GeneNet™ Focused siRNA Libraries

Cat. #s SI616PA-1 - SI636PA-1
SI616VA-1 - SI636VA-1

User Manual

Store kit at –70°C on receipt

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

(ver. 080731)

Contents

I. Introduction and Background

A. Overview

This manual provides information describing genetic screening with System Biosciences' (SBI's) GeneNet™ siRNA 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™ siRNA libraries into target cells, select target cells with a specific phenotype, and identify siRNAs 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.

B. Functional Screening with siRNA 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 entire classes of genes with siRNA libraries targeting every gene is facilitating progress in the area of functional genomics and systems biology. There are two main strategies for using siRNA libraries for genetic screening experiments (Figure 1).

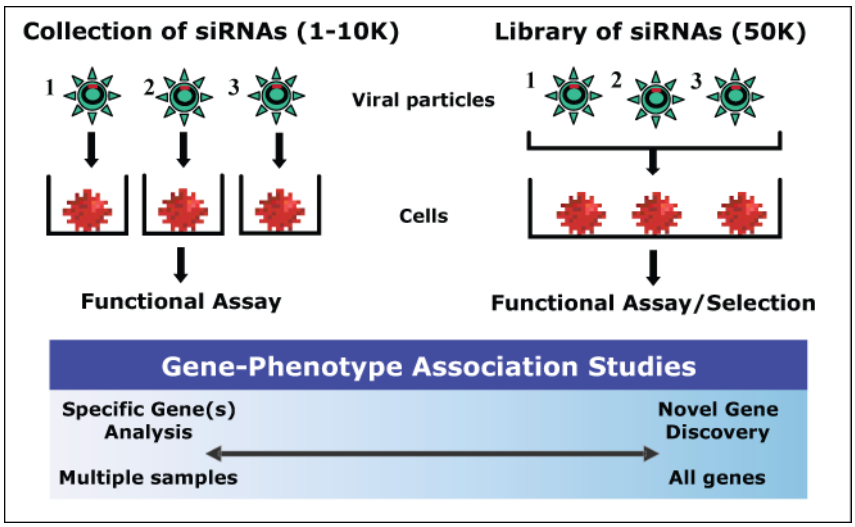

Fig. 1. On the left, the conventional approach to analyze function with siRNA—using a collection of gene-specific siRNAs to knockdown specific genes in separate reactions to observe the effect. On the right, a library-based approach where pooled siRNA constructs targeting a large number of genes are simultaneously introduced into a cell population that is then screened for those siRNA molecules that knock down genes involved in a particular phenotypic response.

The first strategy relies on the development of a collection of siRNA 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 siRNAs (see References, Genetic Screens with siRNA 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 size companies.

In the second strategy, a library encoding a pooled set of siRNAs 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 very high complexity siRNA libraries for the discovery of genes involved in specific phenotypes. Moreover, such pre-made pooled siRNA libraries also enable comprehensive cost effective loss-of-function genetic screens to be performed by small research groups.

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 (11-13). In order to achieve long-term knockdown, stable transcription of siRNA can be achieved by viral siRNA 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 siRNA libraries into recipient cells (see Appendix, Lentiviral Delivery Vectors). Based on lentiviral delivery technology, SBI has developed a set of novel research tools for genetic screen experiments including focused and genome-wide lentiviral siRNA libraries.

C. GeneNet™ siRNA Library Features

SBI has developed the next generation of user-friendly genetic screening technology with several novel features that significantly extend the application of this technology for high-throughput functional genomics studies:

- ***Biosafe third generation lentiviral (HIV-based) siRNA Vectors*** with puromycin selection (or copGFP reporter) and RNA polymerase III H1 promoter shRNA expression cassette for the expression of shRNA constructs (see Figure 2).

- ***Lentiviral siRNA 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 siRNA libraries greatly facilitates complex genetic selection schemes and allows the identification of cellular targets linked directly to phenotypes.

- ***Comprehensive high complexity siRNA libraries*** comprised of a redundant set of siRNAs (3-5 siRNAs per transcript) to provide reliable knockdown for known human kinase, phosphatase or apoptosis-related genes.

- ***Ready-to-use siRNA 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 siRNA Expression Vectors

Lentiviral expression vectors are the most effective vehicles for transducing and stably expressing different effector molecules (siRNA, 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 siRNA vector construct into viral particles, you can obtain highly efficient transduction and heritable expression of siRNA—even with the most difficult to transfect cells, such as primary, stem, and differentiated cells. Endogenously expressed siRNA 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 transfections or use of an adenoviral delivery system (Gould, 2003, Cann, 2000). SBI offers GeneNet™ siRNA libraries constructed in a third generation biosafe HIV-based lentivector originally developed for gene therapy applications (Poeschla, 2003; Sodroski, J.G., 1997, 1999; Federico, 2003; Heiser, 2004; Machida, 2003). As shown in Figure 2, the lentiviral expression vector contains the genetic elements (LTR, psi, RRE, cPPT, WPRE) required for packaging, transduction, stable integration of the expression constructs into genomic DNA, and expression of the siRNA effector sequences (H1 promoter) and drug selectable marker (CMV promoter and Puro gene). The siRNA constructs packaged in pseudoviral particles can infect (or 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. For more detailed descriptions, sequence information of our siRNA cloning and packaging vectors, and packaging protocol which is used at SBI, please refer to the "Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells" user manual, available on SBI's web site (www.systembio.com).

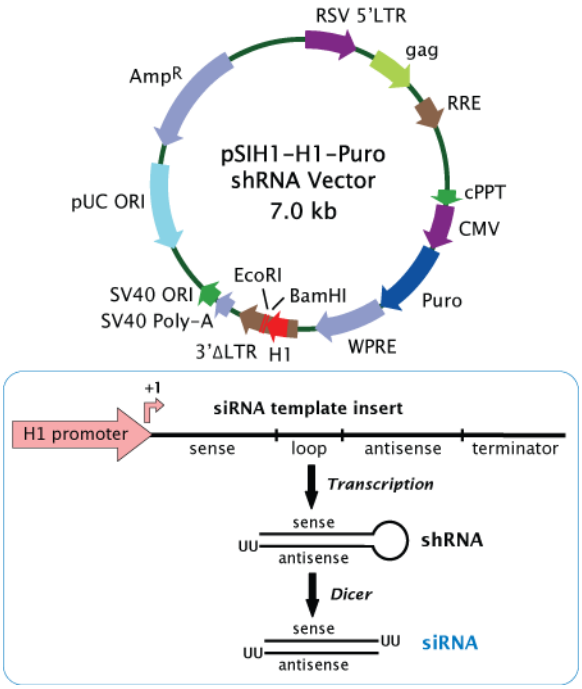

Fig. 2. Top: functional map of the pSIH1-H1-Puro shRNA Vector used in the construction of HIV-based GeneNet™ siRNA Libraries. Bottom: expanded view of the shRNA expression cassette, transcribed shRNA, and processed siRNA product.

