



# **GeneNet™ Lentiviral shRNA Libraries**

**Cat. # SI2XXB-1,SI6XXB-1**

## ***User Manual***

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**Store kit at -70°C on receipt**

(ver. 6 -080511)

**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.**

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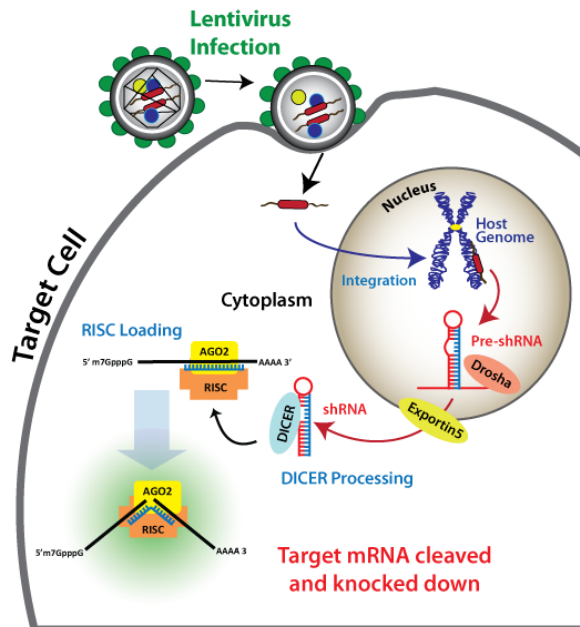
# I. Introduction and Background

## 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

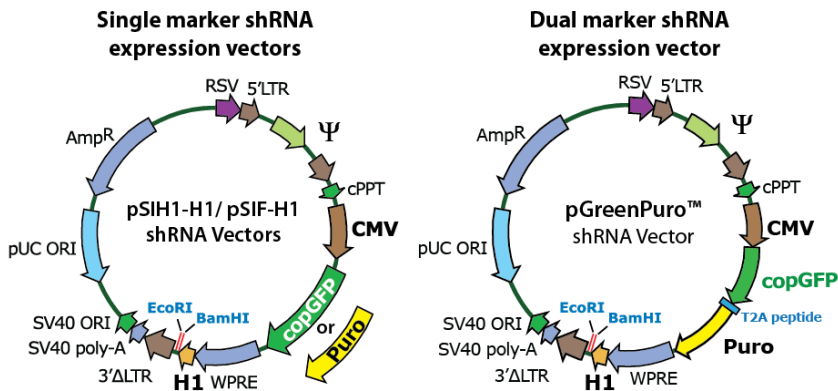
The next generation of user-friendly genetic screening technology that includes genome-wide shRNA libraries has been developed at SBI with several novel features that significantly extend the application of this technology for high-throughput functional genomics studies:

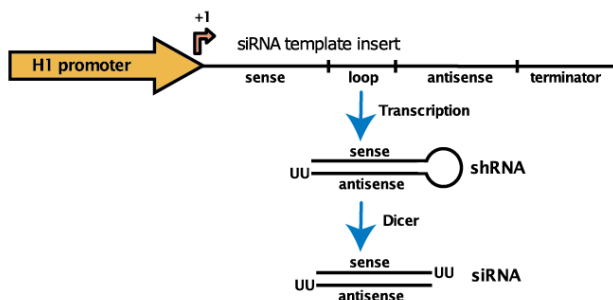
- ***Biosafe third generation lentiviral (HIV and FIV-based) shRNA Vectors*** with puromycin selection (or copGFP reporter) and the RNA polymerase III H1 promoter shRNA expression cassette for the expression of shRNA constructs.
- ***Lentiviral shRNA transduction system*** that significantly extends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

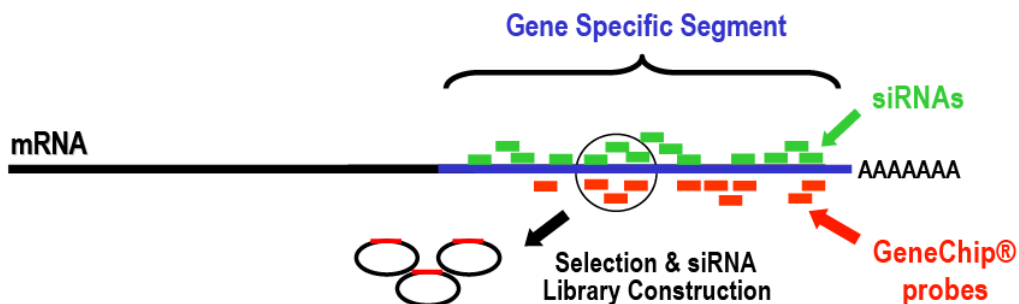
| <b>Criteria</b>            | <b>siRNA Sequence</b>                           | <b>GeneChip Probes</b>             |
|----------------------------|---|------------------------------------|
| <b>Size (nucleotides)</b>  | 19-29   | 25                                 |
| <b>Homology</b>            | Unique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

