GeneNet™ Lentiviral shRNA Libraries

Cat. # SI2XXB-1,SI6XXB-1

User Manual

Store kit at –70°C on receipt

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

(ver. 6 -080511)

Contents

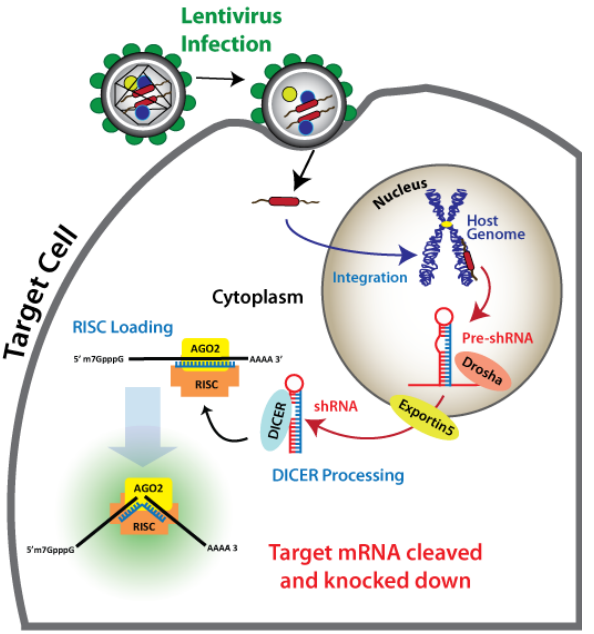
I. Introduction and Background

A. Overview

This manual provides information describing genetic screening with System Biosciences's (SBI's) GeneNet™ shRNA libraries cloned in Lentiviral Expression Vectors and pre-packaged in VSV-G pseudotyped viral particles. Specifically, it provides recommendations and instructions on how to transduce packaged GeneNet™ shRNA libraries into target cells, select target cells with a specific phenotype, and identify shRNAs and corresponding target genes which induce the specific phenotype. Before using the reagents and material supplied with this product, please read the entire user manual.

Please refer to the associated Product Analysis Certificate (PAC) for Viral titers of the Libraries and for the Positive transduction control.

ShRNAs are short hairpin RNAs that have a sequence of RNA that makes a tight hairpin turn. The shRNA hairpin structure is cleaved by Dicer into siRNA, which is then binds to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs to which the corresponding siRNA hybridizes. ShRNAs and the resulting siRNAs can be used to silence gene expression via RNA interference.



B. Functional Screening with shRNA Libraries

Gene silencing by small interfering double-stranded RNAs (siRNAs) is becoming a powerful tool for functional analyses of the genes associated with specific biological processes in cells. Scaling up this approach to the entire genome with shRNA libraries targeting every gene is facilitating progress in the area of functional genomics and systems biology. There are two main strategies for using genome-wide shRNA libraries for genetic screening experiments.

The first strategy relies on the development of a collection of shRNA molecules for each individual target gene with subsequent functional analysis through inactivation of a single gene at a time. Though this strategy provides an efficient tool to study the functions of individual genes and can be used in combination with many biological assays, it is very expensive and labor-intensive for genome-wide screens. Despite this time consuming process, this strategy was successfully applied for the functional analyses of thousands of genes, based on collections of non-verified or partially verified shRNAs (see References, Genetic Screens with shRNA libraries, in Section IV of this manual). These large-scale projects represent the first attempts to apply global loss-of-function genetic screens to mammalian cells. Unfortunately, such projects require significant resources that are only plausible for research consortiums or medium-to-large sized companies.

In the second strategy, a library encoding a pooled set of shRNAs designed for all target genes is prepared, introduced into a population of identical cells, and a functional selection is applied. Cells exhibiting the desired phenotypic changes are isolated and the siRNA constructs, presumably inducing the phenotypes, are recovered by PCR and identified by sequence analysis or microarray hybridization. The main advantage of the second strategy is the possibility of creating a very high complexity genome-wide shRNA library for all genes (including ESTs) in the genome, with application for unbiased (to any specific set of pre-selected genes) discovery of genes involved in specific phenotypes. Moreover, such pre-made pooled shRNA libraries would also allow comprehensive cost effective loss-of-function genetic screens to be performed by small research groups. The main disadvantage of genetic screens using shRNA libraries is the requirement for recipient cells with desired phenotypic changes to be selected from a pool of unaffected cells; for example, by selection based on cell survival, appearance of specific markers, or induction of reporter constructs, cell morphology or behavior, etc.

Efficient delivery and stable expression of siRNA effector molecules in a wide range of recipient cells are critical factors for knockdown technology. Suppression of protein levels by exogenous synthetic siRNA or siRNA expressed from plasmid vectors is transient and the levels of targeted gene products typically recover in several days following transfection (Lockhart, 1996; Lorens, 2001; Michiels, 2003). In order to achieve long-term permanent levels of siRNA in the cell, stable transcription of shRNA can be achieved by viral shRNA constructs integrated into genomic DNA of target cells. From a

practical standpoint, lentiviral vectors are an optimal delivery system for stable and effective (up to 100%) transduction of gene-specific RNA interference constructs and complex shRNA libraries into recipient cells (see Appendix, Lentiviral Delivery Vectors). Based on lentiviral delivery technology, SBI has developed a novel research tool for genetic screen experiments -–the genome-wide lentiviral shRNA library.

C. GeneNet™ shRNA Libraries

The next generation of user-friendly genetic screening technology that includes genome-wide shRNA libraries has been developed at SBI with several novel features that significantly extend the application of this technology for high-throughput functional genomics studies:

- ***Biosafe third generation lentiviral (HIV and FIV-based) shRNA Vectors*** with puromycin selection (or copGFP reporter) and the RNA polymerase III H1 promoter shRNA expression cassette for the expression of shRNA constructs.

- ***Lentiviral shRNA transduction system*** that significantly extends the application of genetic screens to primary cell lines, stem cells, cells isolated from organisms (blood cells, tissue biopsies), or even directly in model organisms (mouse). The high efficiency of transduction and physiological way of delivery achieved by the use of lentiviral shRNA libraries greatly facilitates complex genetic selection schemes and allows the identification of cellular targets linked directly to phenotypes.

- ***Genome-wide high complexity shRNA libraries*** comprised of a redundant set of shRNAs (3-5 shRNAs per transcript) to provide reliable knockdown for each known human or mouse gene.

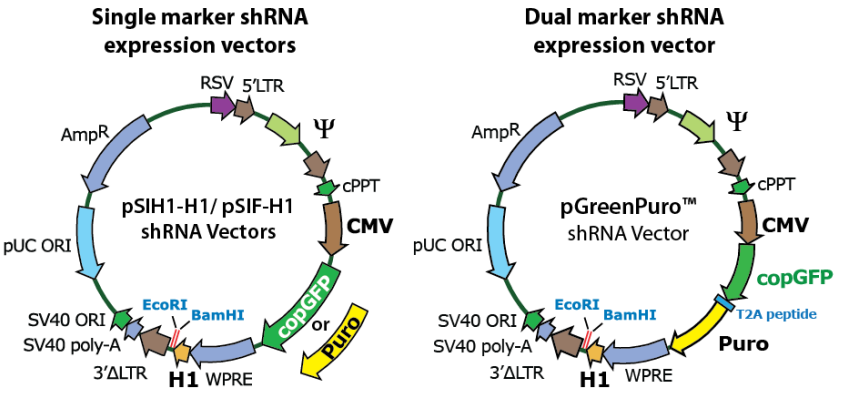
- ***Ready-to-use shRNA libraries*** pre-packaged as VSV-G pseudoviral particle stocks that have passed stringent controls for the absence of replication-competent virus contamination. This significantly adds to the convenience and safety by eliminating the need for researchers to work with complicated packaging cell line technology.

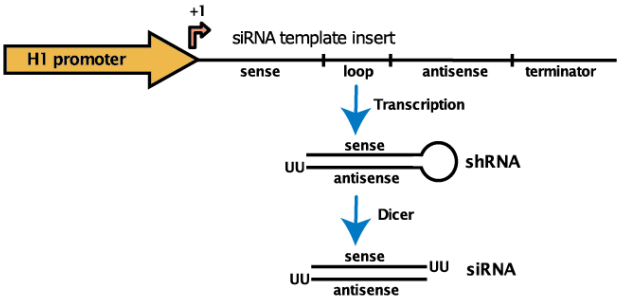
- ***Post-screening identification of siRNA sequences using microarrays.*** The sequences of siRNA templates are selected according to corresponding probe sequences on the Affymetrix GeneChip® Arrays. Using the same sequences for the siRNA and microarray allows high-throughput identification of siRNA effectors modulating a specific phenotype with the microarrays.

Lentiviral shRNA Expression Vectors

Lentiviral expression vectors are the most effective vehicles for transducing and stably expressing different effector molecules (shRNA, cDNA, DNA fragments, antisense, ribozymes, etc.) in almost any mammalian cell, including non-dividing cells and whole model organisms (Cann, 2000). As with standard plasmid vectors, it is possible to introduce lentiviral effector constructs in plasmid form into the cells with low-to-medium efficiency using conventional transfection protocols. However, by packaging the lentiviral shRNA vector construct into viral particles, you can obtain highly efficient transduction and heritable expression of shRNA, even with the most difficult to transfect cells, such as primary, stem, and differentiated cells. Endogenously expressed shRNA effectors provide long-term silencing of the target gene and allow the researcher to generate cell lines and transgenic organisms with a stable knockdown phenotype for functional studies. Moreover, lentiviral delivery does not produce the non-specific cell responses typically associated with chemical transfection or use of an adenoviral delivery system (Gould, 2003, Cann, 2000).

SBI offers GeneNet™ shRNA libraries constructed in both HIV-based and FIV-based lentivectors. SBI's lentivectors are a third generation of lentivectors developed for gene therapy applications (Poeschla, 2003; Sodroski, 1997, 1999; Federico, 2003; Heiser, 2004; Machida, 2003). The lentiviral expression vector contains the genetic elements (LTR, GAG, RRE, cPPT, WPRE) required for packaging, transduction, stable integration of the expression constructs into genomic DNA. It also contains the siRNA effector sequences driven by the H1 promoter. Puromycin resistance, GFP expression, or both is driven by the CMV promoter. The shRNA constructs packaged in pseudoviral particles can transduce target cells and express siRNA and reporter molecules, but they cannot replicate within target cells because the viral structural genes are absent and the LTRs are designed to be self-inactivating upon transduction.





Design of shRNA templates

Despite the development of many algorithms for prediction of functional synthetic shRNAs (Vickers 2003; Khvorova 2003; Reynolds 2004), the selection of efficient shRNA sequences that target mRNA still remains a challenging problem. There is no reliable algorithm to predict the efficacy of different siRNAs. The principal prediction criteria, which are used to select siRNA sequences that most likely knock down a target gene, are summarized in the table below. It is interesting to note that these criteria are very similar to those used to predict the most efficient short hybridization probes for microarrays (Lokhard 1996). Perhaps this similarity is not surprising since both siRNA and expression profiling technologies are based on hybridization of antisense oligonucleotides (target or antisense strand of siRNA) with the complementary sequence in mRNA (or the probe sequence immobilized on the array).

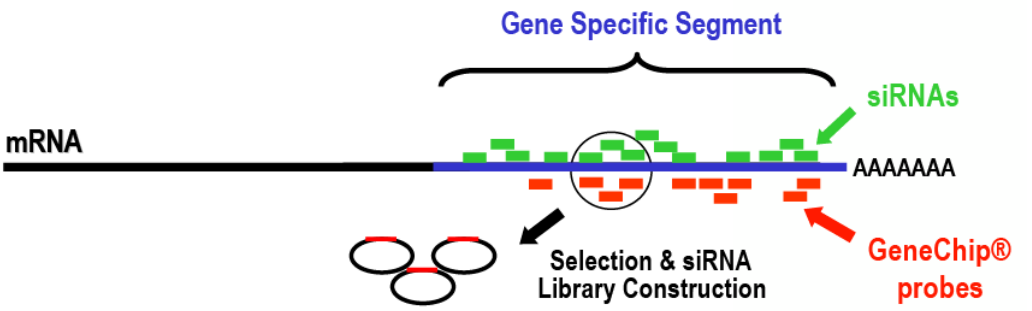
Criteria	*siRNA Sequence*	*GeneChip Probes*
Size (nucleotides)	19-29	25
Homology	Unique < 70%	Unique < 70%
GC Content	40-50	40-60
Specific Sequences	< 4 A,T < 5 G	< 6 A, T < 5 G
Secondary Structure	<0 kcal / mol	<0 kcal / mol
Other	GC rich 5' AT rich 3' ends in target mRNA	Yes, based on experimental data

To take advantage of this finding, we designed shRNA template sequences for our GeneNet™ shRNA Libraries that, based on known parameters, should work well to silence the targeted genes as well as hybridize to Affymetrix GeneChip® Arrays. When we tested siRNA constructs expressing sequences targeting p53, p73, and CD71 genes and designed to hybridize to Affymetrix arrays, we found that at least 50% of these

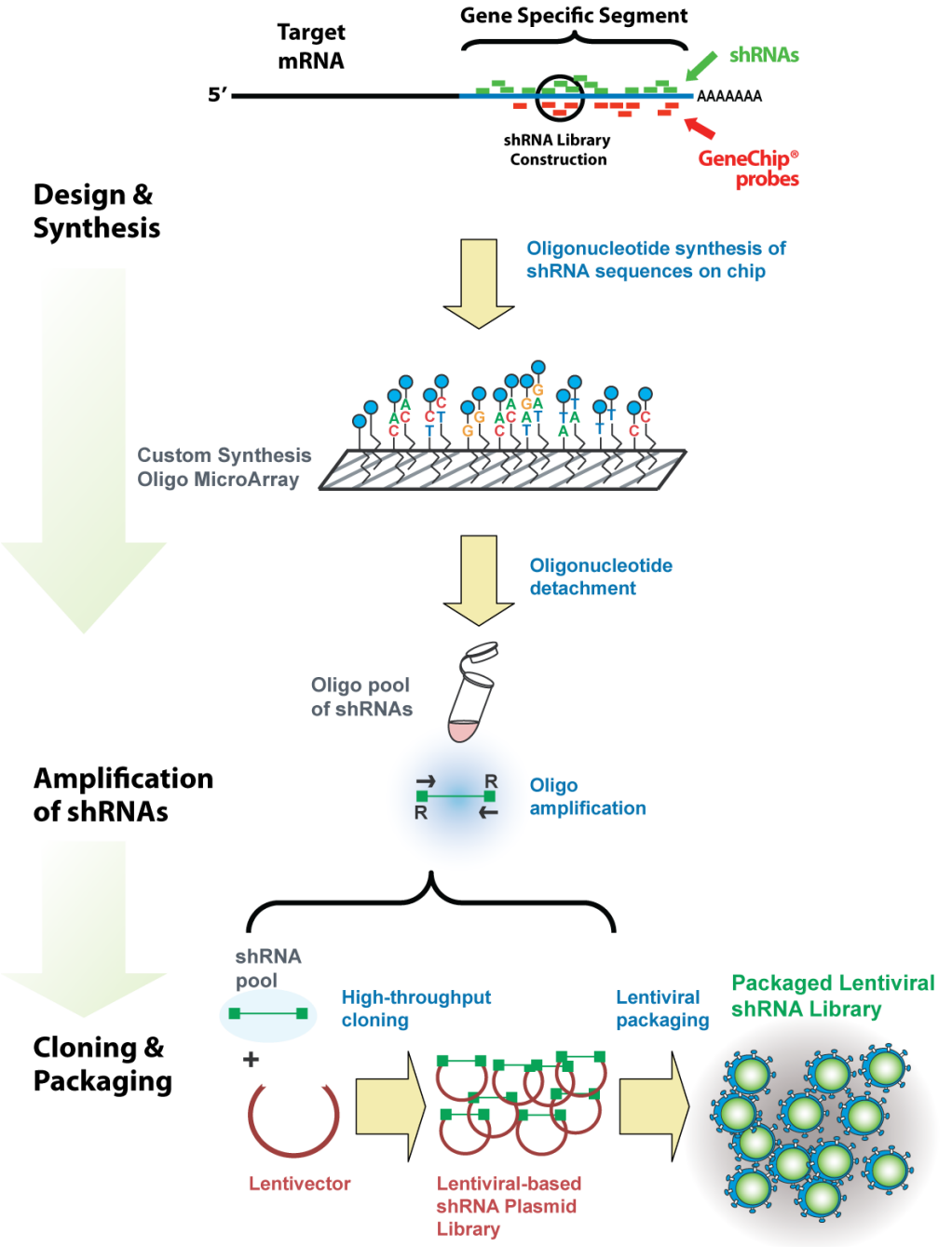
siRNAs could efficiently silence the target mRNAs (*i.e.*, reduce expression by more than 70%). These data confirm that GeneChip® probe sequences share considerable similarity with efficient siRNA sequences. Moreover, using sequences similar to the probes on the GeneChip® Array enables the use of the microarray as a simple readout tool for analysis of siRNA recovered from selected cell populations.