Design of siRNA templates

Despite the development of many algorithms for prediction of functional synthetic siRNAs (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 shRNAs. The principal prediction rules, which are used to select siRNA sequences most likely to knock down a target gene, are summarized in Figure 3. It is interesting to note that these rules 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).

To take advantage of this finding, we designed siRNA template sequences for our GeneNet™ siRNA 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 shRNA 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.

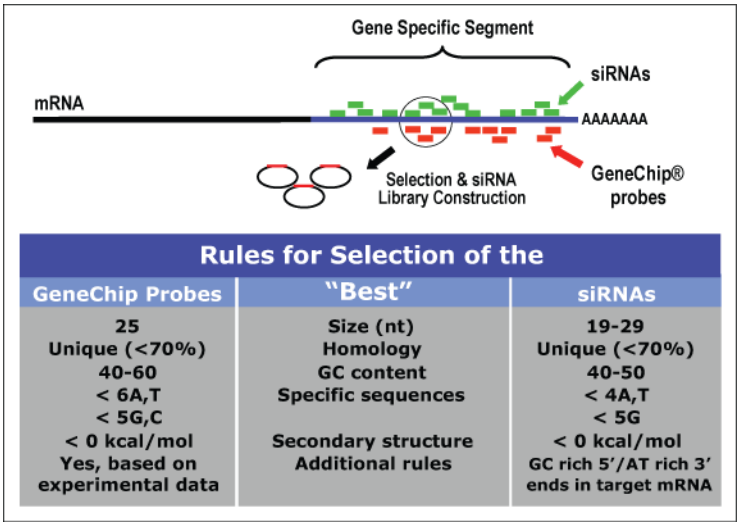

Fig. 3. The rules for selecting siRNA sequences that are likely to effectively silence target genes of interest are similar to rules used to select short probe sequences that are effective for microarray hybridization.

Construction and Quality Control of siRNA libraries

The high complexity GeneNet™ Lentiviral siRNA Libraries were constructed as diagrammed in Figure 4 and described below:

- We selected a set of target genes for each GeneNet™ Library (*e.g.*, for the Human Kinase library, we selected 919 human kinase genes represented on the GeneChip® Human Genome U133+2.0 Array)

- For each target gene, we designed 3-5 siRNA template oligonucleotides that will express 27-mer shRNAs targeting each of the mRNA sequences. The siRNAs were designed based on rules developed by SBI for selection of the most efficient siRNAs (see Figure 3). Since results mentioned previously indicate that our algorithm yields about 50% functional siRNA sequences, 3-5 siRNA 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 has additional 5'- and 3'-flanking sequences for directional cloning into a lentiviral siRNA expression vector.

- After synthesis, the siRNA 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 (Figure 4).

- The ligated siRNA library was then transfected into competent *E. coli* cells, grown as independent colonies on LB agar plates, and the total siRNA library in plasmid DNA form was purified from the pool of independent ampicillin-resistant bacterial colonies.

- The pseudoviral-packaged siRNA library was then produced by co-transfection of the plasmid siRNA library with the pPACK Packaging Plasmid mix (SBI Cat. # LV500A-1) into 293TN cells (SBI Cat. # LV900A-1).

- Quality control analysis of constructed siRNA libraries is 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 (2 mutations in 100 nucleotides). In addition, in order to test the representation of siRNA inserts in the pseudoviral packaged siRNA library, we reverse transcribe the viral RNA and amplify the siRNA inserts using flanking vector primers (see Appendix for PCR primer map). As a control, we amplify the siRNA inserts from the plasmid library used in the packaging step. Both samples are then hybridized to microarrays and compared in order to ensure representation is maintained after packaging. An example of this type of analysis is shown in Figure 5, in the graph on the left. Furthermore, we verify that each GeneNet™ siRNA Library can be efficiently transduced and expressed in target cells without significant loss of representation by amplifying siRNA inserts from pseudoviral RNA isolated from a packaged GeneNet™ siRNA library and from total

RNA of target cells transduced with the same library. As seen in the sample data shown in Figure 5, the packaging and transduction steps do not significantly affect representation of siRNA templates. Moreover, since the amplification is done using RT-PCR, this confirms that siRNA inserts are effectively expressed from the genomic DNA of target cells transduced with the packaged siRNA library.

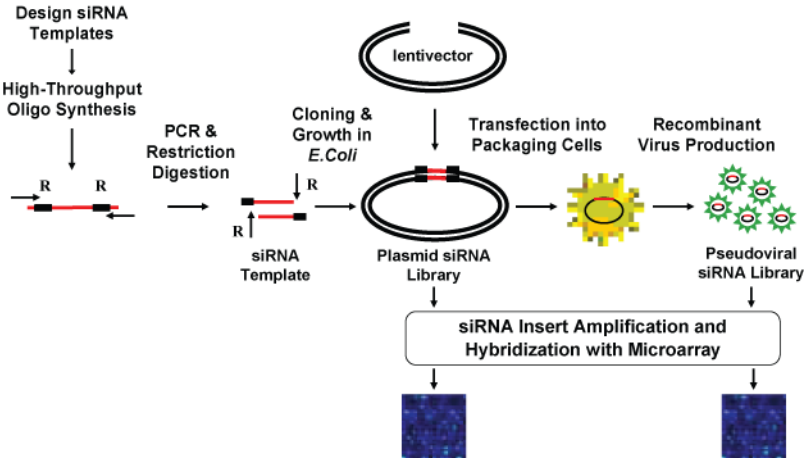

Fig. 4. Flowchart showing the steps in the construction of GeneNet™ siRNA Libraries. siRNA templates are synthesized, mixed as a pool, amplified by PCR, and cloned into the vector after digestion. After propagation in *E. coli*, the plasmid siRNA library is transfected into HEK 293 packaging cells, and transduction-ready packaged viral particles containing siRNA are produced. For quality analysis, siRNA inserts are amplified with primers flanking the siRNA sequences, and the representation of the siRNA inserts is analyzed by hybridization with a microarray.

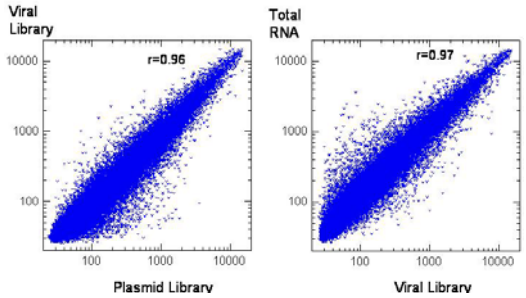

Fig. 5. Sample data of QC analysis of the GeneNet™ Human 8.5K siRNA Library in the pFIV-H1-copGFP vector. Scatter plot analysis of siRNA insert representation in the siRNA library in plasmid form, siRNA library packaged in pseudoviral particles, and in total RNA isolated from H1299 cells transduced with the 8.5K siRNA library. Biotinylated siRNA targets from each sample were amplified and hybridized to an Affymetrix Human Genome Focus Array.