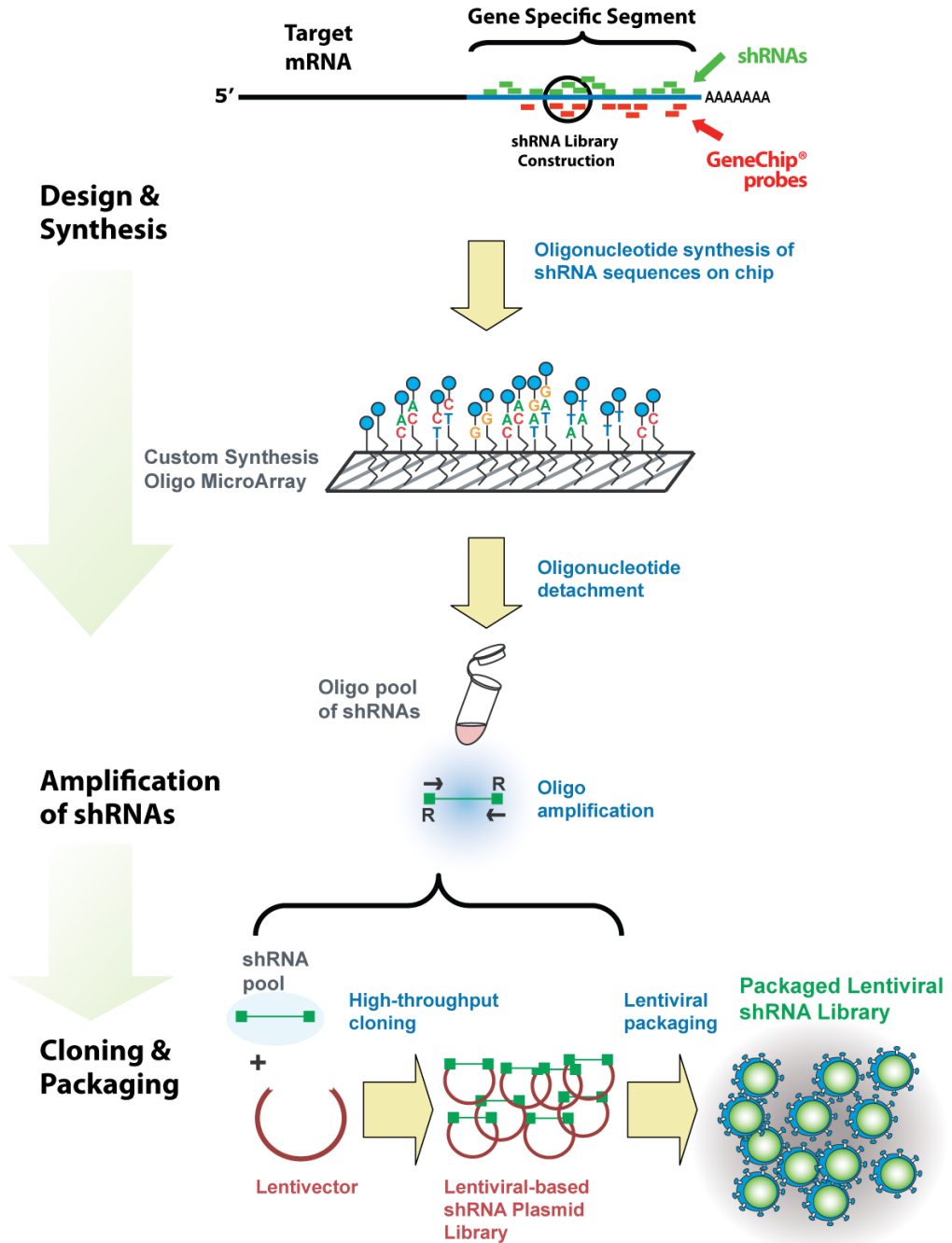
siRNAs could efficiently silence the target mRNAs (*i.e.*, reduce expression by more than 70%). These data confirm that GeneChip® probe sequences share considerable similarity with efficient siRNA sequences. Moreover, using sequences similar to the probes on the GeneChip® Array enables the use of the 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.



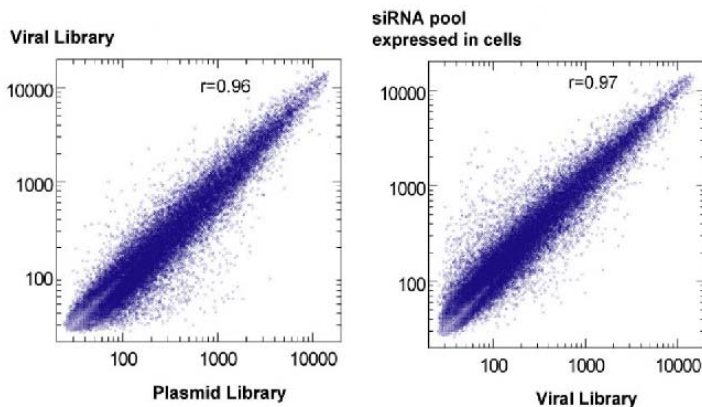
3. After synthesis, the shRNA template oligonucleotides were amplified by PCR using primers complementary to the additional flanking 5'- and 3'-sequences, digested with the appropriate restriction enzymes, and ligated to the corresponding linearized cloning vector.
4. The ligated shRNA library was then 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.



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.

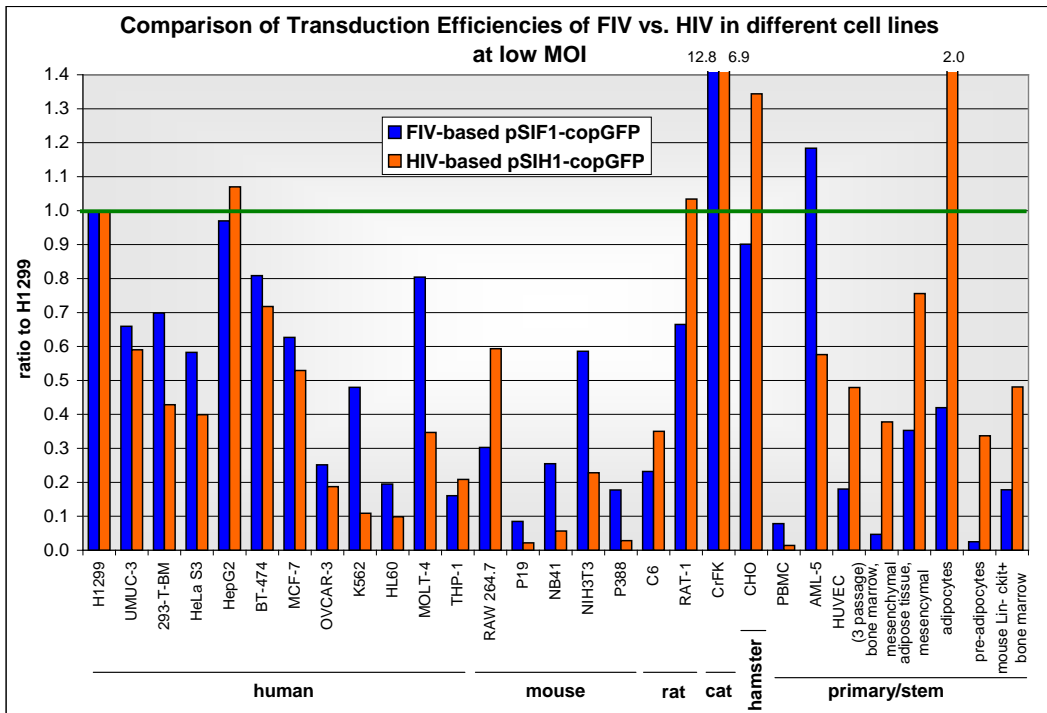
In addition, in order to test the representation of shRNA inserts in the pseudoviral packaged shRNA library, we reverse transcribed the viral RNA and amplified the shRNA inserts using flanking vector primers. As a control, we amplify the shRNA inserts from the plasmid library used in the packaging step. 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](http://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](http://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          | $2 \times 10^7$    | HG-U133+ 2.0         | 38,500    | 47,400 |
| SI222B-1  | Mouse 40K     | pSIF1-H1-Puro | 150,000          | $2 \times 10^7$    | MG-430 2.0           | 34,000    | 39,000 |
| SI606B-1  | Human 50K     | pSIH1-H1-Puro | 200,000          | $2 \times 10^7$    | HG-U133+ 2.0         | 38,500    | 47,400 |
| SI622B-1  | Mouse 40K     | pSIH1-H1-Puro | 150,000          | $2 \times 10^7$    | 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 <sup>6</sup> 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 <sup>6</sup> 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  $-70^{\circ}\text{C}$  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<sup>9</sup> 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](http://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** ( $>2 \times 10^5$  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** ( $>2 \times 10^5$  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](http://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](http://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](http://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' $\Delta$ LTR 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:**