Construction and Quality Control of shRNA libraries

1. We selected a set of target genes for each GeneNet™ Library (*e.g.*, for the Human 50K library, we selected all human genes including ESTs (about 47,000 transcripts) represented on the GeneChip® Human Genome U133+2 Array).
2. For each target gene, we designed 3-5 shRNA template oligonucleotides that express 27-mer siRNAs targeting each of the mRNA sequences. The shRNAs were designed based on criteria developed by SBI for selection of the most efficient siRNAs. Since our algorithm yields about 50% functional siRNA sequences, 3-5 shRNA per target mRNA should silence about 90% of target genes for the library. Each target sequence was also designed to hybridize with probe oligos on Affymetrix GeneChip® Arrays and have additional 5'- and 3'-flanking sequences for directional cloning into a lentiviral shRNA expression vector.



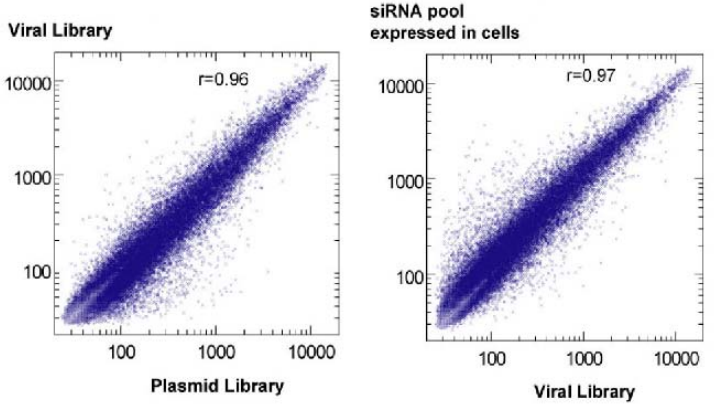
3. After synthesis, the shRNA template oligonucleotides were amplified by PCR using primers complementary to the additional flanking 5'- and 3'-sequences, digested with the appropriate restriction enzymes, and ligated to the corresponding linearized cloning vector.
4. The ligated shRNA library was then transfected into competent *E. coli* cells, grown as independent colonies on LB agar plates, and the total shRNA library in plasmid DNA form was purified from the pool of independent ampicillin-resistant bacterial colonies.
5. The pseudoviral-packaged shRNA library was then produced by co-transfection of the plasmid shRNA library with the pPACK Packaging Plasmid mix into 293TN cells.


Target mRNA
Gene Specific Segment
shRNAs
5′
AAAAAAA
shRNA Library Construction
GeneChip® probes
Design & Synthesis
Oligonucleotide synthesis of shRNA sequences on chip
Custom Synthesis Oligo MicroArray
Oligonucleotide detachment
Oligo pool of shRNAs
Amplification of shRNAs
R
R
Oligo amplification
shRNA pool
High-throughput cloning
Lentiviral packaging
Packaged Lentiviral shRNA Library
+
Lentivector
Lentiviral-based shRNA Plasmid Library
Cloning & Packaging

6. Quality control analysis of constructed shRNA libraries was performed by sequence analysis of randomly selected clones (>20 from each library). Sequencing results show an insert rate >90% with <10% concatemeric inserts. In addition, all inserts have the expected sequence with less than a 2% mutation rate.

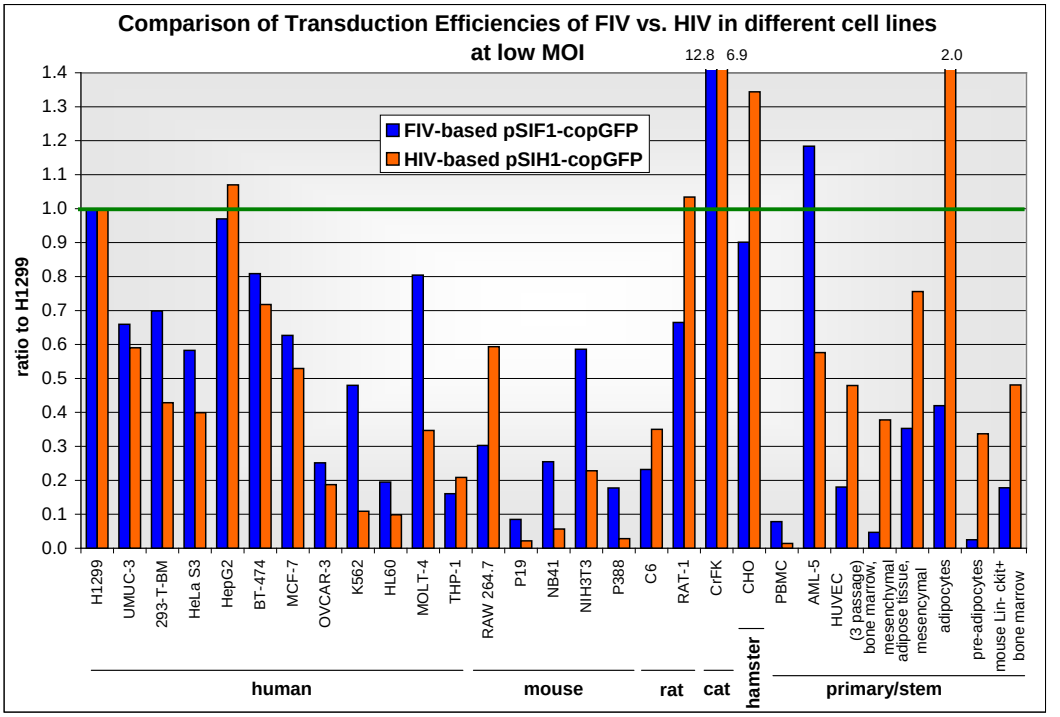
   In addition, in order to test the representation of shRNA inserts in the pseudoviral packaged shRNA library, we reverse transcribed the viral RNA and amplified the shRNA inserts using flanking vector primers. As a control, we amplify the shRNA inserts from the plasmid library used in the packaging step. Both samples were then hybridized to microarrays and compared in order to ensure representation was maintained after packaging.

   An example of this type of analysis is in the graph on the left. Furthermore, we verified that each GeneNet™ shRNA Library can be efficiently transduced and expressed in target cells without significant loss of representation by amplifying shRNA inserts from pseudoviral RNA isolated from a packaged GeneNet™ shRNA library and from total RNA of target cells (HT1080) transduced with the same library. As seen in the sample data, the packaging and transduction steps do not significantly affect representation of shRNA templates. Moreover, since the amplification is done using RT-PCR, this confirms that shRNA inserts are effectively expressed from the genomic DNA of target cells (HT1080) transduced with the packaged shRNA library.



Delivery of Packaged GeneNet™ shRNA Library into Target Cells

Pantropic VSV-G pseudotyped viral particles containing the RNA copy of the GeneNet™ shRNA library can be efficiently used to deliver and stably express shRNA and reporter sequences in a wide range of mammalian target cells. In order to provide guidelines for the use of lentivector delivery systems, we compared transduction efficiencies of packaged HIV-based and FIV-based vectors in 27 different cell types.



These data clearly indicate that unlike commonly used cancer cell lines (like H1299, HeLa, HeK295, HepG2, etc.) which can be effectively transduced by lentivector constructs, some cell types (mouse Lin- ckit+ bone marrow, P19, PBMC, HL60, P388) are more resistant to infection. More efficient transduction of "resistant" cell types may be possible by using a higher concentration of pseudoviral particles per cell in order to achieve the same MOI, but not in all cases. It is important to mention that HIV-based and FIV-based lentivectors have different tropism. For example, the FIV-based shRNA constructs are more effective at infecting several of the tested mouse cell lines (P19, NB41, NIH3T3, P388) and some of the blood cells (MOLT-4, K562, T-cells from AML patient). The HIV-based system is more effective at infecting stem and primary cells (HUVEC, bone marrow, adipose).

Pseudotyped lentiviruses have been successfully used to infect many other cell types, including neuronal, dendritic, endothelial, retinal, pancreatic, hepatic, aortic smooth muscle cells, airway epithelia, skin fibroblasts, macrophages, etc. Lentivectors have also been used successfully for *in vivo* delivery and expression of transgenes in muscle, brain, airway epithelium, liver, pancreas, retina, and skin. For a more complete list of cells or tissues, which have been successfully transduced with lentivectors, please see the Appendix, Section A.

D. Product Description and List of Components

The table below outlines the general features of the available GeneNet™ shRNA Libraries and indicates the compatibility of each library with the latest version of the Affymetrix GeneChip® Array (www.affymetrix.com). The most updated list of shRNA libraries available in different vectors can be found on SBI's website at www.systembio.com.

Catalog #	shRNA Library	Vector	shRNA Complexity	Quantity (ifu/kit)	Compatible GeneChip®	Number of	
						Genes	mRNAs
SI206B-1	Human 50K	pSIF1-H1-Puro	200,000	2×10^7	HG-U133+ 2.0	38,500	47,400
SI222B-1	Mouse 40K	pSIF1-H1-Puro	150,000	2×10^7	MG-430 2.0	34,000	39,000
SI606B-1	Human 50K	pSIH1-H1-Puro	200,000	2×10^7	HG-U133+ 2.0	38,500	47,400
SI622B-1	Mouse 40K	pSIH1-H1-Puro	150,000	2×10^7	MG-430 2.0	34,000	39,000

The shRNA libraries are provided in ready-to-use, pre-packaged in VSV-G pseudotyped viral particle format or as plasmid library, which you can package in pseudoviral particles in your cell culture facility. Depending on the complexity of the library, different amounts of pseudoviral particles (infection units, or ifu) are provided in the kit. The GeneNet™ shRNA Library Kits provide enough VSV-G pseudotyped pre-packaged shRNA library for 2-3 transductions for the most commonly used cell lines with an MOI of 1-2.

Packaged GeneNet™ shRNA Library Components

GeneNet™ shRNA Libraries (HIV-based), Cat. #s SI602B-1, SI606B-1, SI622B-1	
500-2000 µl	GeneNet™ shRNA Library, pre-packaged in pseudoviral particles
50 µl	cDNA Synthesis GNH Primer (10 µM)
50 µl	Fwd GNH (Forward) PCR Primer (10 µM)
50 µl	Rev GNH (Reverse) PCR Primer (10 µM)
200 µl	NFwd-Bio (Nested Forward Biotinylated) PCR Primer (10 µM)
200 µl	NRev GNH (Nested Reverse) Universal PCR Primer (10 µM)
20 µl	Positive Control DNA (plasmid shRNA library used for packaging step) (1ng/µl)
50 µl	pSIH1-H1-siLuc-copGFP Packaged Positive Transduction Control (>1×10^6 ifu)
1	CD with gene/shRNA list and data analysis program compatible with Affymetrix data file

GeneNet™ shRNA Libraries (FIV-based) Cat. #s SI202B-1, SI206B-1, SI222B-1	
500-2000 µl	GeneNet™ shRNA Library, pre-packaged in pseudoviral particles
50 µl	cDNA Synthesis GNF Primer (10 µM)
50 µl	Fwd GNF (Forward) PCR Primer (10 µM)
50 µl	Rev GNF (Reverse) PCR Primer (10 µM)
200 µl	NFwd-Bio (Nested Forward Biotinylated) PCR Primer (10 µM)
200 µl	NRev GNF (Nested Reverse) Universal PCR Primer (10 µM)
20 µl	Positive Control DNA (plasmid shRNA library used for packaging step) (1ng/µl)
50 µl	pSIF1-H1-siLuc-copGFP Packaged Positive Transduction Control (>1×10^6 ifu)
1	CD with gene/shRNA list and data analysis program compatible with Affymetrix data file

Additional comments on product components:

- GeneNet™ shRNA Library and pSIH1 / pSIF1-H1-siLuc-copGFP Packaged Positive Transduction Control are provided as frozen VSV-G pseudotyped viral particles in DMEM/25mM HEPES pH 7.4 or in sterile PBS. The total number of infection units (ifu) and concentration (the titer) were determined by measuring copy number of integrated lentiviral constructs in genomic DNA of transduced HT1080 cells using the UltraRapid Lentiviral Titering Kit and may vary for different lots of each library. ***The exact ifu, titer, and volume for each GeneNet™ Library are indicated on the corresponding Product Analysis Certificate.***
- RT-PCR primers are provided to amplify biotinylated hybridization targets comprising shRNA inserts from total cellular RNA (or alternatively from genomic DNA) and to be used for hybridization with the corresponding Affymetrix GeneChip® Array. The sequence of the PCR primers depends on the library vector (HIV-based or FIV-

based). The specific sequences of the PCR primers along with the map of the amplified region can be found in the Appendix. The Nested Reverse Primers have a phosphate at the 5'-end for selective degradation of the sense strand in amplified shRNA targets with Lambda exonuclease.

- The GeneNet™ shRNA Library Kit is shipped on dry ice and should be immediately stored at –70°C upon receipt. **Avoid thawing and refreezing of pseudoviral particles.** Each freeze-thaw cycle causes reduction of the titer by 20-30%. Properly stored pseudoviral particles are stable for 6 months from the date received.
- The list of target genes and shRNA inserts differs for each shRNA Library product. This information is supplied on the compact disc included with each library kit.
- The Positive Control DNA included in the kit is the plasmid form of the GeneNet™ shRNA Library. This DNA was used for production of the packaged GeneNet™ shRNA libraries. The positive control DNA can therefore be used to optimize and troubleshoot your RT-PCR and microarray hybridization steps. The hybridization pattern generated from this Positive Control DNA reflects the abundance level of all shRNA inserts in the packaged library and can be used as a universal reference to compare with recovered shRNA templates from your transduced target cells.

E. Additional Required Materials

For Transduction of shRNA library into target cells

- Dulbecco's Modified Eagle's Medium (D-MEM)
  (high glucose with sodium pyruvate and glutamine; Invitrogen, Cat. # 11995073)
- Fetal Bovine Serum (Invitrogen, Cat. # 16000036)
- Puromycin (Sigma, Cat. # P8833)
- Penicillin/Streptomycin (Invitrogen, Cat. # 15070063)
- Trypsin-EDTA (Sigma, Cat. # T3924)
- TransDux™ (SBI, Cat. # LV850A-1)
- Tissue Culture Plates and Related Tissue Culture Supplies

For Purification of total RNA and genomic DNA from target cells

- For simultaneous purification of total RNA and genomic DNA: TRIzol Reagent (Invitrogen, Cat. # 15596-026)
- For purification of total RNA: RNeasy Mini Kit (QIAGEN, Cat. # 74104)
- For purification of Genomic DNA: DNAeasy Kit (QIAGEN, Cat. # 69504)

For Reverse Transcription of total RNA from target cells

- Reverse Transcriptase (Recommended: M-MLV Reverse Transcriptase (10 U/μl), Epicentre, Cat. # M6125H with 10X Reverse Transcription buffer and DTT; or M-MLV Reverse Transcriptase (200 U/μl), Invitrogen, Cat. # 28025-013 with 5X Reverse Transcription buffer and DTT)
- dNTP set, 100 mM (Amersham, Cat. # 27-2035-01). Before using, mix together the four dNTP to make a final concentration of 10 mM of each dNTP.

