CELLECTA

Cellecta Human Genome-Wide Pooled Lentiviral shRNA Libraries

HT RNAi Genetic Screens

User Manual

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Contents

A. Background

The Human Genome-Wide pooled lentiviral shRNA library (hGW) targets all (19,276) protein-encoding genes. It covers all genes in the human genome, and each gene is targeted by 8 hairpins. We have incorporated clonal barcodes to enable you to track growth, differentiation, or migration of specific cells containing a specific shRNA throughout your experiment. The hGW consists of three modules, each covering 6,500 genes. Since each gene is targeted by 8 hairpins, there are a total of 55,000 hairpins per module. The modules are made with non-overlapping barcodes so that they can be combined to form a complete genome-wide shRNA library.

Each hairpin in the hGW includes a clonal barcode, which facilitates HT sequencing data analysis, identification of functional shRNAs, and allows for tracking of specific shRNAs in individual cells. The barcodes can be read by HT sequencing on the Illumina platform. Identified barcodes can be converted to lists of genes/ shRNAs using our enumerated barcode data analysis software. The inclusion of clonal barcodes allows for identification of shRNAs without the need for amplification or sequencing of the hairpins themselves, which can be cumbersome due to the secondary structure present in them. For more information on how the clonal barcodes were built and how they are useful, please read: http://www.cellecta.com/millions-of-defined-sequenceable-barcodes-for-clonal-cell-tracking-2/.

Each 55K hGW library module also includes a panel of internal controls. The control block consists of 2 shRNA sequences for PSMA1, 2 shRNA sequences for RPL30, and 4 shRNA sequences for luciferase. Each shRNA sequence is replicated 5 times with 5 different barcodes. This 5-replicate internal control is useful for assessing internal noise, because the 5 replicates for each shRNA should elicit the same phenotype.

The protocols below provide the instructions on how to package the plasmid form of the hGW into viral particles and guidelines for the preparation of barcoded probes for high-throughput (HT) sequencing and analysis of raw sequencing data sets. Please read the entire user manual before proceeding with your experiment. For a description of the theories behind using pooled shRNA lentiviral libraries, information on transduction of target cells, viral targeting, or for examples of positive and negative screens using pooled lentiviral libraries, please read the **Pooled Lentiviral shRNA Library Screening Reference Manual.**

The protocols and methods apply specifically to Human Genome-Wide Modules 1-3. *To ensure you have the latest version of this user manual, please visit http://www.cellecta.com/resources/protocols/.*

IMPORTANT: The barcode sequences in the Human Genome-Wide Modules do not overlap, therefore these modules can be combined in any step of the procedure including HT Sequencing.

Library	Vector	Target Genes	# mRNA	# shRNA	Catalog #
Human Module 1	pRSI16	Signaling Pathways	6,500	55,000	HGW-M1-P2 (plasmid) HGW-M1-V8 (reg. titer virus) HGW-M1-V9 (high titer virus)
Human Module 2	pRSI16	Disease-Associated and Drug Targets	6,500	55,000	HGW-M2-P2 (plasmid) HGW-M2-V8 (reg. titer virus) HGW-M2-V9 (high titer virus)
Human Module 3	pRSI16	Cell Surface, Extracellular, DNA Binding	6,500	55,000	HGW-M3-P2 (plasmid) HGW-M3-V8 (reg. titer virus) HGW-M3-V9 (high titer virus)
Human Modules 1-3	pRSI16	All Modules	19,276		HGW-P2 (plasmid) HGW-V8 (reg. titer virus) HGW-V9 (high titer virus)

NOTE: The module names for hGW 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 associated with each module for detailed information regarding which genes are present in each specific module on our website www.cellecta.com.