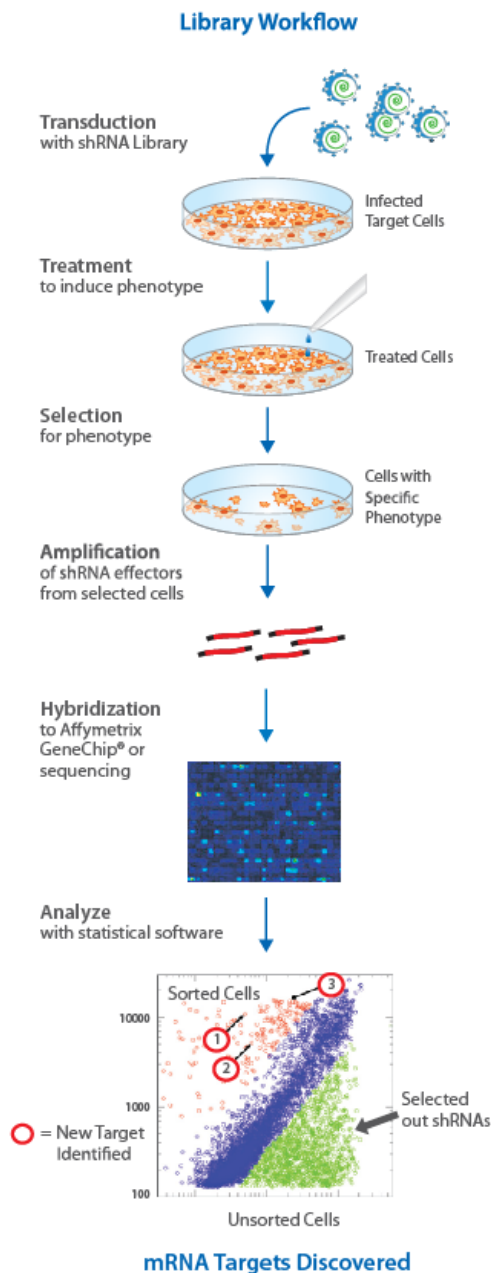
**MOI** (multiplicity of infection): The ratio of infectious pseudoviral particles (ifu) to the number of cells being infected.  $\text{IFU} / \# \text{ cells} = \text{MOI}$

**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.

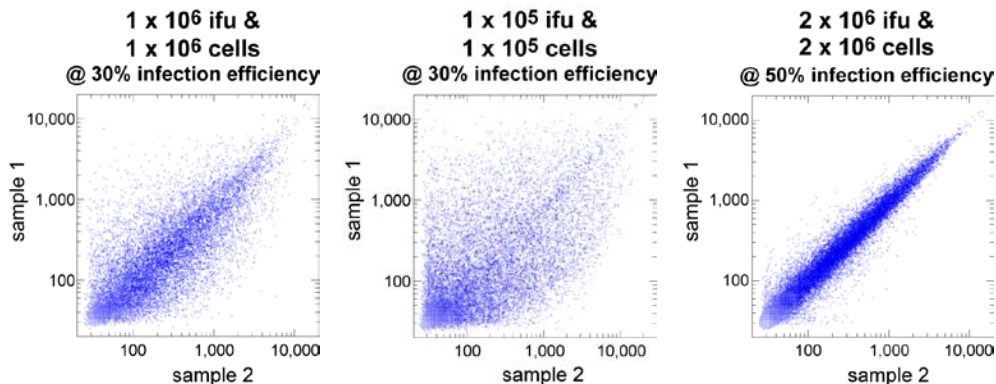
3. Combine culture medium with TransDux (LV850A-1) to a 1X final concentration.
4. Add virus to each well and swirl to mix. Add increasing amounts of virus to different wells at varying MOIs (~1, 2, 5, 10 and 20, etc.) to optimize the transduction.

### 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  $2 \times 10^6$  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  $5 \times 10^5$  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  $1 \times 10^6$  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<sub>2</sub> 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  $1 \times 10^7$  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<sub>2</sub> 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  $2 \times 10^6$  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

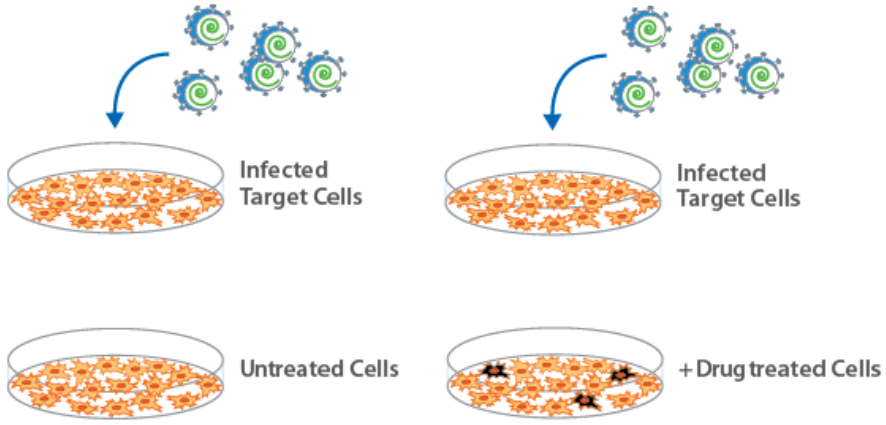
Pools of cells that are stably transduced with GeneNet™ shRNA library constructs can be optionally enriched before selection step by FACS (copGFP vectors) or by resistance to the antibiotic puromycin (Puro vectors). shRNA constructs are usually stably integrated into genomic DNA two days following infection. 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](http://www.systembio.com/rnai-libraries/human-genome-wide/#product_37_tab_1_3)