For PCR Amplification of shRNA inserts

- Taq DNA polymerase (Recommended: Titanium™ Taq DNA Polymerase (50X), Clontech, Cat. # 639208 with 10X Titanium buffer)
- dNTP set (Amersham, Cat. # 27-2035-01)
- Thermal Cycler (DNA Engine, MJ Research, Cat. # PTC-200)
- 2.5% 1X TAE Agarose gel

For Lambda Exonuclease treatment of biotinylated shRNA targets

- Lambda Exonuclease (Recommended: Lambda Exonuclease (10 U/μl), New England BioLabs, Cat. # M0262S with 10X ExoLambda buffer)

For Purification of amplified shRNA inserts

- PCR purification kit (Recommended: QIAquick PCR Purification Kit, QIAGEN, Cat. # 28106)

For Hybridization of shRNA targets with Affymetrix GeneChip®

- For Human 50K Libraries: Human Genome U133+2.0 GeneChip® Array (Affymetrix, Cat. # 900470)
- For Mouse 40K Libraries: Mouse Genome 430 2.0 GeneChip® Array (Affymetrix, Cat. # 900495)
- Reagents for standard hybridization, washing, and staining of Affymetrix GeneChip® Arrays

F. Additional Supporting SBI Products and Services

- **Custom Hybridization and Analysis for GeneNet™ shRNA Libraries** (Cat. # CS902A-1)
  You provide cell samples transduced with SBI's GeneNet™ shRNA Library. We purify RNA/DNA, determine MOI, generate hybridization targets, hybridize them with the corresponding GeneChip® microarray, and provide you results of data analysis.

- **Custom shRNA Libraries** (Cat. # CS901A-1)
  You provide a list of the 100-50,000 genes for any organism with GenBank accession numbers. We design shRNAs, clone them in any of SBI's shRNA

Lentivectors, and provide you the shRNA library in plasmid and/or packaged form with all necessary supporting information.

- **Custom shRNA Constructs in Lentivectors** (Cat. # CS900A-1)
  You provide names of the genes with GenBank accession numbers. We design shRNAs, clone them in any of SBI's shRNA Lentivectors, and provide you the shRNA construct in plasmid and/or packaged form with all necessary supporting information.

- **Plasmid GeneNet™ shRNA Libraries**
  For production of packaged HIV or FIV-based GeneNet™ shRNA Libraries in your cell culture facility. The amount of plasmid is enough in order to produce at least 10^9 ifu of packaged pseudoviral particles. A complete protocol is available in the "Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells" user manual located on SBI's website (www.systembio.com).

  HIV-Based:
  - GeneNet™ Human 50K Plasmid shRNA Library in pSIH1-H1-Puro (200 μg), Cat. # SI606PB-1
  - GeneNet™ Mouse 40K Plasmid shRNA Library in pSIH1-H1-Puro (200 μg), Cat. # SI622PB-1

  FIV-Based:
  - GeneNet™ Human 50K Plasmid shRNA Library in pSIF1-H1-Puro (200 μg), Cat. # SI206PB-1
  - GeneNet™ Mouse 40K Plasmid shRNA Library in pSIF1-H1-Puro (200 μg), Cat. # SI222PB-1

- **293TN Human Kidney Producer Cell Line** (SBI, Cat. # LV900A-1)
  For packaging of plasmid GeneNet™ shRNA Libraries and lentivector constructs

- **pPACKH1™ Lentivector Packaging Kit** (Cat. # LV500A-1)
  For packaging of HIV-based lentivector expression constructs.

- **pPACKF1™ Lentivector Packaging Kit** (Cat. # LV100A-1)
  For packaging of FIV-based lentivector expression constructs.

- **pSIH1-H1·siLuc-copGFP Packaged Positive Transduction Control** (>2×10^5 ifu) (Cat. # LV601B-1) *(included with GeneNet™ shRNA Libraries in pSIH Vectors)*
  Packaged positive control HIV-based lentivector allows you to measure transduction efficiency in target cells based on percent of GFP-positive cells. The H1·siLuc lentivector also expresses an shRNA targeting Luciferase.

- **pSIF1-H1·siLuc-copGFP Packaged Positive Transduction Control** (>2×10^5 ifu) (Cat. # LV201B-1) *(included with GeneNet™ shRNA Libraries in pSIF Vectors)*
  Packaged positive control FIV-based lentivector allows you to measure transduction efficiency in target cells based on percent of GFP-positive cells. The lentivector also expresses an shRNA targeting Luciferase.

- **Global UltraRapid Lentiviral Titering Kit** (Cat. # LV961A-1, human and mouse compatible)
  The Global UltraRapid Lentiviral Titer Kit is designed to rapidly determine the titers of pseudoviral particles that are generated with SBI's HIV and FIV lentiviral vectors or libraries. It allows users to measure the copy numbers of integrated lentiviral constructs in genomic DNA of transduced target cells.

- **shRNA Cloning and Expression Lentivectors** (many)
  These HIV and FIV-based single-promoter shRNA cloning vectors allow you to clone and express shRNA constructs for positive control genes, which are involved in your biological mechanism of interest and will be enriched for (depleted) in the phenotypical selection step. For a list of currently available vectors, please visit our website at www.systembio.com.

- **cDNA Cloning and Expression Lentivectors** (many)
  These HIV and FIV-based cDNA cloning vectors allow strong and ubiquitous expression of your gene of interest involved in your biological pathway of interest. Choose from copGFP or puromycin selection markers. For a list of currently available vectors, please visit our website at www.systembio.com.

- **pGreenFire™ Transcriptional Reporter Lentivectors** (many)
  HIV and FIV-based transcriptional reporter vectors, available in plasmid form or pre-packaged in pseudoviral particles. These vectors allow the creation of stable reporter cell lines, which measure activation of specific signaling pathways and can be used as a read-out system in genetic screen experiments with GeneNet™ shRNA libraries. For a list of currently available vectors, please visit our website at www.systembio.com.

G. Safety Guidelines

SBI's lentiviral vectors are efficient gene transfer vehicles, as used for research applications, because of their stable integration in non-dividing and dividing cells and long-term transgene expression. Along with our understanding that lentiviral vectors offer solutions for research applications, biosafety concerns have uncovered risks due to insertional mutagenesis, the generation of replication competent lentiviruses and vector mobilization.

Both HIV-based and FIV-based lentivector systems are designed to maximize their 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 (in HIV-based vectors) and CMV promoter (in FIV-based vectors) upstream of 5'LTR in the lentivector allow efficient Tat-independent production of viral RNA, reducing the number of viral genes 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 that lack packaging signals. The packaging plasmids share no significant homology to any of the expression lentivectors, pVSV-G expression vector, or any other vector in order to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (*gag, pol, rev)* will be present in the packaged viral genome, as they are expressed from packaging plasmids lacking packaging signal. Therefore, the lentiviral particles generated are replication-incompetent.
- Produced pseudoviral particles will carry only a copy of your expression construct.

The choice of SBI's lentiviral system for experimental studies is driven by functional considerations, including increased productivity and transduction efficiency. The design of SBI's biosafe vectors has benefited researchers allowing them to conduct experimental studies with lower risk. Currently, SBI's vectors combine improved safety features (that decrease the risk of recombination and vector mobilization) with increased transduction efficiency.

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/bmbl4/bmbl4s3.htm.
It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and to always follow standard microbiological practices, which include:

- Wear gloves and a lab coat when handling the lentiviral vectors, pseudoviral particles, or transduced cells.
- Always work with pseudoviral particles in a Class II laminar flow hood.
- Perform all procedures carefully to minimize splashes, spills or the production of aerosols.
- Decontaminate work surfaces at least once a day or after any spill of viable material.
- Decontaminate all cultures, stocks, and other regulated wastes before disposal by an approved decontamination method, such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area should be placed in a durable, leak-proofed, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.

II. Protocol

A. Procedure Outline and General Comments

GeneNet™ shRNA libraries provide a high-throughput functional genomics approach that focuses on the identification of genes responsible for various biological processes. For general information and background on working with lentiviral technology, we recommend the General Reviews listed in the Reference Section, particularly Cann (2000) and Buchschacher et al. (2000).

The diagram below outlines the general steps required for the discovery of genes modulating a specific phenotype with the pre-made GeneNet™ shRNA library, including transduction into target cells, selection of cells with desired phenotype, and identification of phenotype-inducing shRNAs and corresponding target genes by hybridization of amplified shRNA cassettes with a GeneChip® Array.

Some key terms used in the protocol:

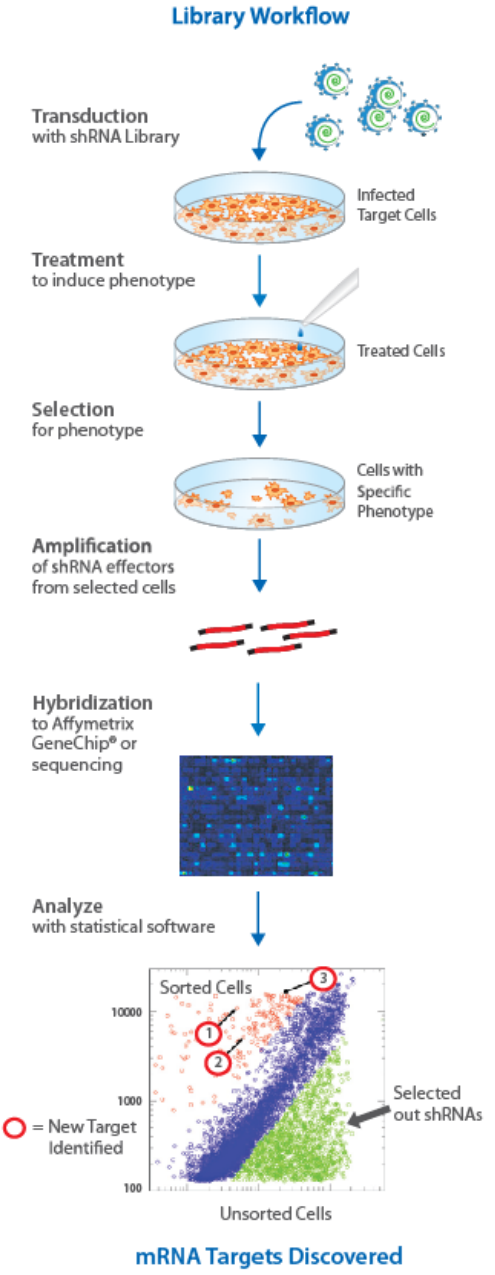
MOI (multiplicity of infection): The ratio of infectious pseudoviral particles (ifu) to the number of cells being infected. IFU/ # cells = MOI

IFU/ml (infectious units per ml): The relative concentration of infection-competent pseudoviral particles.

Transduction Efficiency: The average copy number of expression constructs per genome of target cell in the infected population.

The overall protocol includes the following steps:

1. Transduce target cells with the GeneNet™ lentiviral shRNA library provided by SBI. The lentiviral constructs integrate into the cellular genome and each cell acquires and expresses one (or a few) unique shRNA library inserts.
2. Select cells with a specific phenotypic trait (*e.g.* resistance to radiation, apoptosis, etc.) and expand surviving cells. Alternatively, select a target cell subpopulation displaying a desired phenotype by FACS or binding to Ab-beads using phenotype-specific markers, cell morphology/behavior, etc.
3. Isolate total RNA and DNA from selected and control cells.
4. Amplify and label the shRNA inserts with biotin by RT-PCR from total RNA isolated from the cells. Alternatively, you can amplify shRNA inserts from genomic DNA.
5. Remove non-biotinylated sense strand of amplified shRNA inserts by treatment with Lambda exonuclease.
6. Hybridize the biotin-labeled amplified shRNA targets with an Affymetrix GeneChip® Array. In some cases, you may alternatively clone and sequence amplified RNA inserts from selected phenotype-specific clones. *This approach, however, is very time-consuming and not suitable if there are a large numbers of different shRNA templates present in the selected cell population.* With the microarray approach, it is possible to identify shRNA effectors with a weak phenotypical effect by analyzing changes in hybridization signals between control and selected target.



B. Optimize Transduction Efficiency with the copGFP Packaged Transduction Control

Pantropic VSV-G pseudotyped viral particles containing the lentiviral shRNA construct can be efficiently used to deliver and stably express shRNA sequences in a wide range of mammalian target cells, but transduction efficiency can vary significantly depending on the target cells (see Appendix A). The packaged pSIH1 or pSIF1-H1-siLuc-copGFP control vector can be used to estimate and optimize transduction conditions for any target cells with the GeneNet™ shRNA Library. After transduction in target cells and integration into genomic DNA, the H1-siLuc-copGFP control vector stably expresses the fluorescent copGFP marker. This way, you can easily measure the percentage of transduced cells using fluorescent microscopy or flow cytometry and calculate copy number. Expression of the copGFP reporter can be measured directly at about 72 hours after transduction. The goal of transduction optimization experiments is to find the concentration of pseudoviral particles which yields a copy number between ~0.5 and 1. Within this range, you would expect that each cell that has been successfully transduced contains only one copy of a given shRNA construct. Above this range (copy number >1), successfully transduced cells may express more than one introduced shRNA construct.

To determine the concentration of pseudoviral particles required to provide a copy number between ~0.5 and 1 for your particular target cells, you should do several transductions with different concentrations of packaged copGFP transduction control. Based on the percentage of GFP-positive cells, determine the transduction efficiency. Use this simple guideline to convert the percentage of GFP positive cells to copy number. The range highlighted in yellow is the target range for copy number.

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

* Please note that copy number cannot be reliably calculated if the percentage of transduced cells is more than 90%.

Caution: You are working with infectious pseudovirus at this stage. Please, follow the recommended guidelines for working with BSL-2 class viruses (see Section I.G for more details).

Day 1

1. Plate 50,000 cells per well in a 24 well plate in cell culture medium.

Day 2

2. Cells should be between 50 to 70% confluent. Aspirate medium from cells.

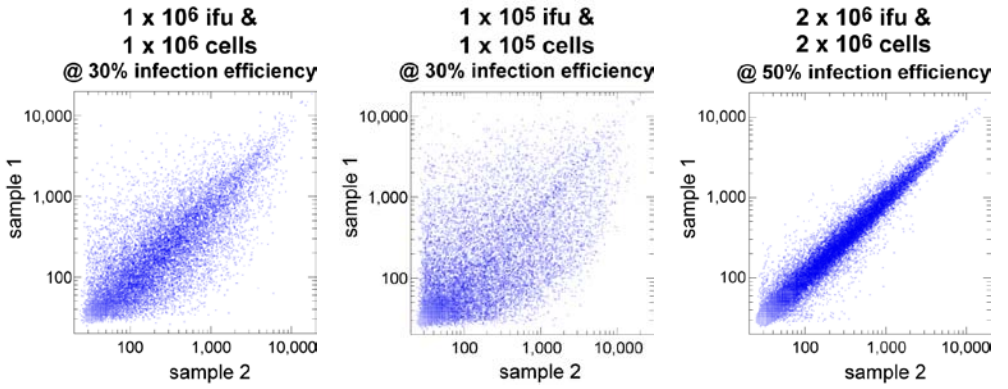
3. Combine culture medium with TransDux (LV850A-1) to a 1X final concentration.
4. Add virus to each well and swirl to mix. Add increasing amounts of virus to different wells at varying MOIs (~1, 2, 5, 10 and 20, etc.) to optimize the transduction.

Day 5

5. 72 hours post-transduction, the viral genome will be integrated into the host cell genome. Look at the cells for reporter expression if the viral construct has a reporter like GFP.
6. Aspirate off medium. Wash each well with PBS.
7. Count the fraction of fluorescent cells by FACS analysis. You may also visualize the cells for copGFP fluorescence, but the results may be less accurate due to inconsistencies in counting methods. Use an average of the fraction of green-glowing cells in 5-10 random fields of view to estimate the overall fraction of fluorescent cells on the plate (*i.e.* the fraction of infected cells). Based on the dilution factor, calculate the final concentration of pseudoviral particles which gives a copy number =1.

C. Transducing shRNA Library into Target Cells

In order to maintain representation of the entire shRNA library, the number of stably transduced cells used for transduction needs to be at least 10-fold greater than the complexity of the shRNA library. For example, you would need to transduce at least $2x10^6$ target cells when using the Human 50K shRNA Library, which has a complexity of about 200,000 cloned shRNA templates. The following data show that infecting less than the recommended amount of cells results in loss of representation of shRNA constructs when comparing duplicate populations of infected cells.