B. hGW Pooled shRNA Library Required Materials

B.1. Included Materials

- **For Plasmid Library purchases**: 200 µg of each plasmid library ordered, in the pRSI16-U6-(sh)-13kCB18-HTS6-UbiC-TagRFP-2A-Puro vector; enough to generate lentivirus for approximately 50-100 screens (depending on cell type)
- **For Virus Library purchases**: Aliquots of virus at the ordered titer, plus a small amount of extra packaged library for titering purposes. Exact number and titer are indicated on the Product Analysis Certificate
- **For Plasmid Library purchases**: 10 µg empty library vector, as a packaging and transduction control; or, after linearization by BbsI/BpiI restriction digest, for cloning individual constructs used to validate hits from your screen
- User Manual and Product Analysis Certificates (http://www.cellecta.com/resources/protocols/)
- List of shRNA and barcode sequences (http://www.cellecta.com/resources/vectors/)
- HT Sequencing QC data of plasmid library (http://www.cellecta.com/resources/vectors/)

The vector map, sequence, feature map, and restriction map can be downloaded from http://www.cellecta.com.

B.2. Materials Available Separately from Cellecta

- Lentiviral packaging 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.)
- Positive control (targeting) lentiviral shRNA constructs (Custom or premade)
- Negative control (non-targeting) lentiviral shRNA constructs (Custom or premade)
- Linearized shRNA expression vector, for cloning individual constructs used to validate hits from your screen
- LentiFuge, lentiviral concentration reagent

The following custom services are available from Cellecta at additional cost. For more information, visit www.cellecta.com, email us at sales@cellecta.com, or call +1-650-938-3910.

Additional Products and Services	Catalog #
Ready-to-Use Packaging Plasmid Mix (250 µg)	CPCP-K2A
LentiFuge™ Viral Concentration Reagent (1000X), for 1 L supernatant	LFVC1
HT Sequencing of hGW Library experimental samples (frozen cells, DNA, or xenograft)	CANA-SQ, CANA-SQD, CANA-SQT
Pre-made or Custom Lentiviral shRNA Constructs (Plasmid or Packaged)	Many
Cloning hGW Module into Custom shRNA library vector	Inquire

B.3. Materials Needed from Other Vendors

- 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 D-MEM comes supplemented with stable L-Alanyl-L-Glutamine dipeptide, addition of fresh glutamine is not necessary.

- HEPES
- $MgCl_2$
- 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 (Mediatech, Cat. # 21-031-CV)
- Trypsin-EDTA (Mediatech, Cat. # 15-040-CV)
- Polybrene® (hexadimethrine bromide) (Sigma-Aldrich, Cat.# 107689)
- 500 ml, 0.2 µm filter units (Fisher Scientific Cat.# 09-741-05 or Thermo Scientific Cat.# 569-0020)
- Tissue Culture Plates and Related Tissue Culture Supplies
- Lipofectamine™ Reagent (Life Technologies, Cat.# 18324-020)
- Plus™ Reagent (Life Technologies, Cat.# 11514-015)
- 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) (GE Healthcare, Cat. # 28-4065-52)
- QIAquick PCR purification kit (QIAGEN, Cat.# 28106)
- QIAquick Gel Extraction Kit (QIAGEN, Cat.# 28706)
- Primer for sequencing shRNA inserts in shRNA constructs (IDT)*: See **Appendix M**
- PCR primers for barcode amplification from genomic DNA (IDT)*: See **Appendix M**
- HT sequencing primers (IDT)*: See **Appendix M**

- 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
NextSeq 500	Sequencing	FC-404-2005	NextSeq 500 v2 Kit

** See Illumina website for information on HiSeq 2500 rapid run kits.*

NOTE: We currently do not support HT sequencing of samples on the Illumina MiSeq.

B.4. Related Services from Cellecta

- Custom Pooled shRNA Library Construction
- RNAi Functional Genetic Screens with Pooled shRNA Libraries, Cat.# CRGS-X
- HT Barcode Sequencing of Cell Pellets, DNA, or Xenografts from RNAi Screen (with Cellecta Library)
- Pre-made and Custom shRNA and CRISPR Constructs
- Linearized shRNA Expression Vectors

C. Packaging Protocol for Pooled Lentiviral shRNA Libraries

The following protocol describes the generation of a packaged Human Genome-Wide pooled lentiviral 55K shRNA library (55K shRNA complexity) using Invitrogen's Lipofectamine™ and Plus™ Reagent. 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 × 10^6 TU/ml. In this protocol, using twenty (20) 15-cm plates, at least 6 × 10^8 TU of total lentiviral particles can be made and then concentrated to up to 100-fold using several described methods. We do not recommend scaling down the lentiviral packaging protocol due to risk of compromising the representation of the shRNA library.