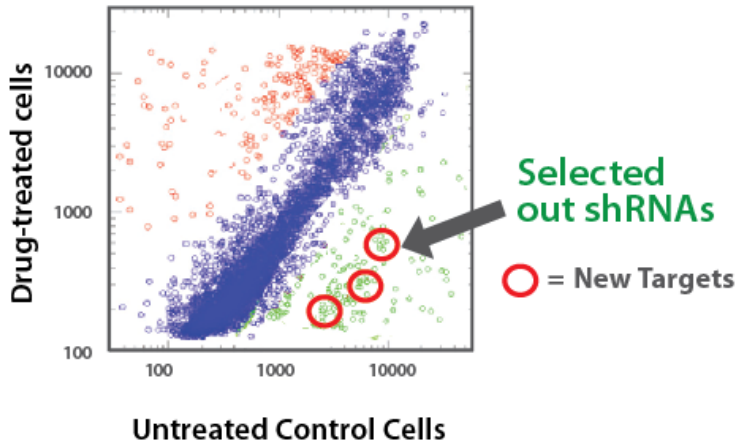
Although the specific protocol and controls may be different depending on the cell type, functional assay, and selection protocol (e.g., FACS, apoptosis induction, toxic chemical survival, etc.), it is critical to carefully design your experiment in order to generate statistically significant data.

### Transduction of shRNA Library



### Amplification & Hybridization

Analyze with statistical software



## 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'-phosphate 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<sup>®</sup> 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 <sub>2</sub> O (add up to 16 μl final volume)                    |
| <hr/> |    |  |
| 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 $\mu$ l | 10X Reverse Transcriptase Buffer |
| 1 $\mu$ l | DTT (100 mM)                     |
| 1 $\mu$ l | dNTP mix (10 mM of each dNTP)    |
| <hr/>     |                                  |
| 4 $\mu$ l | Total volume                     |

- e. Mix contents by vortexing, and spin the tube briefly in a microcentrifuge.
- f. Aliquot 4  $\mu$ l of cDNA synthesis Master Mix into each tube from Step 1.c, and mix contents by gently pipetting up and down.
- g. Add 1  $\mu$ l (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  $\mu$ l aliquot from each test tube and transfer to a new 0.5 ml reaction tube. For the positive control, aliquot 10  $\mu$ l 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  $\mu$ l 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 $\mu$ l | Deionized H <sub>2</sub> O   |
| 10 $\mu$ l | 10X Titanium Taq PCR buffer  |
| 2 $\mu$ l  | 50X dNTP mix (10 mM of each dNTP)                                    |
| 2 $\mu$ l  | Fwd GNF/GNH (Forward) PCR Primer (10 $\mu$ M)                        |
| 2 $\mu$ l  | Rev GNF/GNH (Reverse) PCR Primer (10 $\mu$ M)                        |
| 2 $\mu$ l  | 50X Titanium Taq DNA polymerase <b>*Do not use any alternatives!</b> |
| <hr/>      |  |
| 90 $\mu$ l | Total volume   |

- b. Mix contents by vortexing, and spin the tube briefly in a microcentrifuge.
- c. Aliquot 90  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l of Master Mix remaining.** Compare your results to those below to confirm that your reactions were successful.
- f. Aliquot 1  $\mu$ l 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  $\mu$ g—repeat previous steps if <10  $\mu$ g.) for hybridization with a GeneChip® Array. Contents in tubes may also be combined to obtain the desired 10  $\mu$ g. **Note: If you use 6  $\mu$ l in 6 tubes, you should still have 89  $\mu$ l 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:

|                 |  |
|-----------------|--|
| <u>Per Tube</u> | (6 tubes per sample now) + Biotin Label                              |
| 66 $\mu$ l      | Deionized H <sub>2</sub> O   |
| 10 $\mu$ l      | 10X Titanium Taq PCR buffer  |
| 2 $\mu$ l       | 50X dNTP mix (10 mM of each dNTP)                                    |
| 10 $\mu$ l      | NRev GNF/GNH (Nested Reverse) Universal Primer (10 $\mu$ M) *        |
| 10 $\mu$ l      | NFwd-Bio (Nested Forward Biotinylated) PCR Primer (10 $\mu$ M)       |
| 2 $\mu$ l       | 50X Titanium Taq DNA polymerase <b>*Do not use any alternatives!</b> |
| <hr/>           |  |
| 100 $\mu$ l     | Total volume per tube  |

- h. Mix contents by vortexing, and spin the tubes briefly in a microcentrifuge.
- i. Aliquot 100  $\mu$ l of the PCR Master Mix into each tube with the 1  $\mu$ l aliquot from Step 2.e, and place them in the hot-lid thermal cycler. **Note: This will give a total volume of 101  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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.

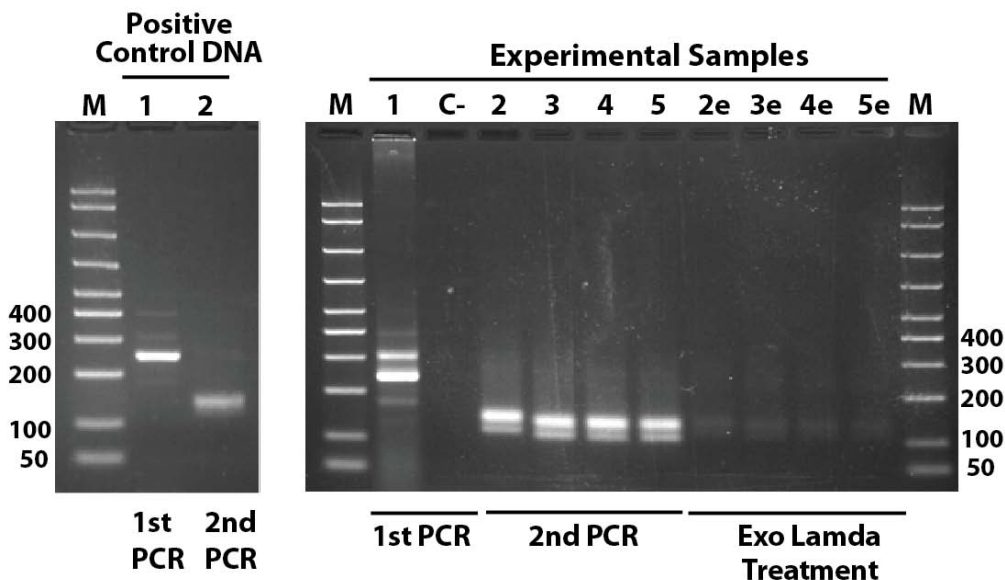
- For maximum PCR product recovery, elute PCR product from the column once with 22  $\mu$ l of elution buffer, followed by a second elution with 22  $\mu$ l of elution buffer. The total volume should be approximately 40  $\mu$ l after elution.
- Combine all eluates from each sample into one tube. The total volume should be about 260  $\mu$ l. Take a 1  $\mu$ l 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  $\mu$ g.