You should also consider that if more than 50% of target cells are infected by the shRNA library, some infected cells will express more than one shRNA construct and may therefore knock down more than one gene simultaneously.

Day 1.

1. Plate target cells in about six (6) 10-cm plates at a density of about 5×10^5 cells per plate 24 hours prior to viral infection. The optimal density of seeding should be adjusted in order to have about 50% confluency level with about 1×10^6 cells per plate at the time of infection (Day 2). Add 10 ml of complete optimal medium (with serum and antibiotics) per plate and incubate cells at 37°C with 5% CO_2 overnight.

Day 2.

2. Quickly thaw the GeneNet™ shRNA Library pseudoviral particles in a water bath at 37°C. Transfer the thawed particles to a laminar flow hood and keep on ice if not used immediately. Dilute an appropriate amount of GeneNet™ shRNA library (usually about 1×10^7 ifu) with 15 ml of complete medium in order to have a final concentration of pseudoviral particles equal to the concentration of packaged copGFP control vector necessary to get MOI between ~0.5 and 1. For extremely fast-growing and metabolizing cell lines, such as 293T, use 3% FBS in the medium. Add TransDux™ to a final concentration of 1x.

   Caution: Only open the tube containing the pseudoviral-packaged GeneNet™ shRNA Library in the laminar flow hood.

   Note: Gently mix the pseudovirus with the medium by rotation or inversion. Do not vortex.

   Note: The remaining pseudoviral stock may be refrozen at –70°C, but it will result in a loss of about 20% of the infection particles.

3. Remove the culture medium from cells. Infect target cells by adding the 3 ml viral stock dilutions to each of the five plates. For one plate (the mock transduction control), add 3 ml of D-MEM medium with TransDux. Incubate cells at 37°C with 5% CO_2 overnight.

Day 4.

4. By day 4, the culture will be confluent. Split 1:3, and continue incubating for 24 hours in complete D-MEM. Plate about 2×10^6 cells in a separate 6-cm plate to determine the copy number in transduced cells.

Day 5. (72 hours after transduction)

5. At this stage, you can confirm that you get a copy number close to ~0.5-1 by measuring the percentage of GFP-positive cells (using FACS or fluorescence microscopy) for shRNA libraries in the copGFP vector. If you have used a GeneNet™ shRNA Library in the Puro vector, the copy number in transduced cells

(6-cm plate from step 4) can be easily determined using SBI's UltraRapid Lentiviral Titering Kit (LV961A-1) (see Section I.E). Alternatively, the percent of stably transduced cells can be calculated based on number of puromycin-resistant colonies.

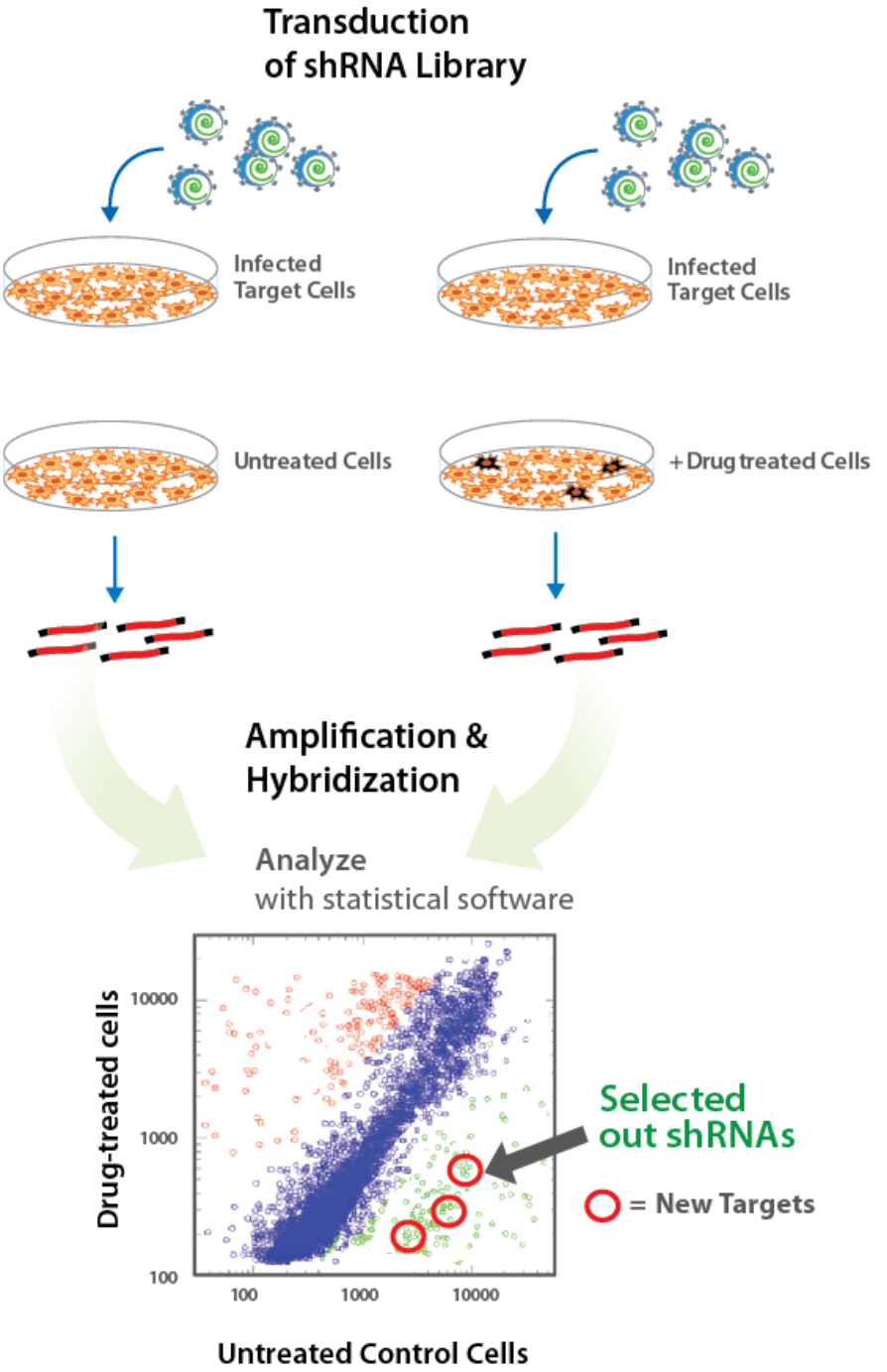
D. Screen Your Target Cells

Pools of cells that are stably transduced with GeneNet™ shRNA library constructs can be optionally enriched before selection step by FACS (copGFP vectors) or by resistance to the antibiotic puromycin (Puro vectors). shRNA constructs are usually stably integrated into genomic DNA two days following infection. Thus, you can often apply an appropriate functional screening protocol 2-3 days after transduction.

Specific screening protocols will vary depending on the biological mechanism you are studying. For general information and examples of successful genetic screening experiments, we recommend that you refer to the "Genetic Screens with shRNA Libraries" section of the bibliography in the References section. An example target screen is also shown below. To review successful screens and the resulting publications, please visit SBI's website:

http://www.systembio.com/rnai-libraries/human-genome-wide/#product_37_tab_1_3

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.


Transduction
of shRNA Library
Infected
Target Cells
Infected
Target Cells
Untreated Cells
+Drug treated Cells
Amplification &
Hybridization
Analyze
with statistical software
Drug-treated cells
10000
1000
100
100
1000
10000
Untreated Control Cells
Selected
out shRNAs
= New Targets

E. Recovering the shRNA templates from selected cells

In order to identify shRNAs from selected target cells with a specific phenotypic trait, you will need to amplify and label shRNA targets with biotin for detection when hybridizing to Affymetrix Arrays. The shRNA template inserts can be amplified from either genomic DNA or from RNA.

Isolation of RNA (please refer to Appendix F for the protocol for starting with genomic DNA)

- In addition to isolating RNA from your samples, you can also isolate RNA from non-transduced target cells. This RNA can be used as a negative control for the amplification, labeling, and hybridization.
- Optional: You can simultaneously isolate total genomic DNA to verify data generated by the total RNA and to measure copy number in the transduced cells.

1. For each fraction of selected and reference cells, detach cells from plates, collect by centrifugation, and wash in PBS. Follow standard protocols for purification of total RNA and DNA from cells. For most cell lines and tissue samples, we recommend using TRIzol Reagent (Invitrogen, Cat. # 15596-026). DNase treatment of RNA samples is not necessary for the follow-up protocol.

2. After isolating total RNA, measure the concentration (*e.g.*, by measuring absorbance at 260 nm) and examine the integrity of the RNA by electrophoresis of a sample on a denaturing formaldehyde agarose/EtBr gel or by using a BioAnalyzer (Agilent Technologies). High quality total RNA samples should appear as two bright ribosomal RNA bands at approximately 4.5 and 1.9 kb and at a ratio of about 2:1. Lower ratios are indicative of degradation.

Reverse Transcribe and Amplify Biotin-labeled shRNA Targets

Lentiviral constructs integrated into genomic DNA produce an alternative transcript from the CMV promoter that is a fusion of the marker gene (copGFP or Puro) with the shRNA sequence. This alternative transcript is used as a template to amplify the shRNA insert. Amplification of the inserts from total RNA requires two rounds of PCR. During the second round of PCR with two nested primers (one primer has biotin residues at the 5'-end and another a 5'-phospate group), the amplified shRNA targets are labeled with biotin, sense strands removed by lambda exonuclease, and biotin-labeled antisense strands are used as hybridization targets for Affymetrix GeneChip® Arrays using standard protocols.

Notes:

- In addition to amplifying and labeling RNA isolated from your samples, you should also include a positive control using 10 ng of the Positive Control DNA that is included with the GeneNet™ Library. The Positive Control DNA included in the kit is the GeneNet™ shRNA Library in plasmid form. This control can be used to optimize and troubleshoot your RT-PCR and array hybridization. Moreover, the hybridization pattern generated from the Positive Control DNA reflects the abundance level of all shRNA inserts in the packaged shRNA library and can therefore be used as a standardizing reference for all shRNA target samples rescued from your control and selected target cells.

- Titanium Taq Poly is **key** to the success of the protocol.

- Running all analytical gels while performing the protocol is important because this can help troubleshoot any discrepancies detected early on in the process rather than later.

- After running the First Round, there should be a bright band to indicate the quality of the product. There should also **only** be one band after the First Round. ***Overcycling will lead to dimers forming.***

- A negative control can also be included with your samples. The negative control should contain RNA isolated from target cells that have not been transduced with the GeneNet™ library.

1. Reverse Transcription - cDNA

Note: The following protocol is optimized for the enzymes and reagents recommended in Section I.E; specifically, Epicentre's M-MLV Reverse Transcriptase and 10X reaction buffer. Other enzymes may require somewhat different conditions.

a. For each sample, combine the following reagents in a 0.5 ml reaction tube:

5-15	μl	Total RNA sample (5-10 μg)* (use up to *50 μg if possible for better results*)
1	μl	cDNA Synthesis GNF/GNH Primer (10 μM)
		Deionized H_2O (add up to 16 μl final volume)
16	μl	Total volume (*50-100 μl if possible for better results*)

* Use 5 μg if the RNA concentration is low.

b. Mix contents and spin the tubes briefly in a microcentrifuge.

c. Incubate the tubes at 72°C in a hot-lid thermal cycler for 2 min, and then reduce the temperature to 42°C.

d. Prepare a cDNA synthesis Master Mix for all reaction tubes, plus one additional tube, using the following proportions. Combine the components in the order shown:

Per Tube	
2 µl	10X Reverse Transcriptase Buffer
1 µl	DTT (100 mM)
1 µl	dNTP mix (10 mM of each dNTP)
4 µl	Total volume

e. Mix contents by vortexing, and spin the tube briefly in a microcentrifuge.

f. Aliquot 4 µl of cDNA synthesis Master Mix into each tube from Step 1.c, and mix contents by gently pipetting up and down.

g. Add 1 µl (10 units) of M-MLV Reverse Transcriptase into each tube, mix the contents by gently pipetting up and down, and place the test tubes back in the thermal cycler.

h. Incubate the tubes at 42°C for 1 hour in a hot-lid thermal cycler.

i. Stop the reaction by heating the tubes at 72°C for 5 min, and then cool to room temperature.

j. When the program is completed, take a 10 µl aliquot from each test tube and transfer to a new 0.5 ml reaction tube. For the positive control, aliquot 10 µl from the Positive Control DNA into a new 0.5 ml tube.

2. Amplification and Biotinylation

The following procedure describes the protocol for amplification of shRNA inserts from cDNA using two rounds of PCR. We have optimized the PCR cycling parameters using Clontech Titanium™ Taq DNA polymerase (see Section I.E) and a hot-lid thermal cycler (DNA Engine, MJ Research, Cat. # PTC-200). These parameters may vary with different polymerase mixes and thermal cyclers. We recommend that you also perform amplification using the Positive Control DNA (10 ng) that is included in the kit. This control can be used to optimize and troubleshoot your RT-PCR and GeneChip® hybridization steps.
Note: You will be using 10 µl of cDNA reaction from the previous step.

a. In the first round PCR (Amplification), prepare enough First Round PCR Master Mix for all reaction tubes, plus one additional tube. Combine the following components in the order shown:

Per Tube	
72 µl	Deionized H_2O
10 µl	10X Titanium Taq PCR buffer
2 µl	50X dNTP mix (10 mM of each dNTP)
2 µl	Fwd GNF/GNH (Forward) PCR Primer (10 µM)
2 µl	Rev GNF/GNH (Reverse) PCR Primer (10 µM)
2 µl	50X Titanium Taq DNA polymerase ***Do not use any alternatives!**
90 µl	Total volume

b. Mix contents by vortexing, and spin the tube briefly in a microcentrifuge.

c. Aliquot 90 µl of the PCR Master Mix into each tube from Step 1.j and place them in the hot-lid thermal cycler. **Total volume should now be 100 µl with cDNA added.**

d. Commence thermal cycling using the following program:

94°C for 2 min
(94°C for 30 sec, 68°C for 1 min), 18 cycles
68°C for 3 min
15°C hold

e. When the program is complete, analyze a 5 µl sample from each tube alongside a 50 bp DNA size marker by running on a 2.5% agarose/EtBr gel in 1X TAE. ***Note: This will leave 95 µl of Master Mix remaining.*** Compare your results to those below to confirm that your reactions were successful.

f. Aliquot 1 µl from each tube into **at least** six new 0.5 ml reaction tubes. You will need about 6 PCR reactions per sample to obtain enough biotin-labeled shRNA target (about 10 µg—repeat previous steps if <10 µg.) for hybridization with a GeneChip® Array. Contents in tubes may also be combined to obtain the desired 10 µg. ***Note: If you use 6 µl in 6 tubes, you should still have 89 µl remaining in case you need to go back and repeat this step later. Make sure to save this until you are sure that the reactions have been successful*.**

g. In the Second Round (PCR), prepare a Second Round PCR Master Mix for all reaction tubes, plus one additional tube, using the following proportions. Combine the components in the order shown:

Per Tube		(6 tubes per sample now) + Biotin Label
66	µl	Deionized H_2O
10	µl	10X Titanium Taq PCR buffer
2	µl	50X dNTP mix (10 mM of each dNTP)
10	µl	NRev GNF/GNH (Nested Reverse) Universal Primer (10 µM) *
10	µl	NFwd-Bio (Nested Forward Biotinylated) PCR Primer (10 µM)
2	µl	50X Titanium Taq DNA polymerase ***Do not use any alternatives!**
100	µl	Total volume per tube

h. Mix contents by vortexing, and spin the tubes briefly in a microcentrifuge.

i. Aliquot 100 µl of the PCR Master Mix into each tube with the 1 µl aliquot from Step 2.e, and place them in the hot-lid thermal cycler. ***Note: This will give a total volume of 101 µl of Master Mix per tube (6 tubes total).***

j. Commence thermal cycling using the following program:

(94°C for 2 min, 50°C for 2 min, 68°C for 1 min), 1 cycle
(94°C for 30 sec, 68°C for 30 sec), 18 cycles
68°C for 3 min
15°C hold

3. Run Gel

When the program is completed, analyze a 1 µl sample from each tube alongside a 50 bp DNA size marker by running on a 2.5% agarose/EtBr gel in 1X TAE. Compare your results to Figure 9 to confirm that your reactions were successful. ***Note: If the yield of expected PCR products is less than those in the positive control sample based on the intensity of the gel bands, perform an additional 2-3 cycles of PCR at (94°C for 30 sec, 68°C for 1 min). Alternatively, you can repeat the second-round PCR starting from a 5 µl aliquot from step 2.e.***

4. PCR Purification

Purify PCR products with QIAGEN's QIAquick PCR Purification kit (see Section I.E) with the following **modifications to the manufacturer's protocol:**

- For each of the **6** PCR reaction tubes, add **six volumes** of PB buffer and bind to a single QIAquick column.
- Perform the wash step **twice**, using 0.5 ml of washing buffer for each wash.