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

C.1. Day 0 – Plate Cells

2. Twenty four (24) hours prior to transfection, plate 12.5 × 10^6 293T cells in each of twenty (20) 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.

C.2. Day 1 – Transfection (Twenty 15-cm plates*)

3. In sterile 50-ml polypropylene tube, mix the Ready-to-use Packaging plasmid mix with the plasmid DECIPHER library and add the plasmid mixture to D-MEM medium without serum or antibiotics. Add the Plus Reagent, mix, and incubate at room temperature for 15 min. See the table below for the volumes to use.

20 X 15-cm plates	**Component**
1200 µl	Ready-to-use Packaging Plasmid Mix (0.5 µg/µl) *
120 µl	Plasmid shRNA Library (1 µg/µl) *
24,000 µl	D-MEM, no FBS, no antibiotics
1200 µl	Plus Reagent
26,520 µl	Total volume

**** IMPORTANT: DO NOT use less than twenty 15-cm plates to package a batch of hGW or 55K library. A smaller amount may cause shRNA insert representation to be adversely affected.***

4. Add Lipofectamine Reagent to D-MEM medium without serum or antibiotics in order to make a convenient master mix according to the table below. Mix gently.

20X plates	**Component**
24,000 µl	D-MEM, no FBS, no antibiotics
1800 µl	Lipofectamine
25,800 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.

C.3. 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.

C.4. 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 (600 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. Proceed to concentration step, or aliquot and store the non-concentrated 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 http://www.cellecta.com/products-and-services/lentiviral-packaging/ for more information.

C.5. Concentrating Virus (Optional)

Although concentrating virus is optional, it is recommended if (1) very high titer virus stock is needed to achieve desired MOI in hard-to-transduce target cells, (2) virus should be suspended in another media (besides DMEM/10%FBS) which is optimal for sensitive target cells, or (3) 18h post-tranduction baseline control is used in your screen (to minimize problems with possible plasmid library carry-over). However, because of the additional manipulation of samples, there is the added risk of contamination and loss of virus.

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

1. Aliquot lentiviral supernatant in clear sterile centrifuge tubes.
2. Add LentiFuge to a final concentration of 5 µg/ml, and incubate for 1 hour at +4°C.
3. Centrifuge at 10,000 rpm for at least 1 hour at +4°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 tubes on ice, resuspend the (in)visible pellet in PBS/10%FBS (or PBS/1%BSA), make aliquots, and freeze at –80°C.

Alternatively, you may concentrate virus by the any of the methods below. However, the yield of virus is superior (~80% recovery) using Cellecta's protocol above.

- Ultracentrifugation at 50,000 g for 90 minutes at +4°C
- Sucrose cushion ultracentrifugation
- PEG precipitation, followed by centrifugation

D. Transduction Protocols, Lentiviral Titer Estimation, and Screening Protocols

For complete protocols on transduction of target cells with pooled lentiviral shRNA libraries, titer estimation, and examples of screening protocols, please see the **Pooled Lentiviral shRNA Library Screening Reference Manual.**

E. Genomic DNA Extraction for Barcode Amplification and HT Sequencing

Identification of shRNA 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 generate digital expression data using Deconvolution software. We currently do not support HT sequencing of samples on the Illumina MiSeq.

Cellecta now offers sample prep, HT sequencing, and analysis services. Please contact us at sales@cellecta.com or visit http://www.cellecta.com/products-services/cellecta-pooled-lentiviral-libraries/next-gen-sequencing-and-analysis/ for more information.

Due to the large amount of cells and resulting genomic DNA, the following protocol is recommended for isolating genomic DNA, rather than using a commercial column-based kit. Use of a commercial column-based kit may result in loss of genomic DNA and loss of representation of barcodes that survived the screening protocol.