## 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  $\mu$ l of the purified PCR product, 34  $\mu$ l of 10x ExoLambda buffer, 39.5  $\mu$ l of Lambda Exonuclease (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  $\mu$ l sample from each tube and a 1  $\mu$ l 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  $\mu$ l), 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  $\mu$ l of elution buffer, followed by a second elution with 22  $\mu$ l of elution buffer. Combine all eluates for each sample into one test tube and concentrate by vacuum centrifugation to a 50  $\mu$ l volume.
- d. Take a 1  $\mu$ l sample from each test tube, dilute it in an appropriate volume of H<sub>2</sub>O, and measure the yield of PCR products using a spectrophotometer at 260 nm. The yield of single-stranded shRNA products should be approximately 10  $\mu$ g 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<sup>-</sup>** – 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.*, Spottfire, 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.

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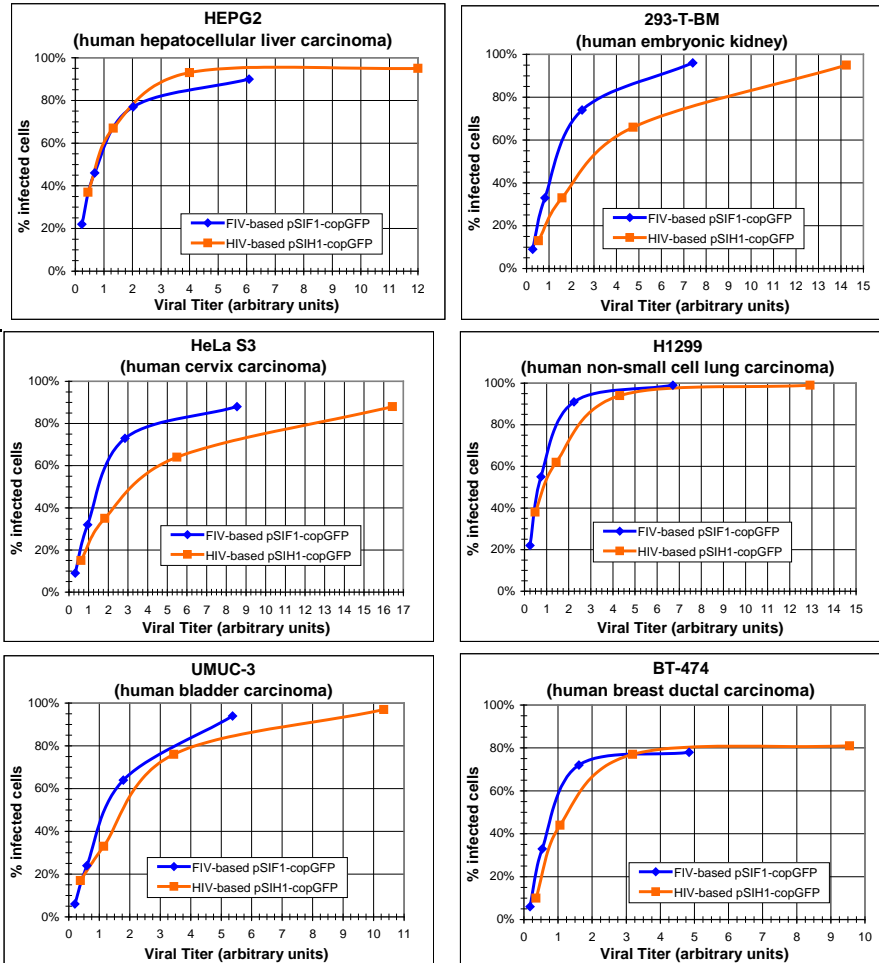
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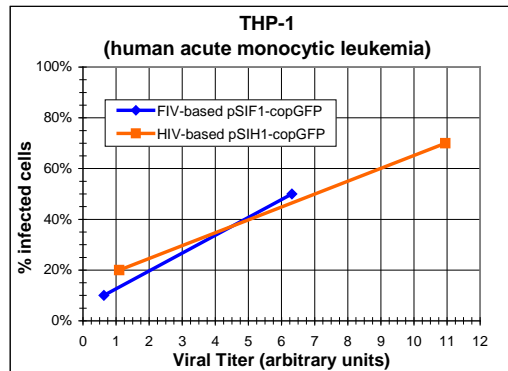
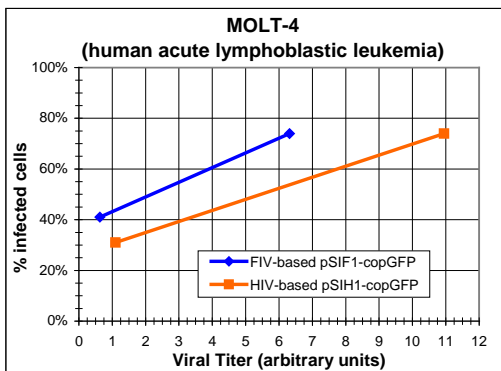
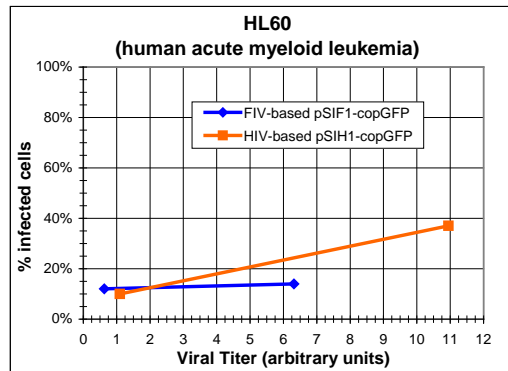
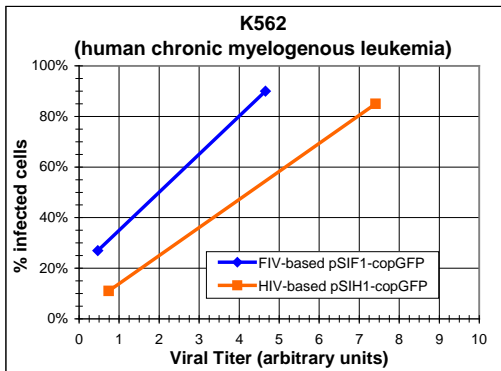
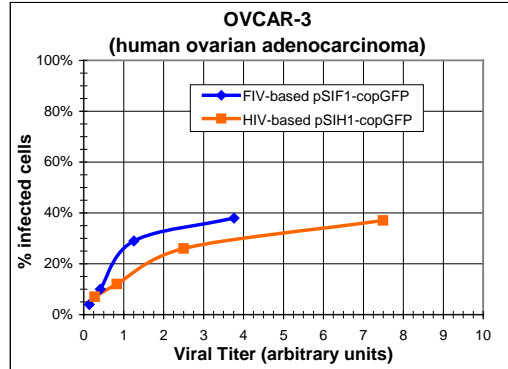
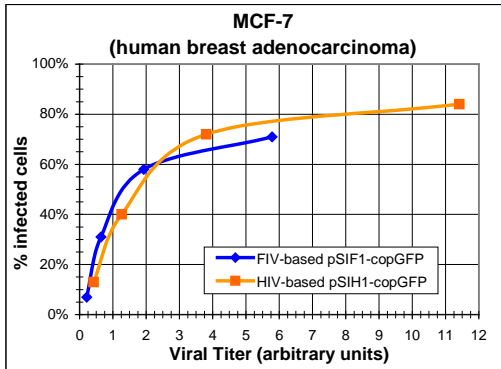
## V. Appendix