D. Product Description and List of Components

Table 1 outlines the general features of the available GeneNet™ siRNA Libraries and indicates the compatibility of each library with the latest version of the Affymetrix GeneChip® Array (www.affymetrix.com). The most updated list of siRNA libraries available in different vectors can be found on SBI's website at www.systembio.com.

Catalog #	siRNA Library	Vector	siRNA Complexity	Compatible GeneChip®	Number of Genes
SI616PA-1	Human Apoptosis	pSIH1-H1-Puro	6,876	HG-U133+ 2.0	579
SI626PA-1	Human Kinase	pSIH1-H1-Puro	10,453	HG-U133+ 2.0	897
SI636PA-1	Human Phosphatase	pSIH1-H1-Puro	2,719	HG-U133+ 2.0	244

Table 1. GeneNet™ Focused siRNA Library Product Line. The list of genes targeted by each library can be found at www.systembio.com.

The siRNA libraries are provided in either ready-to-use, pre-packaged viral particle format (VSV-G pseudotyped) or as a plasmid library, that you can use to package pseudoviral particles (see Supporting SBI products, Section I.F). Depending on the complexity of the library, different amounts of pseudoviral particles (infection units, or ifu) are provided in the kit. The GeneNet™ siRNA Library Kits provide enough VSV-G pseudotyped pre-packaged siRNA library for 2-3 transductions for the most commonly used cell lines with an MOI of 1-2.

Packaged GeneNet™ siRNA Library Components

GeneNet™ siRNA Libraries (HIV-based) [Packaged]	
100-200 µl	GeneNet™ siRNA 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 siRNA library used for packaging step) (100 pg/µl)
25 µl	pSIH1-H1·siLuc-copGFP Packaged Positive Transduction Control
1	CD with gene/siRNA list and data analysis program compatible with Affymetrix data file

Plasmid GeneNet™ siRNA Library Components

GeneNet™ siRNA Libraries (HIV-based) [Plasmid]	
100-200 µl	GeneNet™ siRNA Library Plasmid DNA (200 µg)
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 siRNA library used for packaging step) (100 pg/µl)
25 µl	pSIH1-H1·siLuc-copGFP Packaged Positive Transduction Control
1	CD with gene/siRNA list and data analysis program compatible with Affymetrix data file

Additional comments on product components:

- Packaged GeneNet™ siRNA Libraries and Positive Transduction Controls are provided as frozen VSV-G pseudotyped viral particles 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 Lentivector UltraRapid Titer PCR Kit (Cat. # LV960A-1) and may vary for different lots of each library. ***The exact ifu, titer, and volume for each GeneNet™ Library is indicated on its corresponding Product Analysis Certificate.***
- RT-PCR primers are provided to amplify biotinylated hybridization targets comprising siRNA inserts from total cellular RNA (or alternatively from genomic DNA) and to be used for hybridization with the corresponding Affymetrix GeneChip® Array. 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 siRNA targets with Lambda exonuclease.
- The GeneNet™ siRNA Library Kit is shipped on dry ice and should be immediately stored at –70°C upon receipt. **Avoid thawing and refreezing of pseudoviral particles!** Each freeze-thaw cycle causes reduction of the titer by 20-30%. Properly stored pseudoviral particles are stable for 6 months from the date received.
- The list of target genes and siRNA inserts differs for each siRNA Library product. This information is supplied on the compact disc included with each library kit.
- The Positive Control DNA (corresponding plasmid siRNA library used for production of pseudoviral particles) is provided in an amount sufficient for 3 control amplification reactions.

E. Additional Required Materials

For Transduction of siRNA 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)
- Polybrene® (hexadimethrine bromide; Sigma, Cat. # H9268)
- 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 siRNA 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 siRNA targets
- Lambda Exonuclease (Recommended: Lambda Exonuclease (10 U/μl), New England BioLabs, Cat. # M0262S with 10X ExoLambda buffer)

For Purification of amplified siRNA inserts
- PCR purification kit (Recommended: QIAquick PCR Purification Kit, QIAGEN, Cat. # 28106)

For Hybridization of siRNA targets with Affymetrix GeneChip®
- Human Genome U133+2.0 GeneChip® Array (Affymetrix, Cat. # 900470)
- Reagents for standard hybridization, washing, and staining of Affymetrix GeneChip® Arrays

F. Additional Supporting SBI Products and Services

- **Custom Hybridization and Analysis for GeneNet™ siRNA Libraries** (Cat. # CS902A-1)
  You provide cell samples transduced with SBI's GeneNet™ siRNA 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 siRNA Libraries** (Cat. # CS901A-1)
  You provide a list of the 100-50,000 genes for any organism with GenBank accession numbers. We design siRNAs, clone them in any of SBI's siRNA Lentivectors, and provide you the siRNA library in plasmid and/or packaged form with all necessary supporting information.

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

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

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

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

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

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

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

- **pSIF1-H1·siLuc-copGFP Packaged Positive Transduction Control** (>2×10^5 ifu) (Cat. # LV201B-1) *(included with GeneNet™ siRNA 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 expresses an siRNA targeting Luciferase.

- **pSIH1-copGFP Packaged Positive Transduction Control** (>2×10^5 ifu), (Cat. # LV600A-1)

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

- **Lentivector UltraRapid Titer PCR Kit** (Cat. # LV960A-1 [for human cells], LV961A-1 [for mouse cells])
  Allows you to measure copy number (MOI) of integrated lentiviral constructs in genomic DNA of target cells after transduction with SBI's GeneNet™ siRNA libraries or with constructs made in any of SBI's FIV or HIV-based lentivectors.

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

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

- **pGreenFire™ Transcriptional Reporter Lentivectors** (many)
  HIV-based transcriptional reporter vectors, available in plasmid form or pre-packaged in pseudoviral particles which co-express destabilized copGFP and firefly luciferase when activated. These vectors allow the easy 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™ siRNA libraries. For a list of currently available vectors, please visit our website at www.systembio.com.

G. Safety Guidelines

SBI's Expression lentivectors together with the pPACK packaging plasmids comprise the third-generation lentiviral expression system. These HIV-based lentivectors are based on the vectors developed for gene therapy applications by Dr. J. G. Sodroski (U.S. patent #s 5,665,577 and 5,981,276).