If you are starting with fewer than 1 million cells, we recommend using the Qiagen QIAamp DNA Micro Kit, according to the manufacturer's instructions, instead of using the protocol here.

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, sonicate to shear DNA into 10-100 kb sized fragments. To prevent cross-contamination, thoroughly wash the ultrasound head with running water and dry with clean paper towel between samples.
4. Add 10 ul of proteinase K, mix and incubate 15 minutes at RT.
5. Add 5 ml Phenol:Chloroform:Isoamyl Alcohol solution, vortex hard and spin down 60 min, +20°C at 8,000 rpm in JA-14 or equivalent rotor (Beckman).
6. 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).
7. 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.
8. 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).

9. Discard supernatant, add 10 ml 70% ethanol, spin down 5 min, +20°C at 8,000 rpm in JA-14 or equivalent rotor.
10. Discard supernatant and air-dry pellet.
11. 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.
12. Incubate 30 minutes at +80°C before spectrophotometer reading.

F. Amplification of shRNA-specific Barcodes from Genomic DNA

An adequate amount of DNA needs to be used in the first amplification to ensure full representation of the barcodes from all the cells isolated from each experimental sample.

For negative screens where DNA was isolated in the previous step from 50 million or more cells, the pooled barcodes should be amplified from 400 µg of genomic DNA.

When amplifying barcodes from samples generated by positive selection screens, use the entire amount of genomic DNA recovered (up to 400 ug) with a proportionally fewer number of 100-µl reactions per sample.

This protocol was optimized using an ABI GeneAmp PCR System 9700 with Titanium Taq DNA polymerase mix (Clontech-Takara). 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 SingleRead 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. HT sequencing of samples on the Illumina MiSeq is not supported.

The goal of the first PCR is to amplify barcodes from genomic DNA. The goal of second PCR, which uses only 5% of volume from the 1st PCR with nested PCR primers, is to separate the amplified barcodes from non-specific PCR products and excess genomic DNA. These extraneous contaminants can interfere with gel purification of the amplified barcodes. Moreover, the nested PCR primers introduce sequences complementary to the oligos immobilized in the Illumina flow cell which are required for sequencing.

Use 10 ng of plasmid shRNA library as an amplification control in the first round of PCR and the PCR product from this amplification for the remaining steps.

F.1. First Round of PCR

The first round of PCR serves to amplify the barcodes remaining in the genomic DNA pool after the phenotypic screen is complete. We recommend not exceeding 100 µg in 100 µl total volume per reaction.

Prepare a master mix according to the table below. For each sample, prepare 4 × 100 µl reactions containing a total of 400 µg of genomic DNA.

___	µl	Genomic DNA (400 µg)
12	µl	Forward 1st round PCR primer* (10 µM)

12	µl	Reverse 1st round PCR primer* (10 µM)
8	µl	50X dNTP Mix (10 mM each)
40	µl	10X Titanium Taq Buffer
___	µl	Deionized water
8	µl	50X Titanium Taq
400	µl	Total volume (Split into 4 x 100 µl test tubes)

94°C, 3 minutes **1 cycle**

94°C, 30 seconds
65°C, 10 seconds } **16 cycles**
72°C, 20 seconds

68°C, 2 min

* Please see Appendix for primer sequences for vectors with HTS6 shRNA cassettes.

F.2. 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. Additionally, the second round PCR primers have complimentary sequence to the immobilized primers in the HT sequencing Illumina flow cells. Amplify each DNA sample with the Forward and Reverse 2nd round primer set* and perform HT sequencing on one sample per lane (in the flow cell) with the GexSeq* primer.

1. Combine together the 4 × 100 µl First Round PCR reactions and use a 5 µl aliquot in the second round of analytical PCR with nested primers in each 100 µl reaction:

5	µl	First Round PCR Product
5	µl	Forward 2nd round PCR primer* (10 µM)
5	µl	Reverse 2nd round PCR primer* (10 µM)
2	µl	50X dNTP Mix (10 mM each)
10	µl	10X Titanium Taq Buffer
71	µl	Deionized water
2	µl	50X Titanium Taq
100	µl	Total volume

Run PCR under the following cycling conditions.