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

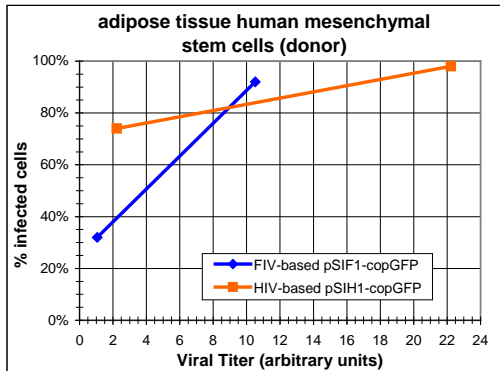
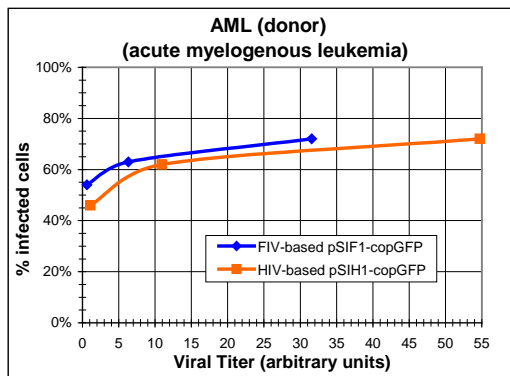
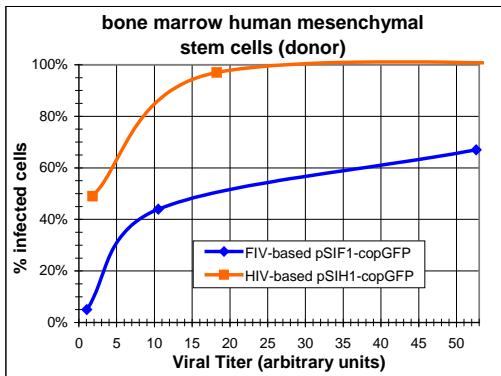
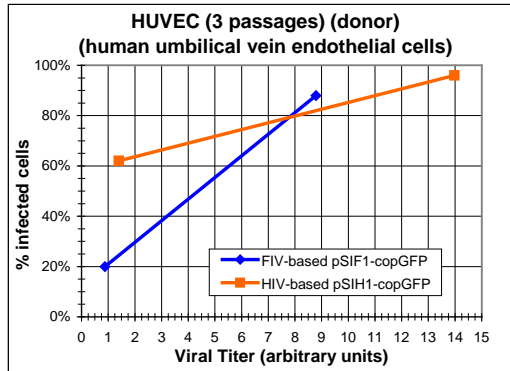
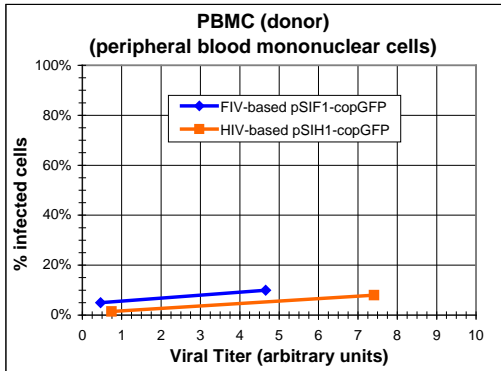
#### Human Cell Lines