Our HIV-based lentivector systems are designed to maximize their biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter (in HIV-based vectors) upstream of the 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*), and the corresponding proteins are expressed from different plasmids lacking packaging signals and share no significant homology to any of the expression lentivectors, pVSV-G expression vector, or any other vector in order to prevent generation of recombinant replication-competent virus.
- None of the viral 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.
- Pseudoviral particles will carry only a copy of your expression construct.

Despite the above safety features, use of lentiviral-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 always follow standard microbiological practices, which include:

- Wear gloves and lab coat all the time when conducting the procedure.
- Always work with pseudoviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area are to be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.
- Decontaminate all cultures, stocks, and other biological wastes before disposal using approved decontamination methods, such as treatment with bleach and autoclaving. Before autoclaving, the biological materials should be placed in a sealed, durable, leakproof container for transport from the laboratory.

II. Protocol

A. Procedure Outline and General Comments

GeneNet™ siRNA 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 flow chart in Figure 6 outlines the general steps required for the discovery of genes modulating a specific phenotype with the pre-made GeneNet™ siRNA library, including transduction into target cells, selection of cells with desired phenotype, and identification of phenotype-inducing siRNAs and corresponding target genes by hybridization of amplified siRNA cassettes with a GeneChip® Array. The overall protocol includes the following steps:

1. Transduce target cells with the GeneNet™ lentiviral siRNA library provided by SBI. The lentiviral constructs integrate into the cellular genome and each cell acquires and expresses one (or a few) unique siRNA library inserts. Measure MOI in the transduced cells.
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 surviving/selected and control cells.
4. Amplify and label with biotin the siRNA inserts by RT-PCR from total RNA isolated from the cells. Alternatively, you can amplify siRNA inserts from genomic DNA.
5. Remove non-biotinylated sense strand of amplified siRNA inserts by treatment with Lambda exonuclease.
6. Hybridize the biotin-labeled amplified siRNA targets with an Affymetrix GeneChip® Array. In some cases, you may alternatively clone and sequence amplified RNA inserts from selected phenotype-specific clones. However, this approach is very time consuming and not suitable if there are a large numbers of different siRNA templates present in the surviving/selected cell population. With the microarray approach, it is even possible to identify siRNA effectors with a weak phenotypical effect by analyzing changes in hybridization signals between control and selected target cells.

Some key terms used in the protocol:

MOI (multiplicity of infection)—the average copy number of lentiviral expression constructs per genome of target cell in the infected cell population.

Pseudoviral titer (ifu/ml): The relative titer (concentration, infection units/ml) of GeneNet™ siRNA libraries or any lentiviral constructs, measured by amplification of the lentivector-specific WPRE region from genomic DNA of infected cells. As a calibration standard, we use DNA from cells infected with a GFP reporter construct at different multiplicity of infection (MOI) based on FACS analysis of the percentage of GFP-positive cells. The Pseudoviral Titer is always specific to a particular cell line.

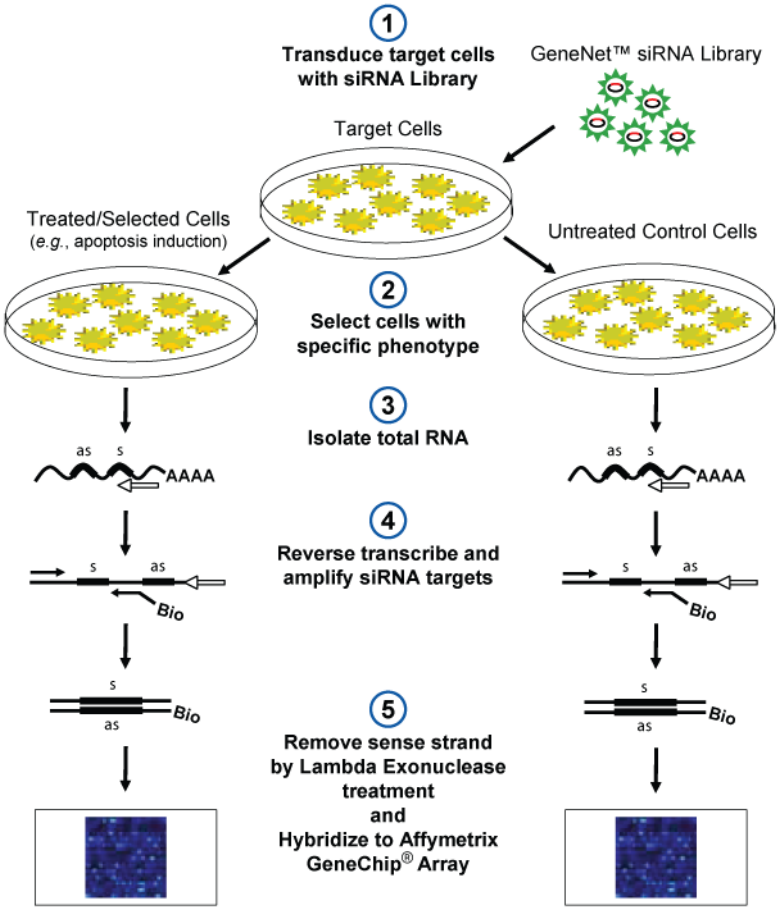

Fig. 6. Outline of functional screening with GeneNet™ siRNA Libraries. **s -** siRNA sense portion; **as -** siRNA antisense portion; **Bio –** biotin, P- 5'-phosphate group.

***Optimize Transduction Efficiency with the copGFP Packaged Transduction Control*.**

Pantropic VSV-G pseudotyped viral particles containing the lentiviral siRNA construct can be efficiently used to deliver and stably express siRNA sequences in a wide range of mammalian target cells, but transduction efficiency can vary significantly depending on the target cells. The packaged pSIH1-H1·siLuc-copGFP control vector provided in the kit (or purchased separately) can be used directly to estimate and optimize transduction conditions for any target cells with the GeneNet™ siRNA Library. The control vector, after transduction in target cells and integration into genomic DNA, stably expresses the fluorescent copGFP marker (and expresses an siRNA targeting Firefly Luciferase), so you can easily measure the percentage of transduced cells using fluorescent microscopy or flow cytometry and calculate MOI. 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 MOI=0.5-1.

Based on our experience, MOI does not depend on the number of cells taken for infection–it depends only on the virus concentration used for infection. To determine the concentration (titer) of pseudoviral particles required to provide MOI=0.5-1 for your particular target cells, you should do several transductions with different concentrations of packaged copGFP transduction control, and based on the percentage of GFP-positive cells, determine the MOI. Please note that MOI does not directly correspond to the percentage of GFP-positive cells. Use this simple guideline to convert % of GFP positive cells to MOI:

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

* - Please note that MOI cannot be reliably calculated if % of transduced cells is more than 90%.

Design and Test Functional Screen.

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 siRNA Libraries” section of the bibliography in the References section. Also, Appendix F includes an example of a GeneNet™ siRNA Library screening for genes involved in radiation-resistance.