94°C, 3 minutes	**1 cycle**
94°C, 30 seconds 65°C, 10 seconds	**10,12 or 14 cycles****
72°C, 20 seconds	
68°C, 2 min	

* Please see Appendix for primer sequences for vectors with HTS6 shRNA cassettes.

****NOTE:** During the PCR, please take a 5 µl aliquot from the tube after 10, 12, and 14 cycles and save it for the next step. The goal is to find the optimal cycle number in order to avoid overcycling of PCR reactions, which can result in the generation of a longer fragment that corresponds to a fusion double barcode product.

2. The amplified barcodes are then analyzed on a 3.5% agarose-1XTAE gel (load 5 µl/lane). The results should reveal a bright band of amplified barcode products (HTS6 cassette: 251-bp). 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 band across all DNA samples from the genetic screen.
3. 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 12-18 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). Prepare a master mix for the second prepration PCR.

X µl	First Round PCR Product
10 µl	Forward 2nd round PCR primer (10 µM)
10 µl	Reverse 2nd round PCR primer (10 µM)
4 µl	50X dNTP (10 mM each)
20 µl	10X Titanium Taq Buffer
Y µl	Deionized water
4 µl	50X Titanium Taq
200 µl	Total volume, Split into 2 x 100 µl reactions

Perform PCR under the following cycling conditions.

94°C, 3 minutes	**1** cycle
94°C, 30 seconds 65°C, 10 seconds	**12 or 14** cycles (the number of cycles that worked the best in the previous step)
72°C, 10 seconds	
68°C, 2 min	

4. 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:
   a. Purify the PCR product with the QIAquick PCR purification kit (QIAGEN) following the manufacturer's protocol. In the last centrifugation step, use a centrifuge spin filter at maximum speed for 5 minutes. This is to dry the membrane completely to avoid ethanol contamination in the purified PCR product.
   b. Separate by electrophoresis in a preparative 3.5% agarose-1XTAE gel.
   c. Cut out band and extract DNA from the gel using the QIAquick gel purification kit (QIAGEN).
   d. Quantitate using A260 nm measurement using NanoDrop spectrophotometer (or equivalent) and adjust concentration to 10nM (~1.8 ng/µl for 251-bp (HTS6) product).

G. HT Sequencing of Pooled shRNA-specific Barcodes on Illumina's GAIIx or HiSeq

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 GexSeq* sequencing primer and following the manufacturer's protocol. The final concentration of GexSeq* 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 depends on the length of the barcode and is 44 for the hGW library.

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).

1. Adjust purified PCR samples to 10nM (1.7 ng/µl) concentration.
2. For cluster generation step – use Illumina Single-Read (SR) flow cell, and for each lane add 2 µl of each sample and add PhiX174 control template based on standard Illumina protocol.
3. For HT sequencing step – Add GexSeq* primer (10 µM, i.e. 20x) to the PhiX174 primer to a final concentration of 0.5 µM.
4. Run HT sequencing reaction for the appropriate number of cycles with GexSeq/PhiX174 primer mix.

See **Required Materials** for a list of recommended Illumina kits for HT Sequencing of samples transduced with a Cellecta library.

* Please see Appendix for HT sequencing primer sequences for vectors with HTS6 shRNA cassettes. For other vectors, refer to the Product Analysis Certificate that came with the product or contact Cellecta.

Cellecta now offers sample prep, HT sequencing, and analysis services. Please contact us at sales@cellecta.com or visit http://www.cellecta.com/products-services/cellecta-pooled-lentiviral-libraries/next-gen-sequencing-and-analysis/ for more information.

H. Barcode Enumeration

Software for conversion of raw sequencing data to number of reads for each barcode for Human Genome-Wide shRNA Libraries is available from Cellecta. Please contact tech@cellecta.com.