- For maximum PCR product recovery, elute PCR product from the column once with 22 μl of elution buffer, followed by a second elution with 22 μl of elution buffer. The total volume should be approximately 40 μl after elution.
- Combine all eluates from each sample into one tube. The total volume should be about 260 μl. Take a 1 μl sample from each test tube, dilute it in an appropriate volume of TE, and measure the yield of PCR products using a spectrophotometer at 260 nm. The expected yield of PCR products should be approximately 15-25 μg.

5. Lambda Exonuclease Treatment

Notes: Overdigestion with lambda exonuclease will lead to product degradation. We also recommend the user to optimize the time on this step.

To remove the sense non-biotinylated strand, we additionally treat all PCR products with exonuclease Lambda. This exonuclease destroys the sense strand with the 5'-phosphate group, leaving the single-stranded biotinylated antisense shRNA strand.

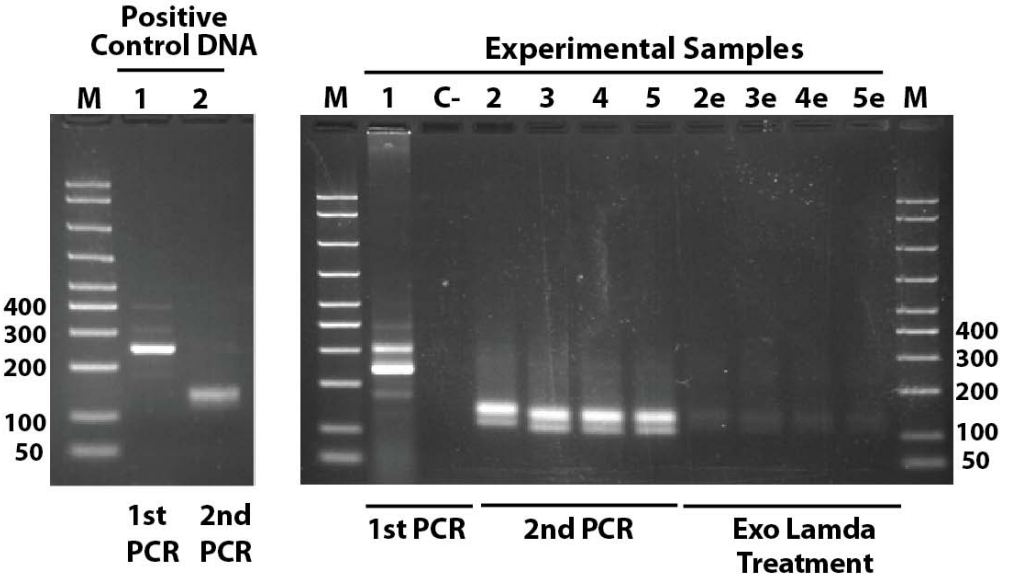
a. For each PCR sample (from step 2.k), into a 1.5 ml tube, add 260 μl of the purified PCR product, 34 μl of 10x ExoLambda buffer, 39.5 μl of Lambda Exonulease (197.6 Units, New England BioLabs, Cat. # M0262S), and incubate at 37°C for 2 hours.

b. When the program is completed, analyze a 1 μl sample from each tube and a 1 μl sample from each tube from Step 2.k) alongside a 50 bp DNA size marker by running on a 3% agarose/EtBr gel in 1X TAE to ensure that the double-stranded PCR product has been degraded. Figure 9 shows results of this analysis.

c. Purify PCR products using QIAGEN's QIAquick PCR Purification kit with the following modifications to the manufacturer's protocol:

   - For the lambda exonuclease reaction (~330 μl), add ten volumes of PB buffer (3.4 ml) and sequentially apply 0.5 ml at a time to three QIAquick columns.
   - Perform the wash step two times (instead of one) using 0.5 ml of washing buffer for each wash.
   - For maximum PCR product recovery, elute PCR product from each column once with 22 μl of elution buffer, followed by a second elution with 22 μl of elution buffer. Combine all eluates for each sample into one test tube and concentrate by vacuum centrifugation to a 50 μl volume.

d. Take a 1 μl sample from each test tube, dilute it in an appropriate volume of H_2O, and measure the yield of PCR products using a spectrophotometer at 260 nm. The yield of single-stranded shRNA products should be approximately 10 μg for all samples.



Analysis of shRNA insert products amplified by RT-PCR from total RNA. In this experiment, an HIV-based GeneNet™ Human 50K shRNA Library in pSIH1-H1-Puro was used to transduce H1299 cells.

1 (with Fwd GNH + Rev GNH primers) – First PCR (step E.2.e);
2 (NFwd-Bio + NRev GNH), **3** (NFwd-Bio + NRev GNH1), **4** (NFwd-Bio + NRev GNH2), **5** (NFwd-Bio + NRev GNH3) – Second PCR (step E.2.j);
2e, 3e, 4e, 5e – Products 2,3,4,5 treated by lambda exonuclease (step E.3.b);
C$^-$ – Negative control (no cDNA synthesis)

The shRNA template recovery procedure enables you to amplify the entire pool of shRNA inserts from the enriched cell population, or to retrieve individual shRNA templates from separate colonies selected by the phenotype-specific screening protocol. For most experiments, microarray analysis provides the most efficient way to analyze enrichment of phenotype-associated shRNAs in the complex shRNA population. The CD included in the kit provides the necessary software for analysis of Affymetrix raw data in order to correlate it with the sequences of the shRNAs present in GeneNet™ shRNA library.

F. Hybridize Biotin-labeled shRNA Targets with GeneChip® Array

Hybridization of GeneChip® Arrays with biotinylated shRNA targets is the most effective way to identify phenotype-associated shRNAs in the wide range of biological systems. The compact disc included in the kit provides the necessary software for

analysis of hybridization data, and it contains the sequences of shRNAs present in the GeneNet™ shRNA library.

Hybridize about 10 μg (minimum required amount is 6 μg) of biotinylated shRNA target with the specific Affymetrix GeneChip® Array required for your particular shRNA library, using the manufacturer's standard protocols and recommended reagents. Use Affymetrix Hybridization buffer with DMSO and hybridize at 45°C overnight.

The software provided with the library on the GeneNet™ shRNA Library Data Analysis Software and Gene List CD will enable you to analyze the hybridization data and create a report file in a format compatible with common spreadsheets and statistical programs. The file lists the intensities of signal, which correspond to the abundance level, for each of the specific shRNA species in the library. The Excel data can be analyzed and presented in conventional formats, such as scatter plots or histograms, using any of the standard statistical analysis software packages (*e.g.*, Systat) or expression data analysis software (*e.g.*, Spotfire, GeneSpring, etc). For more information, please see the documentation included with the software.

An example of a scatter plot analysis of the representation of shRNA inserts involved in radiation resistance in HT1080 cells transduced with a GeneNet™ Human 1.5K shRNA Library is shown in the Appendix.

III. Troubleshooting

A. Inefficient Transduction of Packaged copGFP Transduction Control or shRNA Library into Target Cells

1. Poor infection efficiency

Target cells have too high or too low density
Plate fewer or more cells in order to have about 50% confluency at infection stage.

Target cell line may be difficult to transduce
Use a higher concentration (less fold dilution) of pseudoviral particles. Optimize the transduction protocol and use as positive control cells HT1080 cell line.

Wrong amount of TransDux™ added during infection stage
TransDux is provided as a 5x solution.

Loss of pseudoviral titer during storage
Ensure storage of the copGFP Packaged Transduction Control stock and packaged GeneNet™ shRNA Library at –70°C. Each freeze-thaw cycle causes reduction of the titer by 20-30%. Use a fresh stock for transduction. Do not keep the stock longer than 6-12 months.

Volume of infecting supernatant is too high
Keep the volume as low as possible to achieve maximal adsorption of viral particles to the cells.

2. Transduction affects target cell viability

Packaged copGFP Control or GeneNet™ shRNA Library affects target cell growth
Use a shorter transduction time to minimize the toxic effect to the target cells. Compare toxicity of HIV-based and FIV-based control constructs, which may be different for your target cells. Try replacing with a similar target cell type.

Polybrene® is toxic for target cells
Use TransDux™ instead of Polybrene.

3. No expression of copGFP reporter (or shRNAs) in target cells

The CMV promoter or H1 (U6) promoter is not functional in target cells
It is a very rare case, but the only way to solve this problem is to change the type of target cells.

B. Low Yield of shRNA Targets

1. General Recommendations

The protocol for generating biotin-labeled shRNA targets includes four main steps: reverse transcription, first-round PCR, second-round PCR and lambda exonuclease treatment. It has been optimized using the specific reagents and kits specified. We recommend reading both the manufacturer's protocols for the respective reagents and our protocol, before doing target preparation experiments. For more detailed troubleshooting of each enzymatic step, you should refer to the manufacturer's protocol.

To effectively troubleshoot the overall shRNA target preparation and hybridization, and identify possible problem steps, it is important to run, in parallel, a positive control using the Positive Control DNA (included with the library kit), and a negative control using RNA purified from target cells that were not transduced with the shRNA library. It is critical to analyze samples from each of the enzymatic steps on an agarose gel alongside the positive and negative controls as references.

2. Poor Efficiency of Reverse Transcription

RNA is of low quality or impurities, which inhibit reverse transcriptase
If you have not already done so, analyze the quality of total RNA by gel electrophoresis. If you used QIAGEN RNeasy purification, try purifying RNA with TRIzol. If you still have a problem with the RNA sample from target cells or cannot amplify PCR product from control RNA, but you can amplify shRNA inserts from positive control DNA, try another lot or supplier of reverse transcriptase.

3. Low yield of PCR product or high level of non-specific amplification

Non-optimal PCR conditions
After the first round of PCR, you may see a weak specific band or weak "smear" depending on the target RNA sample. However, the second amplification should produce a clear band with minimal smearing. If this defined band is not present, you may need to optimize the PCR. The yield and quality of PCR products depends significantly on the quality of PCR reagents, amplification parameters, PCR machine, and quality or nature of your cDNA samples. Always run PCR of your target samples alongside with the Positive Control DNA (plasmid shRNA library) and negative control cDNAs. It is very critical to use "hot-start" Taq DNA polymerase with high enzymatic activity and previously test other PCR reagents using positive controls included in the manufacturer's kit.

If, after optimizing the PCR reaction, you continue to generate a smear after the second round or in the negative control RNA, try using a "touchdown" PCR protocol in the first round of PCR by starting the cycling with a higher annealing temperature than specified in the standard protocols, then gradually reducing the annealing temperature in successive cycles until the recommended temperature is reached. For example, try the following parameters:

94°C for 2 min
(94°C for 30 sec, 72°C for 30 sec), 5 cycles
(94°C for 30 sec, 70°C for 30 sec), 5 cycles
(94°C for 30 sec, 68°C for 30 sec), 15 cycles
68°C for 3 min
15°C hold

If you do see a specific PCR product with the expected size, but its intensity is less than expected or is significantly weaker than in the positive control DNA, try adding an additional 3-5 PCR cycles at (94°C, 30 sec; 68°C, 30 sec). We do not recommend doing more than 25 cycles for the first or second round PCR. Cycling over 25 rounds often produces a high percentage of side products that can produce poor quality hybridization results.

Loss of the shRNA target during purification
Repeat purification using another column or another lot of binding buffer. Scale-up the PCR reaction and use additional QIAquick purification columns per sample if necessary. The binding capacity of one QIAquick column is 5-10 μg of PCR product. If your yield is more than 5 μg of PCR product in one PCR reaction, using two columns per reaction could recover more PCR product.

C. Weak Hybridization Signals

1. Not enough biotinylated shRNA target

Check the concentration and repeat the hybridization with a higher amount of biotin-labeled shRNA targets.

2. shRNA target is not biotinylated

Repeat PCR with another lot of NFwd-Bio Primer (contact SBI).

3. Poor hybridization

The conditions for hybridization are not optimal. The hybridization should follow standard Affymetrix procedures. Follow the troubleshooting guidelines recommended by Affymetrix.

D. Data Analysis Problems

1. General Recommendations

In the report file produced by the GeneNet™ software, you can find the estimated background value. Based on our experience, data points with an intensity value two times greater than background may be considered as reliable data points.

IV. References

General references:

Abbas-Terki, Blanco-Bose, N. Deglon, Pralong, W., and Aebischer, P. (2002) Lentiviral-mediated RNA interference. Hum. Gene Ther. 13:2197-2201.

Aza-Blanc, P., Cooper, C.L., Wagner, K., Batalov, S., Deveraux, Q.L., and Cooke, M.P. Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. Mol. Cell (2003)12: 627-637.

Berns, K., Hijmans, E.M., Mullenders, J., Brummelkamp, T.R., Velds, A., Helmerlks, M., Kerkhoven, R.M., Madiredjo, M., Nijkamp, W., Weigelt, B., Agami, R., Ge, W., Cavet, G., Linsley, P.S., Beijrsbergen, R.L., and Bernanrds, R. (2004) A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature 428: 431-437.

Buchschacher, G.L., and Wong-Staal, F. (2000) Development of lentiviral vectors for gene therapy for human diseases. Blood. 95:2499-2504.

Burns, J.C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J.K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to a very high titer and efficient gene transfer into mammalian and non-mammalian cells. Proc. Natl. Acad. Sci. USA. 90:8033-8034.

Cann, A.J.(ed). (2000) RNA Viruses. A Practical Approach. Oxford Univ. Press.

Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. (1998) A third-generation lentivirus vector with a conditional packaging system. J. Virol. 72:8463-8471.

Gould, D.J. and Favorov, P. (2003) Vectors for the treatment of autoimmune diseases. Gene Therapy 10:912-927.

Khvorova, A., Reynolds, A., and Jayasena, D. (2003) Functional shRNAs and miRNAs exhibit strand bias. Cell 115: 209-216,505.

Lee, N.S., Dohjima, T., Bauer, G., Li, H., Li, M-J., Ehsani, A., Salvaterra, P., and Rossi, J. (2002) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nature Biotechnol. 20:500-505.

Lockhart, D., Dong, H., Byrne, M.C., Follettie, M.T., Gallo, M.V., Chee, M.C., Mittmann, M., Wang, C., Kobayashi, M., Horton, H., and Brown, E.L.(1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat. Biotech. 14: 1675-1680.

Lorens, J.B., Sousa, C., Bennett, M.K., Molineaux, S.M., and Payan, D.G. The use of retroviruses as pharmaceutical tools for target discovery and validation in the field of functional genomics. Curr. Opin. In Biotechnol. (2001) 12: 613-621.

Michiels, F., Es, H., and Tomme, P. One step further towards real high- throughput functional genomics. Trends in Biotechol (2003) 21: 147-152.

Morgan, R.A., Cornetta, K. and Anderson, W.F. (1990) Application of the polymerase chain reaction in retroviral-mediated gene transfer and the analysis of gene-marked human TIL cells. Hum. Gene Ther. 1:135-149.

Paddison, P.J., Silva, J.M., Conlin, D.S., Sclabach, M., Li, M., Aruleba, S., Balija, V., O'Shaughnessy, A., Gnoj, L., Scolbe, K., Chang, K., Westbrook, T., Cleary, M., Sachldanandam, R., McCombie, W.R., Elledge, S., and Hannon, G.J. (2004) A resource for large-scale RNA-interference-based screens in mammals. Nature 428:427-431.