Although the specific protocol and controls may be different depending on the cell type, functional assay, and selection protocol (*e.g.*, FACS, apoptosis induction, toxic chemical survival, etc.), it is critical to carefully design your experiment in order to generate statistically significant data. With this in mind, consider the following suggestions when setting up your experiment:

Model Phenotype Selection with Positive Control siRNA Construct(s)
Before performing a large scale genetic screening with the GeneNet™ siRNA Library, we suggest making several shRNA constructs designed against a particular target gene that is known to suppress the desirable phenotypic changes in the target cells. Then, by packaging and transducing these positive control shRNA constructs into target cells, you can optimize the protocol for selection/enrichment of cells with induced phenotypic changes (see below) for your experiment with the GeneNet™ siRNA library. As negative control cells, which should not be enriched during the selection steps, you can use target cells infected by the copGFP Packaged Transduction Control. Packaged gene-specific siRNA constructs can be generated using SBI's siRNA Cloning and Expression Lentivectors in conjunction with the appropriate pPACK Lentivector Packaging Kit, or they can be ordered as a Custom siRNA construction service (see Section F). The packaged positive control shRNA construct can also be added to the GeneNet™ siRNA library in a ratio of about 1 ifu to 1,000 ifu of the library in order to monitor enrichment of the positive control construct during the selection step. This can be accomplished by RT-PCR using positive control gene-specific sense primer and Nested Reverse primer provided in the kit. Unfortunately, this approach can only be used for biological processes with well-characterized mechanisms.

Optimize the selection/enrichment protocol. The quality of genetic screen data will significantly depend on the design and conditions used for the phenotype-specific selection (enrichment) step. A high enrichment level of target cells with a specific trait will help to identify siRNA constructs that are significantly enriched above the inevitable background level of non-enriched siRNA inserts. The best results are generated when the phenotype-positive cells can be selected and expanded as separate colonies. As an alternative to microarray analysis, the siRNA inserts can be recovered from clones by PCR, cloned into a TA cloning vector, and identified by sequencing.

In most cases, transduced cells can be used for most phenotypic screens at approximately two days after infection. However, this is a general observation based on anecdotal observation and the time it takes the lentiviral expression cassette to integrate and express. For certain genes and selections, the knockdown effect may take significantly longer to manifest.

Use Reference Control Cells. As a control for the genetic screen, it is important to use cells infected with the siRNA library but not selected (enriched) for a specific phenotype or induced (treated) by a phenotype-inducing agent, etc. There are many options that can be considered for the selection of appropriate reference control cells, depending on your biological system. The main purpose of the reference control is to have target cells with a non-enriched population of siRNA inserts treated under similar conditions to those of target cells selected for a specific phenotype. This control is particularly important to use as a standard to measure the relative levels and reproducibility in duplicate samples of each siRNA template species in the transduced cell population without selection. Without this control, it is difficult to

determine which siRNA species are over-represented in the target cells after selection step.

Design the experiment with at least duplicate samples. Due to variations in cell cultivation/treatment conditions, transduction efficiency, RNA purification, siRNA insert amplification, hybridization, quality of microarray, etc. you may expect some variation in intensities of microarray hybridization signals. The best solution for addressing potential irreproducibility issues is to consider having duplicate samples for each population of phenotype-selected and reference control cells.

Transducing siRNA Library into Target Cells.

The target cells are transduced with the siRNA library by simply thawing the pseudoviral particles, diluting in growth media, and adding the diluted virus to target cells at the desired titer of pseudoviral particles based on an optimized protocol developed with the copGFP packaged transduction control. Based on our experience, the most successful genetic screen experiments with GeneNet™ siRNA libraries require MOI=0.3-1. We do not recommend performing genetic screen experiments with an MOI less than 0.1-0.2, unless you increase the number of transduced cells used for the genetic screen.

Efficiency of transduction of target cells (MOI) with siRNA libraries with Puro selection can be measured by amplification of the lentivector-specific WPRE region from genomic DNA of transduced cells using SBI's Lentivector Rapid Titer PCR kit. Although you may use puromycin selection to determine MOI for lentivectors with the Puro selection marker, based on our experience the data are very subjective, not accurate, and significantly depend on selection conditions, cells, etc.

Pools of cells that are stably transduced with GeneNet™ siRNA library constructs can be enriched before selection step by resistance to the antibiotic puromycin (Puro Vectors). For most functional screens, we do not recommend this selection (or enrichment) step if a significant portion of target cells (at least 10-20%) are expressing siRNA constructs. Puromycin (or FACS) enrichment steps, due to massive cell death, may significantly reduce complexity of transduced siRNA library in the target cells and as a result will not allow the use of hybridization analysis for identification of phenotype-specific siRNAs.

siRNA 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.

The number of stably transduced cells used for functional screening needs to be at least 10-fold greater than the complexity of the siRNA library. For example, you would need to transduce at least 500,000 target cells when using the Human 8.5K siRNA Library, which has a complexity of about 40,000 cloned siRNA templates.

The following data in Figure 7 show that infecting less than the recommended amount of cells results in loss of representation of siRNA constructs when comparing duplicate populations of infected cells.

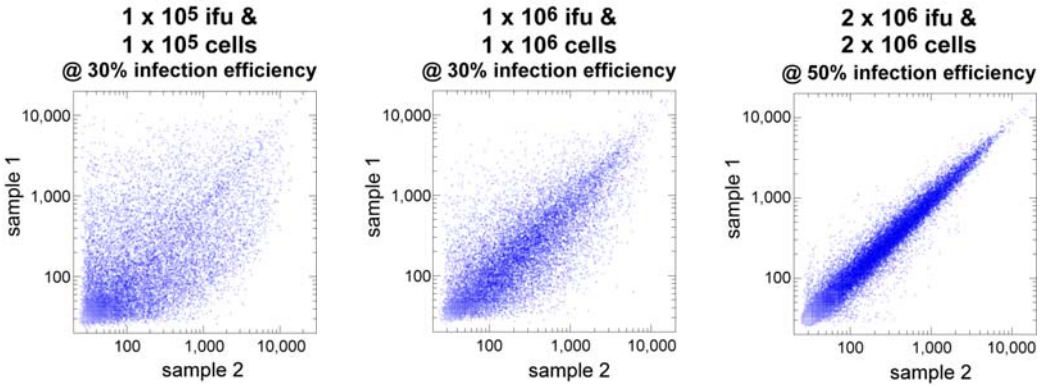

Fig. 7. Representation analysis of siRNA inserts in duplicate H1299 cells infected with the Human 8.5K siRNA Library in pFIV-H1-Puro. Different numbers of cells, as indicated, were infected with H.8.5K siRNA library at MOI=1 in duplicate. siRNA inserts from each duplicate cell sample (sample 1 & sample 2) were amplified and hybridized to the Affymetrix Human Genome Focus Array and compared by scatter plot. In order to ensure library representation, we recommend that in a genetic screening experiment, you use at least 1×10^6 infected cells (2×10^6 cells @ 50% infection efficiency in this example).