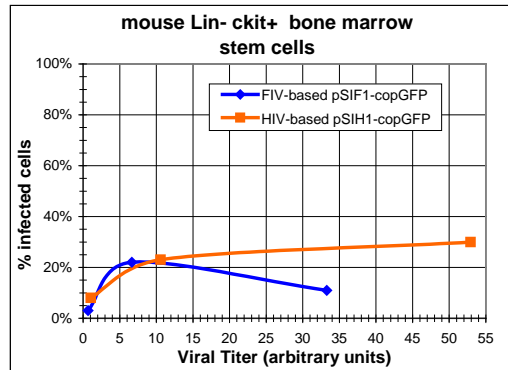
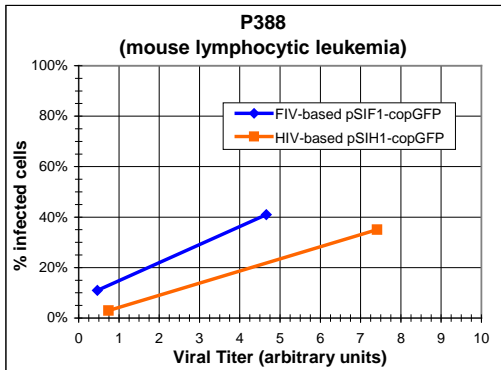
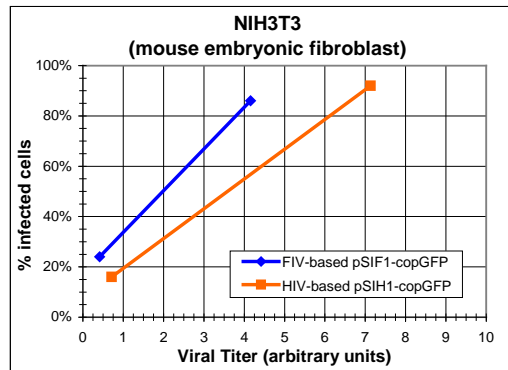
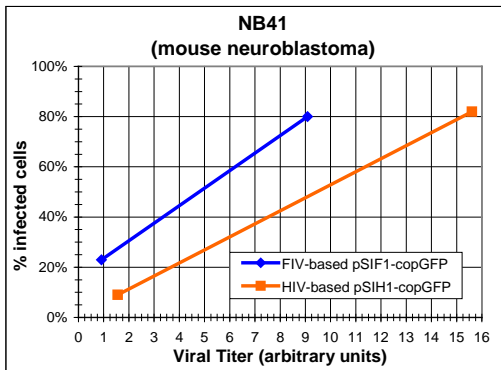
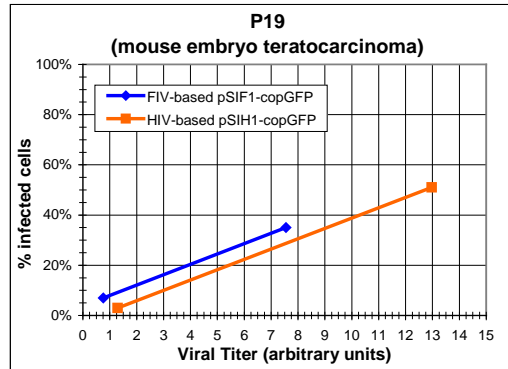
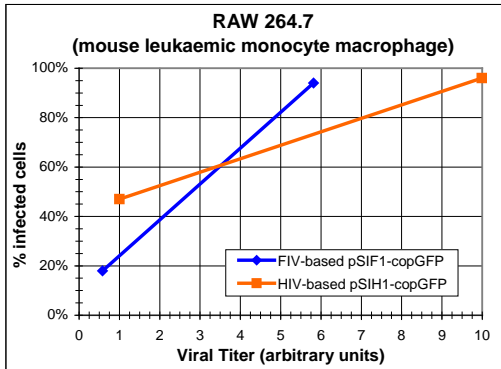
**Human Cell Lines (cont'd)**



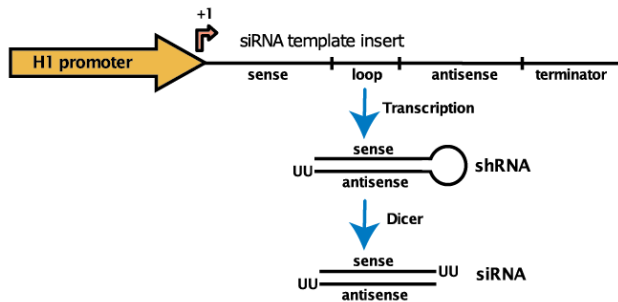
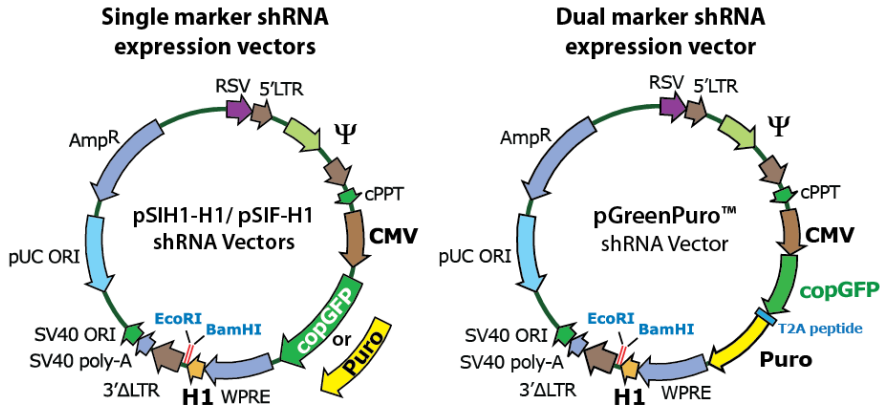
**Human Primary/Stem Cell Lines**



**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)

| <u>Feature</u>  | <u>Location*</u> | <u>Function</u>  |
|-----------------|------------------|--|
| 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   |
| WPPE            | 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 <i>E. coli</i>   |
| AmpR            | 5584-6444 (C)    | Ampicillin resistant gene for selection of the plasmid in <i>E. coli</i>   |

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

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

| <u>Feature</u>  | <u>Location*</u> | <u>Function</u>  |
|-----------------|------------------|--|
| 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 <i>E. coli</i>   |
| AmpR            | 5743-6603 (C)    | Ampicillin resistant gene for selection of the plasmid in <i>E. coli</i>   |

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

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

| <u>Feature</u>  | <u>Location*</u> | <u>Function</u>  |
|-----------------|------------------|--|
| 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   |
| WPRES           | 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 <i>E. coli</i>   |
| AmpR            | 5019-5879 (C)    | Ampicillin resistant gene for selection of the plasmid in <i>E. coli</i>   |

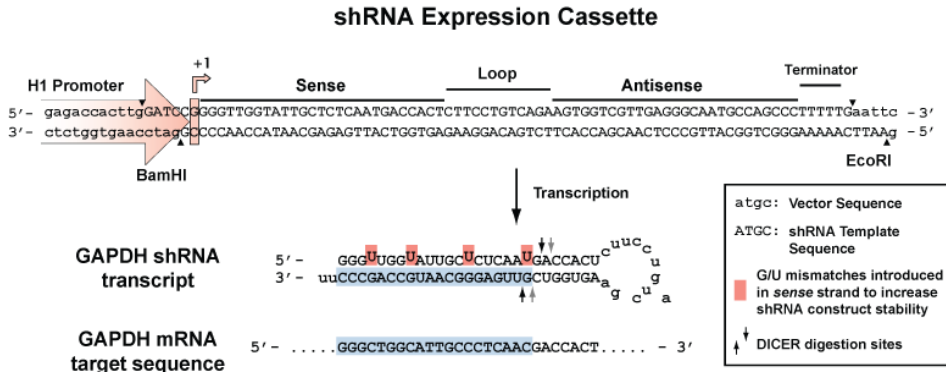
\* The notation (C) refers to the complementary strand.