I. Troubleshooting: Difficulties with Probe Preparation and HT Sequencing

I.1. No PCR Product

Problem: Incorrect primers or bad reagents used, or missing reagents, or low transduction of target cells, or poor DNA prep with PCR inhibitors.

Solutions:

Include 10 ng of plasmid library DNA as a positive control. If it produces the correct amplification product, the problem lies with absent or low numbers of barcodes (e.g. low MOI, or problems with the transduction efficiency) or impurities in genomic DNA which block barcode amplification. If the positive control works, dilute the genomic DNA 2-5 fold and repeat the amplification step using 180 µg of genomic DNA in several PCR test tubes.) If not, confirm use of the correct primers and reagents.

Verify that primer sequences are correct. Please see **Appendix M**.

I.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 proper GexSeq Sequencing primer (see **Appendix M**), 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 help with using hGW Pooled Lentiviral shRNA Libraries, please email technical support at tech@cellecta.com 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:

1. What was the lentiviral titer, and what was the total number of TU packaged?
2. How was the virus concentrated? *(if applicable)*

Transducing Target Cells:

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

RNAi Screen:

1. Could you briefly explain your experiment?
2. How many infected cells were used?

Sample Preparation & HT Sequencing

1. Describe the protocol you used to amplify the barcodes.
2. What HT sequencing system and which Illumina HT Sequencing Kits did you use?
3. How much PCR product was used for HT Sequencing?
4. How many sequences were read per sample?
5. Would you be able to send us the raw data so that it may help us diagnose the issue?

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
General Information: info@cellecta.com
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Orders: orders@cellecta.com

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

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

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) are 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/biosafety/publications/bmbl5/bmbl5_sect_iv.pdf

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:

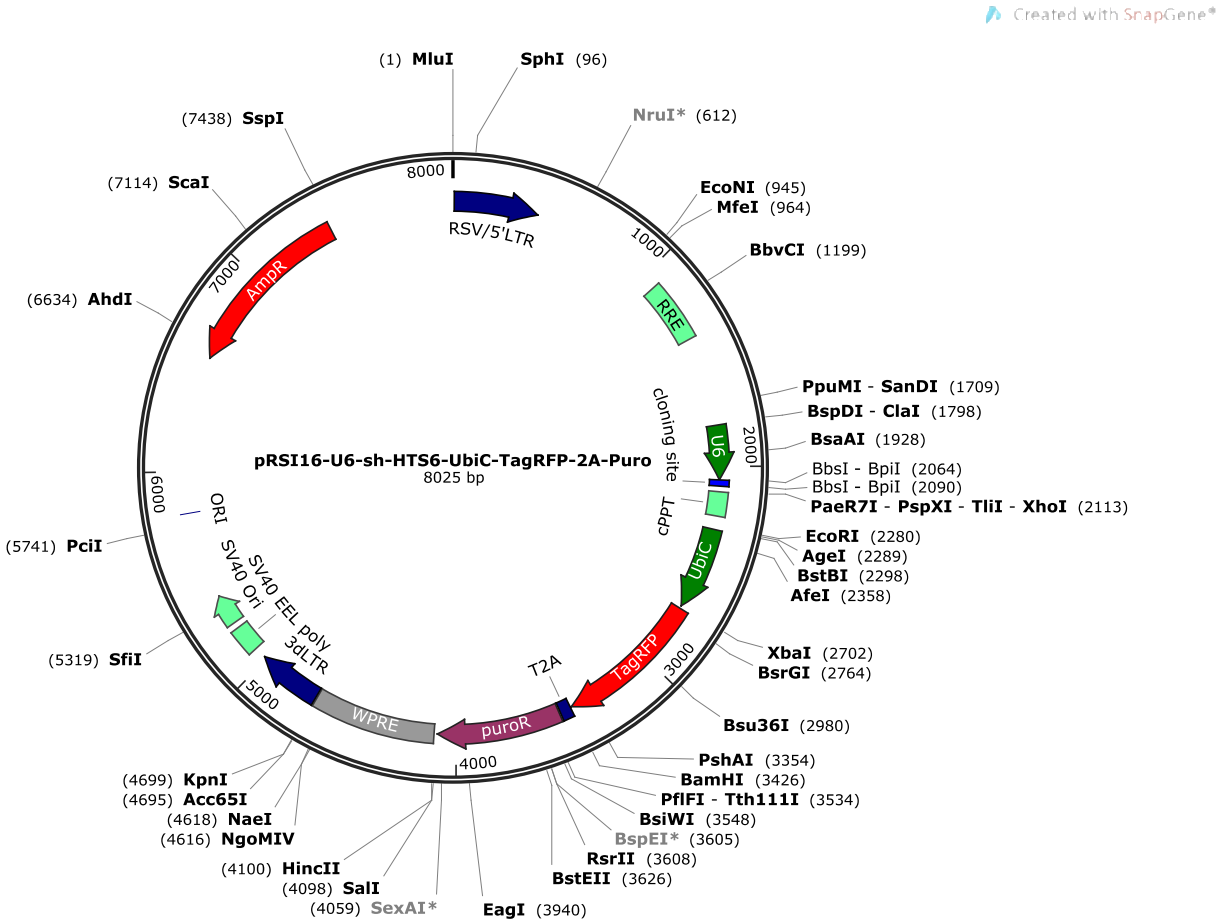
- Wear gloves and lab coat at all times when conducting the procedure.
- Always work with lentiviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any 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. References