Pfeifer, A., Kessler, T., Yang, M., Baranov, E., Kootstra, N., Cheresh, D.A., Hoffman, R.M. and Verma, I.M. (2001) Transduction of liver cells by lentiviral vectors: Analysis in living animals by fluorescence imaging. Mol. Ther. 3:319-322.

Qin, X.F., An, D.S., Chen, I.S., and Baltimore, D. (2003) Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc. Natl. Acad. Sci. USA 100:183-188.

Quinn, T.P., and Trevor, K.T. (1997) Rapid quantitation of recombinant retrovirus produced by packaging cell clones. Biotechniques 23:1038-1044.

Reynolds, A., Leake, D., Scaringe, S., Marshall, W., Boese, Q., and Khvorova, A. RNA interference: mechanistic implications and rational shRNA design. Nat. Biotech.(2004) 22: 326-330.

Robinson, I.B., and Gudkov, A.V. Genetic suppressor elements in the characterization and identification of tumor suppressor genes. In Methods in Molecular Biology, Tumor Suppressor Genes: Pathways and Isolation Strategies (Ed.Wafik, S.E.) Humana Press Inc., Totowa, NJ. (2002) 222: 411-434.

Sui, G., Soohoo, C. Affar, E.B., Gay, F., Forrester, W.C., and Shi, Y. (2002)
A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. U.S.A 99:5515-5520.

Viskers, T.A., Koo, S., Bennett, C.F., Crooke. S.T., Dean, N.M., and Baker, B.F. Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. J. Biol. Chem. (2003) 278(9): 7108-7118.

Zheng, L., Liu, J., Batalov, S., Zhou, D., Orth, A., Ding, S., and Schultz, G. An approach to genomewide screens of expressed small interfering RNAs in mammalian cells. Proc. Natl. Acad. Sci. (2004)101: 135-140.

Wiznerowicz, M., and Trono, D. (2003) Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. J. Virology 16: 8957-8961.

Lentiviral delivery vector reviews:

Curran MA, Nolan GP. Nonprimate lentiviral vectors. Curr Top Microbiol Immunol. 2002; 261: 75-105.

Curran MA, Nolan GP. Recombinant feline immunodeficiency virus vectors. Preparation and use. Methods Mol Med. 2002; 69: 335-50.

Loewen N, Barraza R, Whitwam T, Saenz DT, Kemler I, Poeschla EM.
FIV Vectors. Methods Mol Biol. 2003; 229: 251-71.

Naldini L. Lentiviruses as gene transfer agents for delivery to non-dividing cells. Curr Opin Biotechnol. 1998 Oct; 9(5): 457-63.

Sauter SL, Gasmi M. FIV vector systems. Somat Cell Mol Genet. 2001 Nov; 26(1-6): 99-129.

Genetic Screens with shRNA libraries:

Aza-Blanc, P., Cooper, C.L., Wagner, K., Batalov, S., Deveraux, Q.L. and Cooke, M.P. (2003) Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. Molecular Cell. 12: 627-637.

Bailey, S.N., Ali, S.M., Carpenter, A.E., Higgins, C. and Sabatini, D. (2006) Microarrays of lentiviruses for gene function screens in immortalized and primary cells. Nature Methods. 3: 117-122.

Berns, K., Hijmans, E.M., Mullenders, J. et al. (2004) A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature. 428: 431-437.

Bortone, K., Michiels, F., Vandeghinste, N., Tomme, P. and van Es, P. (2004) Functional screening of viral shRNA libraries in human primary cells. Drug Discovery World. Fall: 20-27.

Brummelkamp TR, Fabius AW, Mullenders J, Madiredjo M, Velds A, Kerkhoven RM, Bernards R, Beijersbergen RL. (2006) An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors. Nat Chem Biol. 2(4):202-206.

Cullen LM, Arndt GM. (2005) Genome-wide screening for gene function using RNAi in mammalian cells. Immunol Cell Biol. 83:217-23.

Downward, J. (2004) Use of RNA interference libraries to investigate oncogenic signaling in mammalian cells. Oncogene. 23: 8376-8383.

Eggert, U.S., Kiger, A.A., Richter, C., Perlman, Z.E., Perrimon, N., Mitchison, T.J. and Field, C.M. (2004) Parallel chemical genetic and genome-wide RNAi screens identify cytokinesis inhibitors and targets. PLOS Biology. 2: 1-8.

Friedman, A. and Perrimon, N. (2004) Genome-wide high-throughput screens in functional genomics. Cur.Opin.Genet. Develop. 14: 470-476.

Huesken, D., Lange, J., Mickanin, C., et al. (2005) Design of a genome-wide shRNA library using an artificial neural network. Nature Biotechnol. 23: 995-1001.

Leung RK, Whittaker PA (2005) RNA interference: from gene silencing to gene-specific therapeutics. Pharmacol Ther.107: 222-39.

Liang Z. (2005) High-throughput screening using genome-wide shRNA libraries. IDrugs. 11: 924-926.

Moffat J, Sabatini DM. (2006) Building mammalian signalling pathways with RNAi screens. Nat Rev Mol Cell Biol. 7:177-187.

Moffat J, Grueneberg DA, Yang X, Kim SY, Kloepfer AM, Hinkle G, Piqani B, Eisenhaure TM, Luo B, Grenier JK, Carpenter AE, Foo SY, Stewart SA, Stockwell BR, Hacohen N, Hahn WC, Lander ES, Sabatini DM, Root DE. (2006) A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell. 124(6):1283-98.

Ngo VN, Davis RE, Lamy L, Yu X, Zhao H, Lenz G, Lam LT, Dave S, Yang L, Powell J, Staudt LM. (2006) A loss-of-function RNA interference screen for molecular targets in cancer. Nature. Mar 29.

Paddison, J.P., Silva, J.M., Conklin, D.S. et al. (2004) A resource for large-scale RNA-interference-based screens in mammals. Nature. 428: 427-431.

Paddison PJ, Schlabach MR, Sheth N, Bradshaw J, Burchard J, Kulkarni A, Cavet G, Sachidanandam R, McCombie WR, Cleary MA, Elledge SJ, Hannon GJ. (2005) Second-generation shRNA libraries covering the mouse and human genomes. Nat Genet. 37:1281-8.

Poulin G, Nandakumar R, Ahringer J. (2004) Genome-wide RNAi screens in Caenorhabditis elegans: impact on cancer research. Oncogene. 23: 8340-8345.

Sachse C, Echeverri CJ.(2004) Oncology studies using shRNA libraries: the dawn of RNAi-based genomics. Oncogene.23: 8384-8391.

Sachse, C., Krausz, E., Kronke, A. et al. (2005) High-throughput RNA interference strategies for target discovery and validation by using synthetic short interfering RNAs: Functional genomic investigations of biological pathways. Methods in Enzymology. 392: 242-277.

Silva, J., Chang, K., Hannon, G.J. and Rivas, F.V. (2004) RNA-interference-based functional genomics in mammalian cells: reverse genetics coming of age. Oncogene. 23: 8401-8409.

Silva JM, Li MZ, Chang K, Ge W, Golding MC, Rickles RJ, Siolas D, Hu G, Paddison PJ, Schlabach MR, Sheth N, Bradshaw J, Burchard J, Kulkarni A, Cavet G, Sachidanandam R, McCombie WR, Cleary MA, Elledge SJ, Hannon GJ. (2005) Second-generation shRNA libraries covering the mouse and human genomes. Nat Genet. 37(11):1281-8.

Sugimoto A. (2004) High-throughput RNAi in Caenorhabditis elegans: genome-wide screens and

functional genomics. Differentiation. 72:81-91.

Vanhecke, D. and Janitz, M. (2005) Functional genomics using high-throughput RNA interference. Drug. Discov. Today. 10: 205-212.

Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, Liu YP, van Duijse J, Drost J, Griekspoor A, Zlotorynski E, Yabuta N, De Vita G, Nojima H, Looijenga LH, Agami R. (2006) A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell. 124(6):1169-81.

Willingham AT, Deveraux QL, Hampton GM, Aza-Blanc P. (2004) RNAi and HTS: exploring cancer by systematic loss-of-function. Oncogene. 23(51):8392-400.

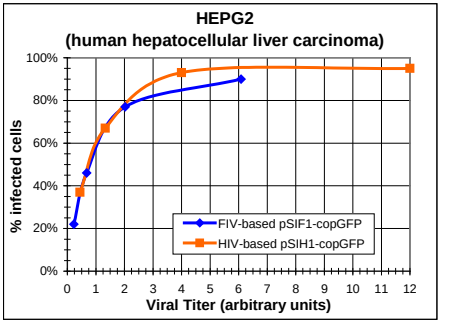
Zheng, L., Liu, J., Batalov, S., Zhou, D., Orth, A., Ding, S. and Schultz, P. (2004) Proc. Natl. Acad. Sci. 101: 135-140.

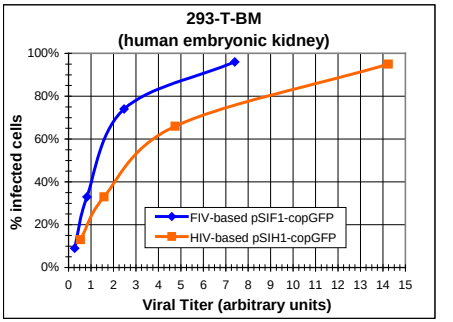
.

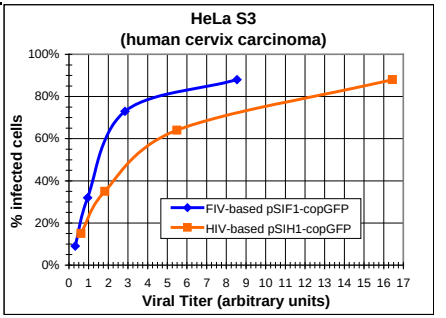
V. Appendix

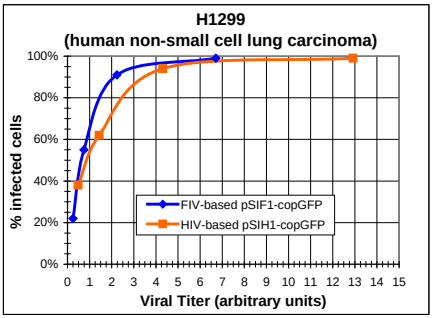
A. Transduction Efficiencies of Different Cell Lines with Increasing Relative Concentration of Viral Particles for HIV-based and FIV-based Lentivectors