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

| <u>Feature</u>  | <u>Location*</u> | <u>Function</u>  |
|-----------------|------------------|--|
| 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 <i>E. coli</i>   |
| AmpR            | 5178-6038 (C)    | Ampicillin resistant gene for selection of the plasmid in <i>E. coli</i>   |

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

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

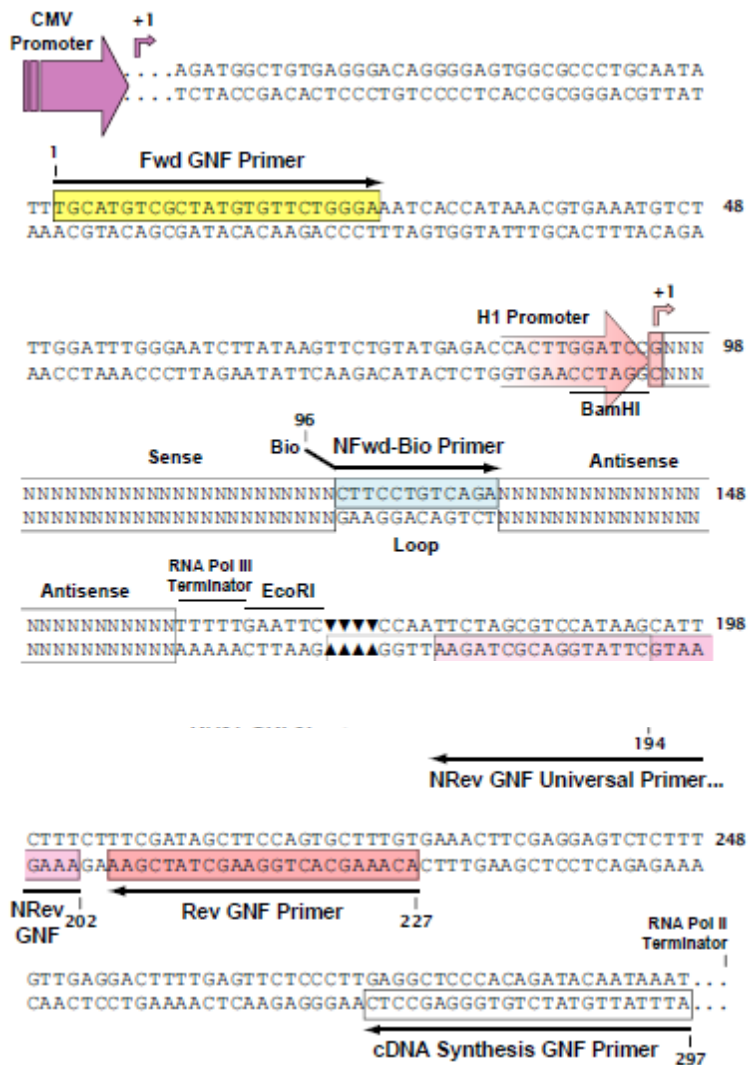


Design of the shRNA expression cassette. The shRNA template sequence is cloned into the shRNA expression cassette which is the same for both pSIH1-H1 and pSIF1-H1 cloning vectors. siRNA template sequences are designed to be directionally inserted between the BamHI and EcoRI nucleotide overhangs (i.e.,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



## 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)**

| <u>Feature</u>  | <u>Location*</u> | <u>Function</u>  |
|-----------------|------------------|--|
| 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 <i>E. coli</i>   |
| AmpR            | 5809-6669 (C)    | Ampicillin resistant gene for selection of the plasmid in <i>E. coli</i>   |

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

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

| <u>Feature</u>  | <u>Location*</u> | <u>Function</u>  |
|-----------------|------------------|--|
| 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 <i>E. coli</i>   |
| AmpR            | 5244-6104 (C)    | Ampicillin resistant gene for selection of the plasmid in <i>E. coli</i>   |

\* 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  $\mu$ l 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  $\mu$ g (5-20  $\mu$ l) of genomic DNA from step F.1 and 10  $\mu$ l of Positive Control DNA, and transfer to new 0.5 ml reaction tubes. In each test tube, adjust the volume to 20  $\mu$ l 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 $\mu$ l | Deionized H <sub>2</sub> O                    |
| 10 $\mu$ l | 10X Titanium Taq PCR buffer                   |
| 2 $\mu$ l  | 50X dNTP mix (10 mM of each dNTP)             |
| 2 $\mu$ l  | Fwd GNF/GNH (Reverse) PCR Primer (10 $\mu$ M) |
| 2 $\mu$ l  | Rev GNF/GNH (Forward) PCR Primer (10 $\mu$ M) |
| 2 $\mu$ l  | 50X Titanium Taq DNA polymerase               |
| <hr/>      |   |
| 80 $\mu$ l | Total volume                                  |

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

- d. Aliquot 80  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{g}$ ) for hybridization with a GeneChip<sup>®</sup> 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 $\mu\text{l}$  | Deionized H <sub>2</sub> O  |
| 10 $\mu\text{l}$  | 10X Titanium Taq PCR buffer   |
| 2 $\mu\text{l}$   | 50X dNTP mix (10 mM of each dNTP)                                     |
| 10 $\mu\text{l}$  | NRev GNF/GNH (Nested Reverse) Universal Primer (10 $\mu\text{M}$ )*   |
| 10 $\mu\text{l}$  | NFwd-Bio (Nested Forward Biotinylated) PCR Primer (10 $\mu\text{M}$ ) |
| 2 $\mu\text{l}$   | 50X Titanium Taq DNA polymerase                                       |
| <hr/>             |   |
| 100 $\mu\text{l}$ | Total volume  |

- h. Mix contents by vortexing, and spin the tubes briefly in a microcentrifuge.
- i. Aliquot 100  $\mu\text{l}$  of the PCR Master Mix into each tube with the 1  $\mu\text{l}$  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  $\mu$ l 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  $\mu$ l 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  $\mu$ l of elution buffer, followed by a second elution with 22  $\mu$ l of elution buffer. Combine all four eluates from each sample into one test tube. The total volume will be about 160  $\mu$ l. Take a 1  $\mu$ l 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  $\mu$ g.

### 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  $\mu$ l of 10X ExoLambda Buffer, 100 units (10-20  $\mu$ l) 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  $\mu$ l sample from each tube and a 1  $\mu$ l 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  $\mu$ l of elution buffer, followed by a second elution with 22  $\mu$ l of elution buffer. Combine all eluates for each sample into one test tube and concentrate by vacuum centrifugation to a 50  $\mu$ l volume.
- d. Take a 1  $\mu$ l sample from each test tube, dilute it in an appropriate volume of H<sub>2</sub>O, and measure the yield of PCR products using a spectrophotometer at 260 nm. The yield of single-stranded shRNA products should be approximately 10  $\mu$ g for all samples. Please refer to Section II.G for information on hybridization of biotin-labeled targets with the GeneChip<sup>®</sup> 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](mailto:tech@systembio.com)

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