For a complete list of References and Product Citations, please see:
http://www.cellecta.com/resources/publications/

M. Appendix

M.1. Lentiviral shRNA Expression Vector Maps*



For sequences and cassette designs for other standard library vectors, please visit the Cellecta website: http://www.cellecta.com/resources/vectors or contact Cellecta at tech@cellecta.com.

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

M.2. HT Sequencing Primers, HTS6 Cassette

(*e.g.* **pRSI16**-U6-(sh)-HTS6-UbiC-TagRFP-2A-Puro)

Amplicon Size, 2nd round PCR: 251 bp

For the Illumina HiSeq and GAIIx Platforms only:

Primer Name	Used for	Sequence (IDT preferred)
F2	1st Round	5'-TCGGATTCGCACCAGCACGCTA-3'
R2	1st Round	5'-AGTAGCGTGAAGAGCAGAGAA-3'
Gex1-NF2	2nd Round	5'-TCAAGCAGAAGACGGCATACGATCGCACCAGCACGCTACGCA-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'

M.3. Common 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	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 (in the hGW vectors).	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

** (c): element on complementary strand*

M.4. hGW Library HT Sequencing Q.C. Data

Complete Plasmid shRNA Library HT sequencing data for all modules is available http://www.cellecta.com/resources. Plasmid HT Sequencing data may be used as negative control (untreated/untransduced/day 0) data for many types of genetic screens.

The shRNA/barcode representation histograms for individual hGW libraries are available on the PAC forms available on the Cellecta website at http://www.cellecta.com/resources/protocols/.

M.5. hGW Library Individual Clone Sequencing Q.C. Data

hGW Libraries in pRSI16 Vector:

hGW Library:	Human M1	Human M2	Human M3
Plasmid Lot #:	12121403	12121403	12121403
Library Complexity (number of clones):	100 x 10^6	100 x 10^6	100 x 10^6
Number of random clones picked:	72	72	72
Correct Structure (with clonal barcode):	>95%	>95%	>95%
Number of clones with at least one mutation, deletion, or insertion:	12	12	12
Mutation / Deletion / Insertion Rate:	0.1%	0.1%	0.1%
Estimated % of Inserts without any mutations, deletions, or insertions in antisense portion and considered to be functional:	>95%	>95%	>95%

M.6. hGW Barcode Analyzer and Deconvoluter

This software is required to convert raw HT sequencing data from hGW library screens into a summary file for subsequent processing, and it includes annotation for every identified gene. Next, data can be processed, edited, normalized, and transformed using your data analysis tool of choice, such as SAS, SPSS, or, for simpler analyses, Microsoft Excel. The software is available for hGW customers by contacting tech@cellecta.com.

N. Terms and Conditions

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>
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