You should also consider that if more than 50% of target cells are infected by the siRNA library, some infected cells will express two (or more) siRNA constructs that may knock down two (or more) genes simultaneously. Some phenotypic screens require simultaneous knockdown of two target genes and could be more efficient at high MOIs of target cells (Berns, 2004; Paddison, 2004), but require more transduced cells for the screen.

Recovering the siRNA templates from selected cells.

In order to identify siRNAs from selected target cells with a specific phenotypic trait, you will need to amplify and label siRNA targets with biotin (for detection when hybridizing to Affymetrix Arrays). The siRNA template inserts can be amplified from either genomic DNA or from RNA. The protocol for amplification from genomic DNA can be found in the Appendix and is useful for some applications, for example for cells where the CMV promoter is not functional. However, amplification of single-copy siRNA templates with a background of 3×10^9 bp of genomic DNA requires starting with a minimum 5 μg of genomic DNA. Also, the PCR is finicky. For this reason, we typically recommend the more robust RT-PCR protocol of amplifying the siRNA inserts from total RNA.

In addition to synthesis of siRNA from the RNA polymerase III H1 promoter, integrated lentiviral constructs also produce an alternative longer transcript from the CMV promoter that is a fusion of the marker gene (copGFP or Puro) with the siRNA sequence. This

transcript terminates at the 3' LTR poly (A) site (see vector maps in the Appendix). Using reverse transcription and two rounds of PCR, siRNA targets can be effectively amplified from total RNA using this CMV promoter-driven transcript as a template. After the second round of PCR with two nested primers (where one primer contains biotin residues at the 5'-end) and removal of the sense strand by lambda exonuclease, the biotinylated siRNA targets can be used directly for hybridization to Affymetrix GeneChip® Arrays.

Identifying the siRNAs/genes inducing a specific phenotype.

The siRNA template recovery procedure enables you to amplify the entire pool of siRNA inserts from the enriched cell population, or to retrieve individual siRNA templates from separate colonies selected by the phenotype-specific screening protocol. After amplification of siRNA inserts from control and selected target cells in order to identify siRNA inducing the specific trait, the PCR product can be cloned into a vector. You should only consider cloning siRNA inserts isolated from selected cells into a vector for sequence analysis as an appropriate strategy if your selection protocol provides you pure clones with a specific phenotype. Unfortunately, most selection protocols usually give you a subpopulation of target cells enriched for a specific phenotype. For most experiments, microarray analysis provides the most efficient way to analyze enrichment of phenotype-associated siRNAs in the complex siRNA population. The compact disc included in the kit provides the necessary software for analysis of Affymetrix raw data and the sequences of the siRNAs present in GeneNet™ siRNA library.

General rules for data analysis developed for gene expression microarrays may also be applied for analysis of your phenotypic screen data. Statistical significance of differences in representation of individual siRNAs can be estimated based on comparative analysis of duplicate samples of selected and reference control cells.

Using the Positive Control DNA.

The Positive Control DNA included in the kit is the plasmid form of the GeneNet™ siRNA Library. This DNA was used for production of the packaged GeneNet™ siRNA 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 siRNA inserts in the packaged library and can be used as a universal reference to compare with recovered siRNA templates from your transduced target cells.

B. Optimize Transduction Efficiency with the pSIH copGFP Packaged Positive Transduction Control

Due to differences in the transduction efficiencies of various cell lines, we recommend that you optimize transduction conditions using the copGFP packaged positive control vector (provided in the kit) for your target cells before proceeding with the GeneNet™ siRNA library. Transduction efficiency mainly depends on virus concentration and does

not depend on the number of cells taken for infection (see section II.A). The goal of the following protocol is to perform several transductions with your target cells at different dilutions of the supplied copGFP packaged control vector in order to find the concentration of pseudoviral particles (dilution factor) which would give an MOI in the range of 0.3 - 1.

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 your target cells in 5 wells of a 24-well plate at a density of about 0.6 - 1×10^5 cells per well 24 hours prior to pseudoviral infection. As an option, we also recommend to plate at least one well with HT1080 cells as a control for transduction efficiency of your target cells. Add 1 ml of complete D-MEM medium (with serum and antibiotics), and incubate cells at 37°C with 5% CO_2 overnight.

Day 2.

2. Prepare complete D-MEM medium plus 10% FBS with Polybrene® to a final concentration of 5 μg/ml.

   Note: Polybrene® is a polycation that neutralizes charge interactions to increase binding between the pseudoviral capsid and the cellular membrane. The optimal concentration of Polybrene® depends on cell type and may need to be empirically determined (usually in the range of 2-10 μg/ml). Excessive exposure to Polybrene® (>12 hr) can be toxic to some cells.

3. Remove culture medium and replace with 0.5 ml of complete D-MEM medium with 10% serum and Polybrene® (from Step 2) in the first five wells. Quickly thaw the copGFP packaged positive control vector in a water bath at 37°C. Infect target cells by adding 1 μl of viral stock into the first well (dilution factor of 500), 2.5 μl of viral stock into the second well (dilution factor of 200), and 10 μl of viral stock into the third well (dilution factor of 50). The fourth well will serve as a mock well control. In the fifth well, add 0.5ml of complete D-MEM medium without Polybrene® in order to estimate Polybrene® toxicity. Incubate cells at 37°C with 5% CO_2 overnight.

Day 3.

4. Remove culture medium and replace with 1 ml of complete D-MEM medium (without Polybrene®). Incubate the cells at 37°C with 5% CO_2 overnight.

Day 4.

5. Split the cells 1:3 to 1:5, depending on the type of cells, and incubate in complete D-MEM for an additional 24 hours.

Day 5.

6. Count the fraction of fluorescent cells by FACS analysis. You may also visualize the cells for copGFP fluorescence, but the results may be inaccurate 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 MOI=1 (about 60-70% of GFP-positive cells).

C. Transduce Target Cells with GeneNet™ siRNA Library

To ensure reproducible and reliable results when using your GeneNet™ siRNA Library, it is critical that you infect enough cells to maintain sufficient representation of each siRNA construct present in the library in the transduced cells. Moreover, the concentration of pseudoviral particles during the transduction step should be the same as you used with the packaged copGFP control vector in order to get MOI=1 (average one copy of integrated siRNA construct per cell). Usually, the number of cells stably transduced with the siRNA library should exceed the complexity of the siRNA library by at least 10 to 20-fold. For example, if the complexity of the Human Kinase siRNA library is approximately 10,000 (*i.e.*, the library contains approximately 10,000 species of siRNA templates) and you can achieve MOI=1 (based on results with the packaged copGFP control vector), then the minimum number of cells that should be transduced is at least 1×10^5. It is typical to get 1×10^6 cells in a 10-cm plate that is about 50% confluent. You need to use proportionally more cells for the transduction step if you did not achieve MOI=1 in your preliminary experiments with the packaged copGFP control vector.

