring delivery of expression constructs into cells.



Characteristic	
Molecular weight, kDa	27
Polypeptide length, aa	237
Fluorescence color	red (orange)
Excitation maximum, nm	555 (use between 530-561)
Emission maximum, nm	584 (use between 580-600)
Quantum yield	0.48
Extinction coefficient, M <sup>-1</sup> cm <sup>-1</sup>	100,000
Brightness*	48.0
Brightness, % of EGFP	148
pKa	3.8
Structure	monomer
Aggregation	no
Maturation rate at 37°C	fast
Photostability	medium
Cell toxicity	not observed
Main advantages	bright red monomeric fluorescent protein
Possible limitations	medium photostability

### Recommended filter sets and antibodies

TagRFP can be recognized using Anti-tRFP antibody (Cat.# AB233-AB234) available from Evrogen. Recommended Omega Optical filter sets are QMAX-Yellow, XF108-2, XF101-2, and XF111-2. TagRFP can also be detected using TRITC filter set or similar.

### Performance and use

TagRFP can be easily expressed and detected in a wide range of organisms. Mammalian cells transiently transfected with TagRFP expression vectors give bright fluorescent signals in 10-12 hrs after transfection. No cytotoxic effects or visible protein aggregation are observed. High pH-stability with pKa=3.8 makes it possible to use TagRFP for imaging in acidic organelles, such as late and recycling endosomes and lysosomes.

\*All information taken from Evrogen's website at [http://www.evrogen.com/products/TagRFP/TagRFP\\_Detailed\\_description.shtml](http://www.evrogen.com/products/TagRFP/TagRFP_Detailed_description.shtml)

## R. Terms and Conditions

### Collecta, Inc. Limited License

Collecta grants the end user (the "Recipient") of the Pooled Lentiviral shRNA Libraries and/or Vector (the "Product") a non-transferable, non-exclusive license to use the reagents for internal research use only as described in the enclosed protocols; in particular, research use only excludes and without limitation, resale, repackaging, or use for the making or selling of any commercial product or service without the written approval of Collecta, Inc. -- separate licenses are available for non-research use or applications. The Product is not to be used for human diagnostics or included/used in any drug intended for human use. Care and attention should be exercised in handling the Product by following appropriate research laboratory practices.

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Collecta's DECIPHER™ shRNA Libraries are covered a fully executed Materials Transfer Agreement (MTA) between Collecta and the receiving institution. Please contact the DECIPHER Project Manager at [info@decipherproject.net](mailto:info@decipherproject.net) for more information.

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