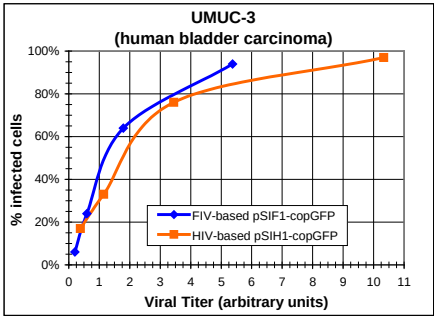
Human Cell Lines

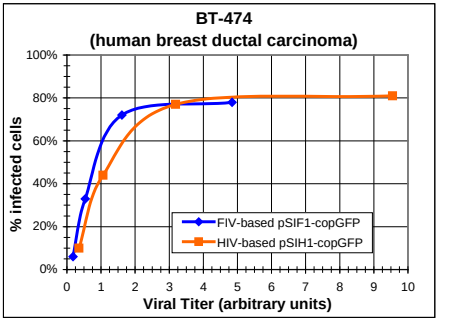




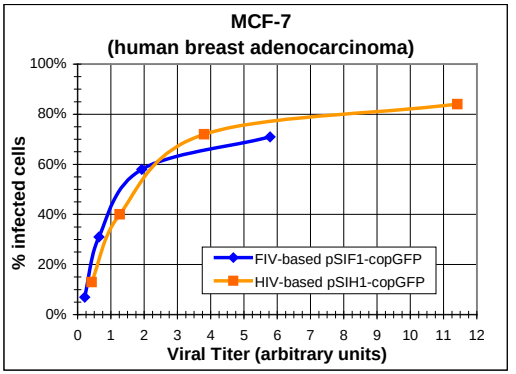


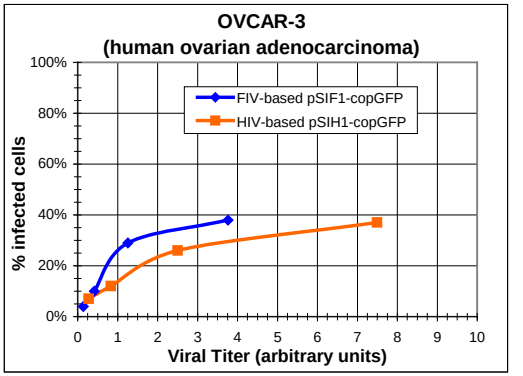


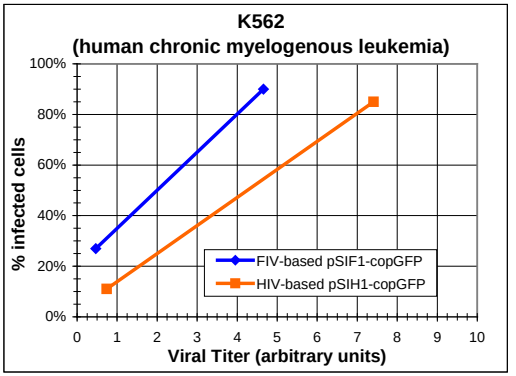


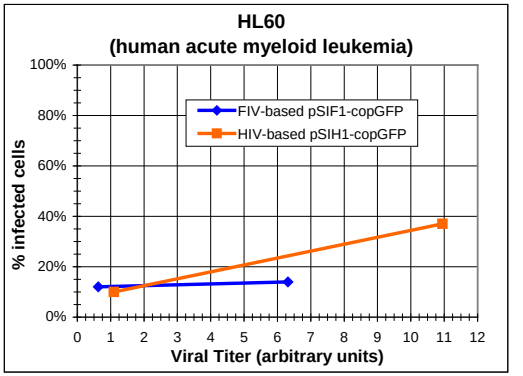


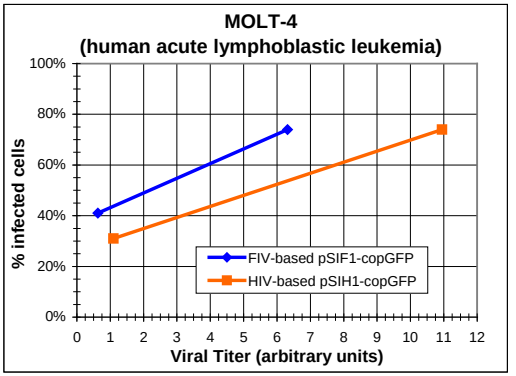
Human Cell Lines *(cont'd)*

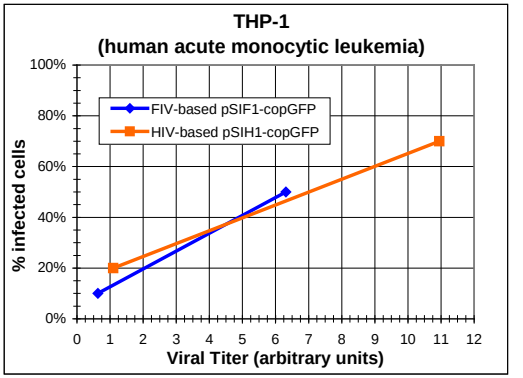




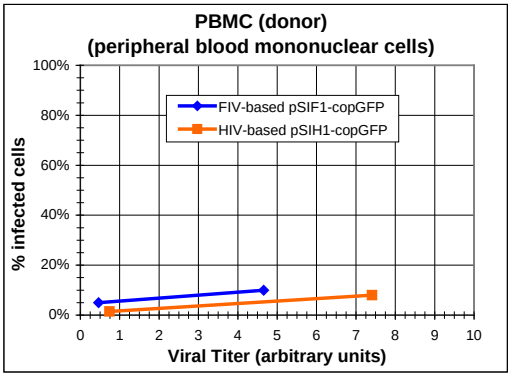


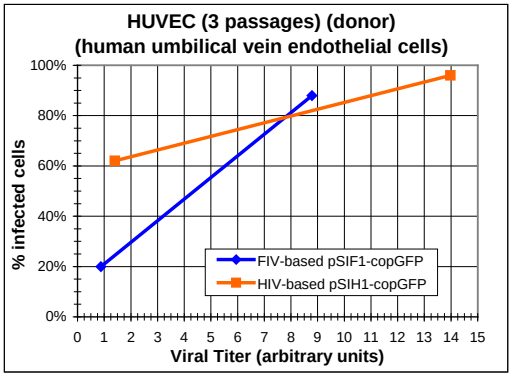


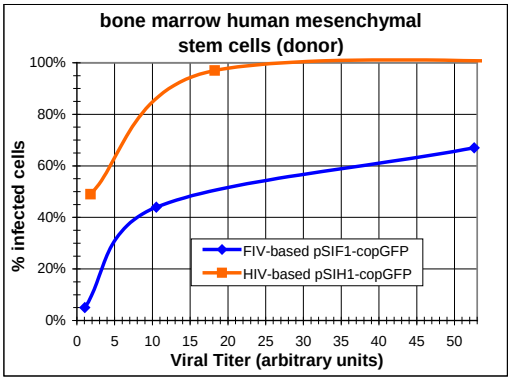


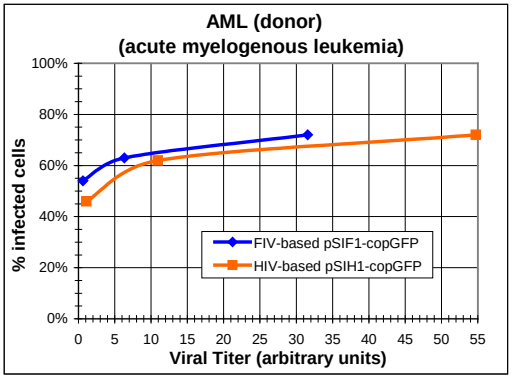


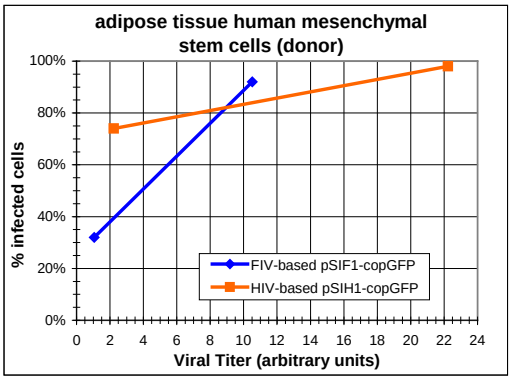
Human Primary/Stem Cell Lines



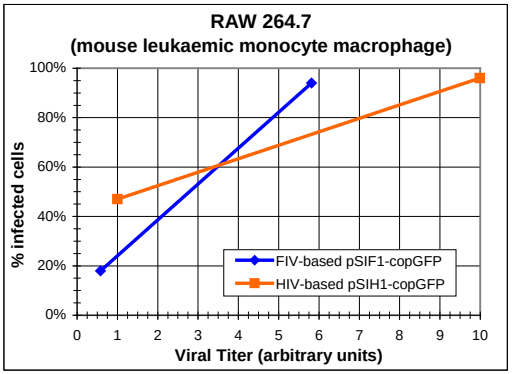


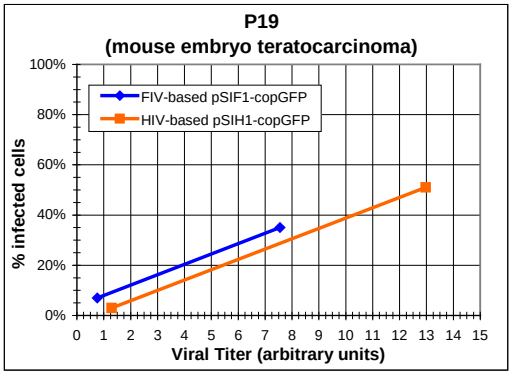


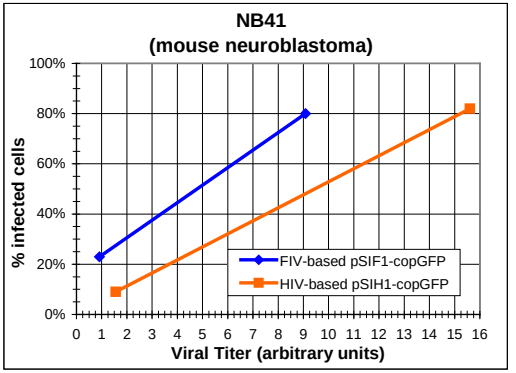


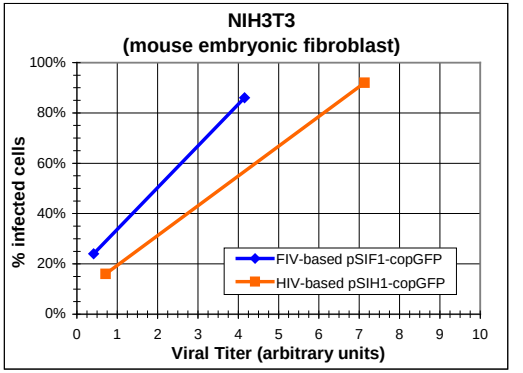


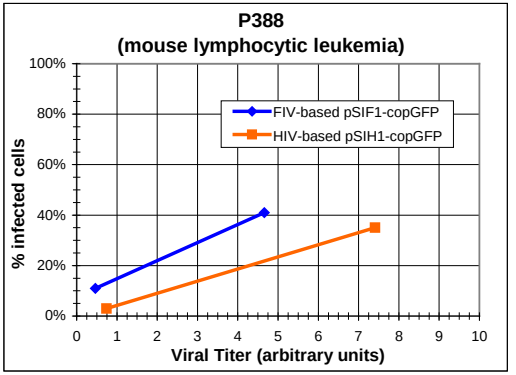
Mouse Cell Lines

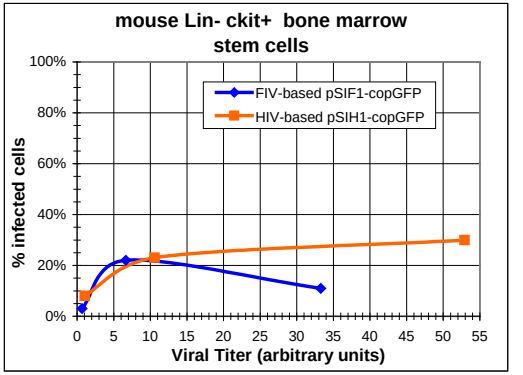




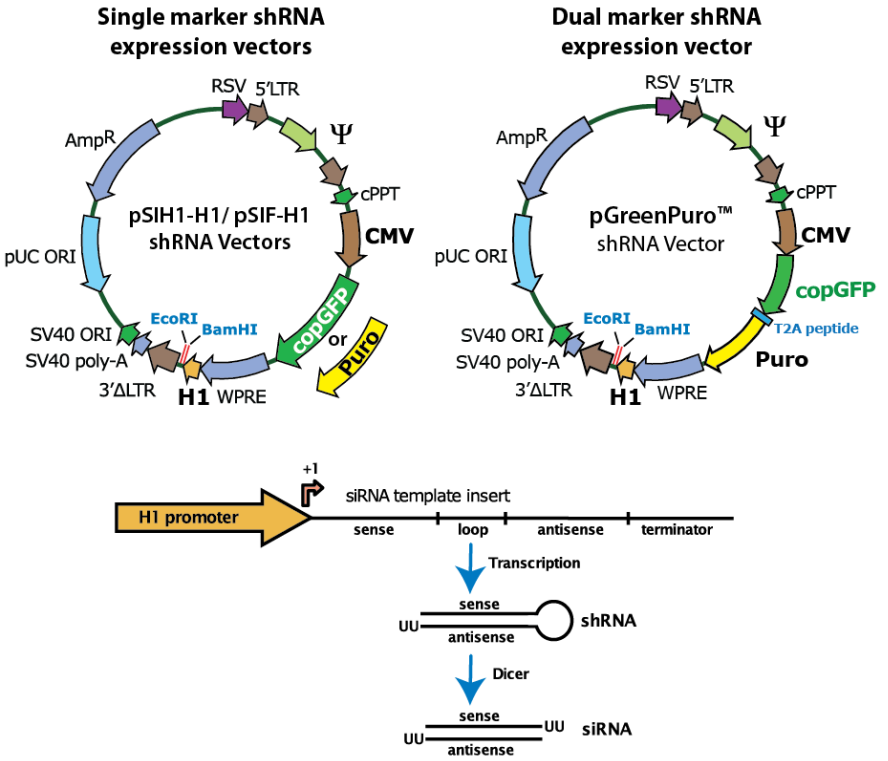








B. Maps and Features of Single-Promoter pSIH- and SIF1-H1 Vectors



The shRNA template sequence is cloned into the shRNA expression cassette which is the same for both pSIH1-H1 and pSIF1-H1 cloning vectors. shRNA template sequences are designed to be directionally inserted between the BamHI and EcoRI nucleotide overhangs (*i.e.*, sticky ends).

The shRNA sense and antisense sequences flank the region coding for the loop structure. In addition, a terminator sequence for the RNA polymerase III is included after the antisense portion. After transcription, a stem-loop-stem shRNA molecule is produced. This molecule is processed by the enzyme, Dicer, to generate a double-stranded siRNA effector.

1. pSIH1-H1-Puro Vector (Cat. # SI500A-1)

Feature	Location*	Function
RSV/5'LTR	7-414	Hybrid RSV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	567-919	Packaging signal
RRE	1076-1309	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1798-1916	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1922-2271	Human cytomegalovirus (CMV)--constitutive promoter for transcription of puromycin
Puro	2279-2878	Puromycin-resistant marker for selection of the transfected/transduced cells
WPRE	2885-3425	Woodchuck hepatitis virus posttranscriptional regulatory element--enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3564-4038	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
H1 RNA promoter	3602-3818	RNA polymerase III promoter for expression of siRNA insert
SV40 Poly-A	4110-4241	Transcription termination and polyadenylation
SV40 Ori	4250-4396	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4766-5439 (C)	Allows for high-copy replication in *E. coli*
AmpR	5584-6444 (C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

* The notation (C) refers to the complementary strand.

2. pSIH1-H1-copGFP Vector (Cat. # SI501A-1)

Feature	Location*	Function
RSV/5'LTR	7-414	Hybrid RSV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	567-919	Packaging signal
RRE	1076-1309	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1798-1916	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1922-2271	Human cytomegalovirus (CMV)--constitutive promoter for transcription of copGFP
copGFP	2279-3037	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/transduced cells
WPRE	3044-3584	Woodchuck hepatitis virus posttranscriptional regulatory element--enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3723-4197	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
H1 RNA promoter	3761-3977	RNA polymerase III promoter for expression of siRNA insert
SV40 Poly-A	4269-4400	Transcription termination and polyadenylation
SV40 Ori	4409-4555	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4925-5598 (C)	Allows for high-copy replication in *E. coli*
AmpR	5743-6603 (C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

* The notation (C) refers to the complementary strand.

3. pSIF1-H1-Puro Vector (Cat. # SI100C-1)

Feature	Location*	Function
CMV/5'LTR	1-415	Hybrid CMV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	762-1011	Packaging signal
RRE	1012-1143	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1150-1391	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1394-1745	Human cytomegalovirus (CMV)--constitutive promoter for transcription of copGFP
Puro	1753-2352	Puromycin-resistant marker for selection of the transfected/transduced cells
WPRE	2359-2947	Woodchuck hepatitis virus posttranscriptional regulatory element--enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3068-3457	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
H1 RNA promoter	3098-3312	RNA polymerase III promoter for expression of siRNA insert
SV40 Poly-A	3545-3676	Transcription termination and polyadenylation
SV40 Ori	3685-3831	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4201-4874 (C)	Allows for high-copy replication in *E. coli*
AmpR	5019-5879 (C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

* The notation (C) refers to the complementary strand.

2. pSIF1-H1-copGFP Vector (Cat. # SI101B-1)

Feature	Location*	Function
CMV/5'LTR	1-415	Hybrid CMV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	762-1011	Packaging signal
RRE	1012-1143	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1150-1391	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1394-1745	Human cytomegalovirus (CMV)--constitutive promoter for transcription of copGFP
copGFP	1753-2511	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/transduced cells
WPRE	2518-3106	Woodchuck hepatitis virus posttranscriptional regulatory element--enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3227-3616	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
H1 RNA promoter	3257-3471	RNA polymerase III promoter for expression of siRNA insert
SV40 Poly-A	3704-3835	Transcription termination and polyadenylation
SV40 Ori	3844-3990	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4360-5033 (C)	Allows for high-copy replication in *E. coli*
AmpR	5178-6038 (C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

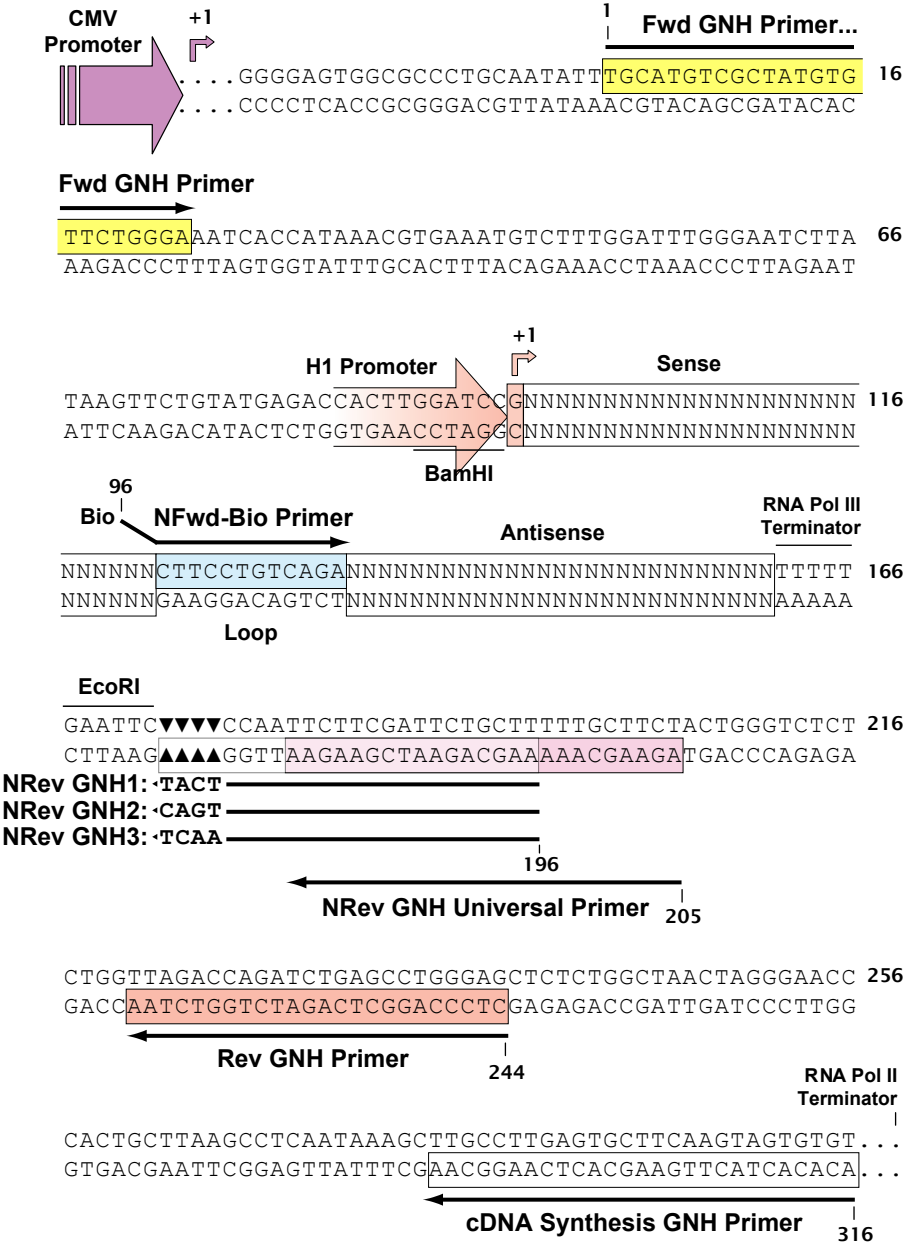
* The notation (C) refers to the complementary strand.