The protocol below provides general guidelines for transduction of a GeneNet™ siRNA library in the target cells with expected MOI=1. Duplicate the number of plates if you have decided to use duplicate samples.

Day 1.

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

Day 2.

2. Quickly thaw the GeneNet™ siRNA 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™ siRNA library (usually about 1×10^7 ifu) with 15 ml of complete medium in order to have a final concentration of pseudoviral particles equal to the concentration of packaged copGFP control vector necessary to get MOI=1. Add Polybrene® to a final concentration of 5 μg/ml. Use less concentration of Polybrene® if you find it toxic to your target cells.

   Caution: Only open the tube containing the pseudoviral-packaged GeneNet™ siRNA 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 20-30% 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 Polybrene. Incubate cells at 37°C with 5% CO_2 overnight.

Day 3.

4. For each plate, remove the culture medium and replace with 10 ml of complete medium (without Polybrene®). Incubate the cells at 37°C with 5% CO_2 overnight.

Day 4.

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

Day 5. (72 hours after transduction)

6. At this stage, you can confirm that you get an MOI close to 0.5-1 by measuring the percentage of GFP-positive cells (using FACS or fluorescence microscopy) for siRNA libraries in the copGFP vector. If you have used a GeneNet™ siRNA Library in the Puro vector, the MOI in transduced cells (6-cm plate from step 5) can be easily determined using SBI's Lentivector UltraRapid Titer PCR Kit (see Section I.E). Alternatively, the percent of stably transduced cells can be calculated based on number of puromycin-resistant colonies, but based on our experience, selection using puromycin resistance usually gives very inconsistent data.

D. Select Target Cells with Specific Phenotype

At this stage, you have generated a library of target cells with a different siRNA expression construct in every cell. In most cases, cells will reveal specific knockdown phenotypes and can be screened about 2-3 days after infection. However, for certain genes which express stable proteins, or for certain selection protocols, cells may require a longer period of incubation before treatment.

As discussed previously, you will need to design the most appropriate protocol for selecting transduced target cells with a specific phenotype (see Section II). For best results, the selection protocol should generate a population of cells enriched greater than 10-fold with your desired phenotype. Use half of the plates you have grown for the phenotype selection step and half of the plates used as reference control cells. Use duplicates if you started with 1×10^7 cells (10 plates).

E. Purify Total RNA and DNA from Target Cells

Notes:

- 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.
- We recommend that you simultaneously isolate total RNA and genomic DNA. The DNA can be used to verify data generated by the total RNA and to measure MOI in 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.

F. Reverse Transcribe and Amplify Biotin-labeled siRNA Targets

As mentioned above, 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 siRNA sequence. This alternative transcript is used as a template to

amplify the siRNA insert. Amplification of the inserts from total RNA requires two rounds of PCR. During the second round of PCR with two nested primers (one primer has biotin residues at the 5'-end and another a 5'-phospate group), the amplified siRNA targets are labeled with biotin, sense strands removed by lambda exonuclease, and biotin-labeled antisense strands are used as hybridization targets for Affymetrix GeneChip® Arrays using standard protocols.

Notes:

- If you would like to recover siRNA targets from genomic DNA, please follow the protocol in the Appendix.
- In addition to amplifying and labeling RNA isolated from your samples, you should also include a positive control using 10 μl of the Positive Control DNA that is included with the GeneNet™ Library. The Positive Control DNA included in the kit is the GeneNet™ siRNA 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 siRNA inserts in the packaged siRNA library and can therefore be used as a standardizing reference for all siRNA target samples rescued from your control and selected target cells.
- 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

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)*
1 μl	cDNA Synthesis GNH Primer (10 μM)
	Deionized H_2O (add up to 16 μl final volume)
16 μl	Total volume

* Use 5 μg if the RNA concentration is low.

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

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

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

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

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

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

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

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

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

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

2. Amplification

The following procedure describes the protocol for amplification of siRNA 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 µl) that is included in the kit. This control can be used to optimize and troubleshoot your RT-PCR and GeneChip® hybridization steps.

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

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

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

c. Aliquot 90 µl of the PCR Master Mix into each tube from Step 1.j and place them in the hot-lid thermal cycler.

d. 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

e. When the program is completed, analyze a 5 µl sample from each tube alongside a 50 bp DNA size marker by running on a 2.5% agarose/EtBr gel in 1X TAE. Compare your results to Figure 9 to confirm that your reactions were successful. Aliquot 1 µl from each tube into four new 0.5 ml reaction tubes. You will need about 4 PCR reactions per sample to obtain enough biotin-labeled siRNA target (about 10 µg) for hybridization with a GeneChip® Array.

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

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

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

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

i. 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

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

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

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

3. Lambda Exonuclease Treatment

To remove 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 siRNA strand.

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

b. When the program is completed, analyze a 1 μl sample from each tube and a 1 μl sample from each tube from Step 2.k) alongside a 50 bp DNA size marker by running on a 3% agarose/EtBr gel in 1X TAE to ensure that the double-stranded PCR product has been degraded. 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 each PCR reaction (test tube), add ten volumes of PB buffer (2 ml) and sequentially apply 0.5 ml at a time to two QIAquick columns.
- Perform the wash step two times (instead of one) using 0.5 ml of washing buffer for each wash.
- For maximum PCR product recovery, elute PCR product from each column once with 22 μl of elution buffer, followed by a second elution with 22 μl of elution buffer. Combine all eluates for each sample into one test tube and concentrate by vacuum centrifugation to a 50 μl volume.