C. Design of the Cloning and Expression Cassette for pSIH1-H1 and pSIF1-H1 Vectors

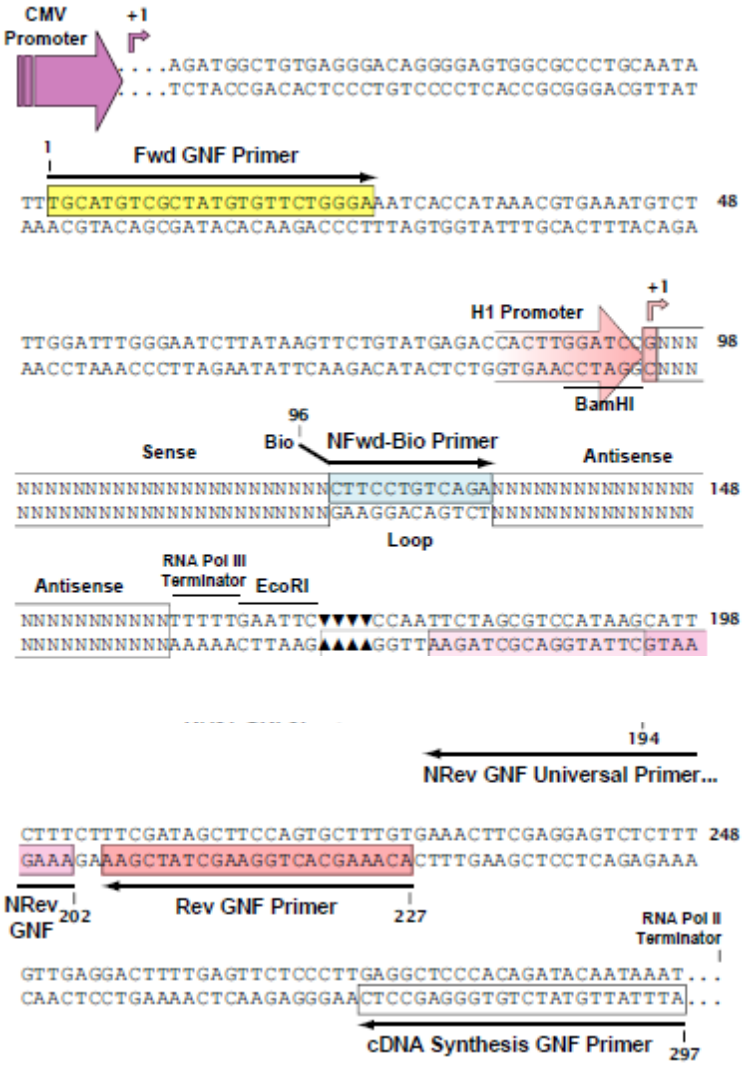
Design of the shRNA expression cassette. The shRNA template sequence is cloned into the shRNA expression cassette which is the same for both pSIH1-H1 and pSIF1-H1 cloning vectors. siRNA template sequences are designed to be directionally inserted between the BamHI and EcoRI nucleotide overhangs (i.e., sticky ends).

The nucleotides for the specific siRNA sequence are shown in capital letters. The siRNA sense and antisense sequences flank the region coding for the loop structure. In addition, a terminator sequence for the RNA polymerase III is included after the antisense portion. After transcription, a stem-loop-stem siRNA molecule is produced. This molecule is processed by the Dicer enzyme to generate a double-stranded siRNA effector.

D. Location and Sequences of Amplification Primers, pSIH1-H1 vectors



Location and Sequences of Amplification Primers, pSIF1-H1 vectors



E. Features of the copGFP Transduction Control Vectors

1. pSIH1-H1·siLuc-copGFP (Cat. # LV601B-1)

Feature	Location*	Function
RSV/5'LTR	7-414	Hybrid RSV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	567-919	Packaging signal
RRE	1076-1309	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1798-1916	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1922-2271	Human cytomegalovirus (CMV)--constitutive promoter for transcription of copGFP
copGFP	2279-3037	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/transduced cells
WPRE	3044-3584	Woodchuck hepatitis virus posttranscriptional regulatory element--enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3723-4263	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
H1 RNA promoter	3761-3977	RNA polymerase III promoter for expression of siRNA insert
siLuc	3979-4053	shRNA targeting Firefly Luciferase
SV40 Poly-A	4335-4466	Transcription termination and polyadenylation
SV40 Ori	4475-4621	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4991-5664 (C)	Allows for high-copy replication in *E. coli*
AmpR	5809-6669 (C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

* The notation (C) refers to the complementary strand.

2. pSIF1-H1·siLuc-copGFP (Cat. # LV201B-1)

Feature	Location*	Function
CMV/5'LTR	1-415	Hybrid CMV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	762-1011	Packaging signal
RRE	1012-1143	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1150-1391	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1394-1745	Human cytomegalovirus (CMV)--constitutive promoter for transcription of copGFP
copGFP	1753-2511	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/transduced cells
WPRE	2518-3106	Woodchuck hepatitis virus posttranscriptional regulatory element--enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3227-3682	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
H1 RNA promoter	3257-3471	RNA polymerase III promoter for expression of siRNA insert
siLuc	3481-3542	shRNA targeting Firefly Luciferase
SV40 Poly-A	3770-3901	Transcription termination and polyadenylation
SV40 Ori	3910-4056	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4426-5099 (C)	Allows for high-copy replication in *E. coli*
AmpR	5244-6104 (C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

* The notation (C) refers to the complementary strand.

F. Protocol for Amplification of shRNA Targets from Genomic DNA (Alternative to Section II.E)

The following protocol describes the amplification of shRNA inserts from genomic DNA of target cells transduced with the GeneNet™ shRNA library. We have optimized the PCR cycling parameters using Clontech Titanium Taq DNA polymerase (see Section I.F) and a hot-lid thermal cycler (DNA Engine, MJ Research, Cat. # PTC-200). These parameters may vary with different polymerase mixes and thermal cyclers. We recommend that you also perform amplification using 10 μl of Positive Control DNA. This control can be used to optimize and troubleshoot your PCR and GeneChip® hybridization steps.

1. Purify Genomic DNA

For each fraction of selected, and reference cells, detach cells from plates, collect and wash in PBS by centrifugation. Follow standard protocols for purification of genomic DNA. For most cell lines and tissue samples we recommend using TRIzol Reagent (Invitrogen, Cat. # 15596-026). Measure the concentration by measuring the absorbance at 260 nm.

2. Amplify shRNA Targets

a. For each sample, aliquot 5 μg (5-20 μl) of genomic DNA from step F.1 and 10 μl of Positive Control DNA, and transfer to new 0.5 ml reaction tubes. In each test tube, adjust the volume to 20 μl by adding the necessary volume of deionized water.

b. Prepare enough First Round PCR Master Mix for all reaction tubes, plus one additional tube by combining the following components in the order shown:

Per Tube	
62 μl	Deionized H_2O
10 μl	10X Titanium Taq PCR buffer
2 μl	50X dNTP mix (10 mM of each dNTP)
2 μl	Fwd GNF/GNH (Reverse) PCR Primer (10 μM)
2 μl	Rev GNF/GNH (Forward) PCR Primer (10 μM)
2 μl	50X Titanium Taq DNA polymerase
80 μl	Total volume

c. Mix contents by vortexing, and spin the tube briefly in a microcentrifuge.

d. Aliquot 80 μl of the PCR Master Mix into each tube from Step 2.a, and place them in the hot-lid thermal cycler.

e. Commence thermal cycling using the following program:

94°C for 2 min
(94°C for 30 sec, 68°C for 1 min), 20 cycles
68°C for 3 min
15°C hold

f. When the program is completed, analyze a 5 μl sample from each tube alongside a 50 bp DNA size marker by running on a 2.5% agarose/EtBr gel in 1X TAE. Compare your results to Figure 13 to confirm that your reactions were successful. Aliquot 1 μl from each tube into four new 0.5 ml reaction tubes. You will need about 4 PCR reactions per sample to obtain enough biotin-labeled shRNA target (about 10 μg) for hybridization with a GeneChip® Array.

g. Prepare a Second Round PCR Master Mix for all reaction tubes, plus one additional tube, using the following proportions. Combine the components in the order shown:

Per Tube	
66 μl	Deionized H_2O
10 μl	10X Titanium Taq PCR buffer
2 μl	50X dNTP mix (10 mM of each dNTP)
10 μl	NRev GNF/GNH (Nested Reverse) Universal Primer (10 μM)*
10 μl	NFwd-Bio (Nested Forward Biotinylated) PCR Primer (10 μM)
2 μl	50X Titanium Taq DNA polymerase
100 μl	Total volume

h. Mix contents by vortexing, and spin the tubes briefly in a microcentrifuge.

i. Aliquot 100 μl of the PCR Master Mix into each tube with the 1 μl aliquot from Step 2.f, and place them in the hot-lid thermal cycler.

j. Commence thermal cycling using the following program:

(94°C for 2 min, 50°C for 2 min, 68°C for 1 min), 1 cycle
(94°C for 30 sec, 68°C for 30 sec), 18 cycles
68°C for 3 min
15°C hold

k. When the program is completed, analyze a 1 μl sample from each tube alongside a 50 bp DNA size marker by running on a 2.5% agarose/EtBr gel in 1X TAE. Compare your results to Figure 13 to confirm that your reactions were successful. If the yield of expected PCR products is less than those in the positive control sample based on the intensity of the gel bands, perform an additional 2-3 cycles of PCR at (94°C for 30 sec, 68°C for 1 min). Alternatively you can repeat the second round PCR starting from a 5 μl aliquot from step 2.f.

l. Purify PCR products with QIAGEN's QIAquick PCR Purification kit (see Section I.E) with the following modifications to the manufacturer's protocol:

- For each PCR reaction (test tube), add six volumes of PB buffer and bind to a single QIAquick column.
- Perform the wash step two times (instead of one), using 0.5 ml of washing buffer for each wash.
- For maximum PCR product recovery, elute PCR product from the column once with 22 μl of elution buffer, followed by a second elution with 22 μl of elution buffer. Combine all four eluates from each sample into one test tube. The total volume will be about 160 μl. Take a 1 μl sample from each test tube, dilute it in an appropriate volume of TE, and measure the yield of PCR products using a spectrophotometer at 260 nm. The expected yield of PCR products should be approximately 15-25 μg.

3. Lambda Exonuclease Treatment

To remove sense non-biotinylated strands, we additionally treated all PCR products with exonuclease Lambda. This exonuclease destroys the sense strand with the 5'-phosphate group, leaving the single-stranded biotinylated antisense shRNA strand:

a. For each PCR sample (from step 2.k), add 20 μl of 10X ExoLambda Buffer, 100 units (10-20 μl) of Exonuclease Lambda (New England BioLabs, Cat. # M0262S) and incubate at 37°C for 2 hours.

b. When the program is completed, analyze a 1 μl sample from each tube and a 1 μl sample from each tube from Step 2.k) alongside a 50 bp DNA size marker by running on a 3% agarose/EtBr gel in 1X TAE to ensure that the double-stranded PCR product has been degraded.

c. Purify PCR products using QIAGEN's QIAquick PCR Purification kit with the following modifications to the manufacturer's protocol:

- For each PCR reaction (test tube), add ten volumes of PB buffer (2 ml) and sequentially apply 0.5 ml at a time to two QIAquick columns.

- Perform the wash step two times (instead of one), using 0.5 ml of washing buffer for each wash.
- For maximum PCR product recovery, elute PCR product from each column once with 22 µl of elution buffer, followed by a second elution with 22 µl of elution buffer. Combine all eluates for each sample into one test tube and concentrate by vacuum centrifugation to a 50 µl volume.

d. Take a 1 µl sample from each test tube, dilute it in an appropriate volume of H_2O, and measure the yield of PCR products using a spectrophotometer at 260 nm. The yield of single-stranded shRNA products should be approximately 10 µg for all samples. Please refer to Section II.G for information on hybridization of biotin-labeled targets with the GeneChip® Array.

G. Technical Support

For more information about SBI products, to download manuals in PDF format, and to get vector map and sequence information, visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or e-mail us at:

System Biosciences (SBI)
265 North Whisman Road
Mountain View, CA 94043
Phone: (650) 968-2200
(888) 266-5066 (Toll Free)
Fax: (650) 968-2277

E-mail: tech@systembio.com

VI. Licensing and Warranty Statement

Limited Use License

Use of the GeneNet™ shRNA Library (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms. SBI reserves the right to decide refund eligibility on a case-by-case basis.

HIV Vector System

This product is for non-clinical research use only. Use of this Product to produce products for resale or for any diagnostic, therapeutic, clinical, veterinary, or food purpose is prohibited. In order to obtain a license to use this Product for these commercial purposes, contact the Office of Research and Technology Ventures at the Dana-Farber Cancer Institute, Inc. in Boston, Massachusetts, USA. This Product or the use of this Product is covered by U.S. Patents Nos. 5,665,577 and 5,981,276 (and foreign equivalents) owned by the Dana-Farber Cancer Institute, Inc.

FIV Vector System

This Product is for non-clinical research use only. Use of this Product to produce products for sale or for any diagnostic, therapeutic, clinical (including pre-clinical), veterinary or high throughput drug discovery purpose (the screening of more than 10,000 compounds per day) is prohibited. In order to obtain a license to use this product for these commercial purposes, contact The Regents of the University of California. This Product or the use of this Product is covered by U.S. Patent No. 6,555,107 owned by The Regents of the University of California.

WPRE Technology

System Biosciences (SBI) has a license to sell the Product containing WPRE, under the terms described below. Any use of the WPRE outside of SBI's Product or the Products' intended use, requires a license as detailed below. Before using the Product containing WPRE, please read the following license agreement. If you do not agree to be bound by its terms, contact SBI within 10 days for authorization to return the unused Product containing WPRE and to receive a full credit.

The WPRE technology is covered by patents issued to The Salk Institute for Biological Studies.

SBI grants you a non-exclusive license to use the enclosed Product containing WPRE in its entirety for its intended use. The Product containing WPRE is being transferred to you in furtherance of, and reliance on, such license. Any use of WPRE outside of SBI's Product or the Product's intended use, requires a license from the Salk Institute for Biological Studies.

This license agreement is effective until terminated. You may terminate it at any time by destroying all Products containing WPRE in your control. It will also terminate automatically if you fail to comply with the terms and conditions of the license agreement. You shall, upon termination of the license agreement, destroy all Products containing WPRE in you control, and so notify SBI in writing.

This License shall be governed in its interpretation and enforcement by the laws of California.

Contact for WPRE Licensing: The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037; Attn: Office for Technology Management; Phone: (858) 435-4100 extension 1275; Fax: (858) 450-0509.

CMV Promoter

The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242.

CopGFP Fluorescent Protein

This product contains a proprietary nucleic acid coding for a proprietary fluorescent protein(s) intended to be used for research purposes only. Any use of the proprietary nucleic acids other than for research use is strictly prohibited. USE IN ANY OTHER APPLICATION REQUIRES A LICENSE FROM EVROGEN. To obtain such a license, please contact Evrogen at license@evrogen.com.

SBI has pending patent applications on various features and components of the Product. For information concerning licenses for commercial use, contact SBI.

Purchase of the product does not grant any rights or license for use other than those explicitly listed in this Licensing and Warranty Statement. Use of the Product for any use other than described expressly herein may be covered by patents or subject to rights other than those mentioned. SBI disclaims any and all responsibility for injury or

damage which may be caused by the failure of the buyer or any other person to use the Product in accordance with the terms and conditions outlined herein.

Limited Warranty

SBI warrants that the Product meets the specifications described in the accompanying Product Analysis Certificate. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

SBI's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

SBI is committed to providing our customers with high-quality products. If you should have any questions or concerns about any SBI products, please contact us at (888) 266-5066.