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

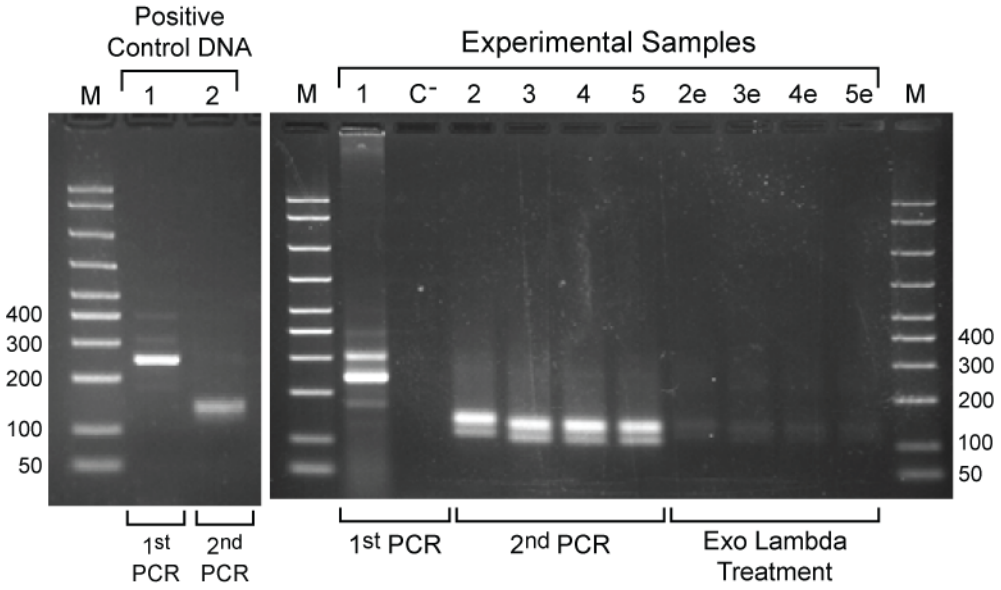

Fig. 9. Analysis of siRNA insert products amplified by RT-PCR from total RNA. In this experiment, an HIV-based GeneNet™ Human 50K siRNA Library in pSIH1-H1-Puro was used to transduce H1299 cells.

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

G. Hybridize Biotin-labeled siRNA Targets with GeneChip® Array

Hybridization of GeneChip® Arrays with biotinylated siRNA targets is the most effective way to identify phenotype-associated siRNAs 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 siRNAs present in the GeneNet™ siRNA library.

Hybridize about 10 μg of biotinylated siRNA target with the specific Affymetrix GeneChip® Array required for your particular siRNA library (see Table 1), using the manufacturer's standard protocols and recommended reagents. Use Affymetrix Hybridization buffer and hybridize at 45°C overnight.

The software provided with the library on the GeneNet™ siRNA Library Data Analysis Software and Gene List CD-ROM will enable you to analyze the hybridization data and create a report file in a format compatible with common spreadsheets (Excel, etc.) and statistical programs. The file lists the intensities of signal—which correspond to the abundance level—for each of the specific siRNA 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.*, SPSS, Systat) or expression data analysis software (*e.g.*, Spotfire, Silicon Genetics, etc).

III. Troubleshooting

A. Inefficient Transduction of Packaged copGFP Transduction Control or siRNA 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 positive transduction control cells (eg. H1299 cells).

Wrong amount of Polybrene® added during infection stage
If Polybrene® is toxic to the target cells, optimize Polybrene® concentration in the range of 1-5 μg/ml.

Loss of pseudoviral titer during storage
Ensure storage of the copGFP Packaged Transduction Control stock and packaged GeneNet™ siRNA 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™ siRNA Library affects target cell growth
Use a shorter transduction time to minimize the toxic effect to the target cells. Try replacing with a similar target cell type.

Polybrene® is toxic for target cells
Optimize the concentration and exposure time to Polybrene® during the transduction step.

3. No expression of copGFP reporter (or siRNAs) 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 siRNA Targets

1. General Recommendations

The protocol for generating biotin-labeled siRNA 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 siRNA 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 siRNA 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 siRNA 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 siRNA 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 siRNA 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 siRNA target

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

2. siRNA 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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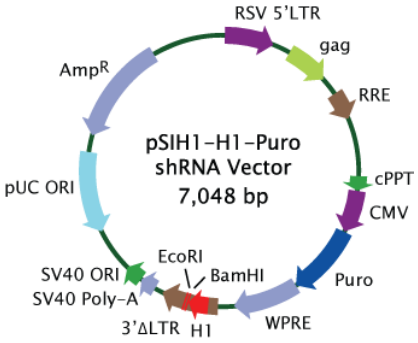
V. Appendix

A. Maps and Features of Single-Promoter pSIH1-H1 Vectors

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



Feature	Location*	Function
RSV/5'LTR	7-414	Hybrid RSV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	567-919	Packaging signal
RRE	1076-1309	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1798-1916	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1922-2271	Human cytomegalovirus (CMV)--constitutive promoter for transcription of puromycin
Puro	2279-2878	Puromycin-resistant marker for selection of the transfected/transduced cells
WPRE	2885-3425	Woodchuck hepatitis virus posttranscriptional regulatory element--enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3564-4038	Required for viral reverse transcription; self-inactivating 3' LTR with deletion in U3 region prevents formation of replication-competent viral particles after integration into genomic DNA
H1 RNA promoter	3602-3818	RNA polymerase III promoter for expression of siRNA insert
SV40 Poly-A	4110-4241	Transcription termination and polyadenylation
SV40 Ori	4250-4396	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4766-5439 (C)	Allows for high-copy replication in *E. coli*
AmpR	5584-6444 (C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

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

B. Design of the Cloning and Expression Cassette for the pSIH1-H1 Vector

H1 Vectors

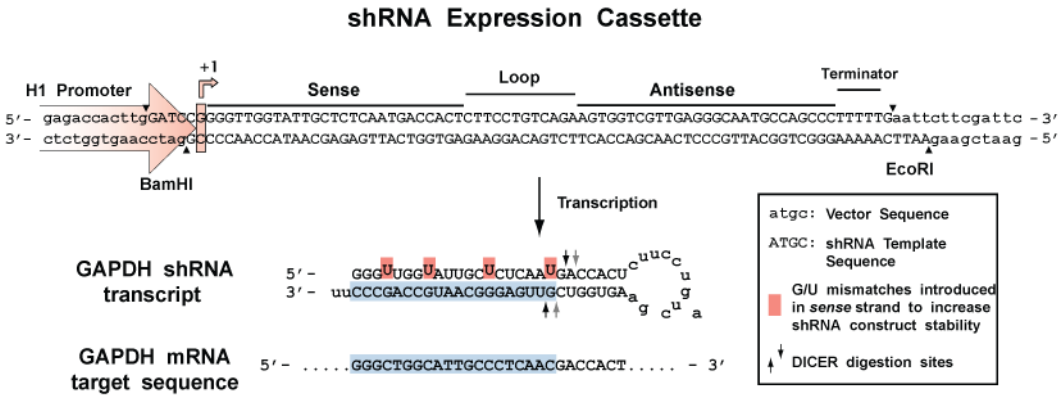


Fig. 10. Design of the shRNA expression cassette. The shRNA template sequence is cloned into the shRNA expression cassette of the pSIH1-H1 cloning vector. siRNA template sequences are designed to be directionally inserted between the BamHI and EcoRI nucleotide overhangs (*i.e.*, sticky ends).

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.

C. Location and Sequences of Amplification Primers

1. pSIH1-H1 Vectors

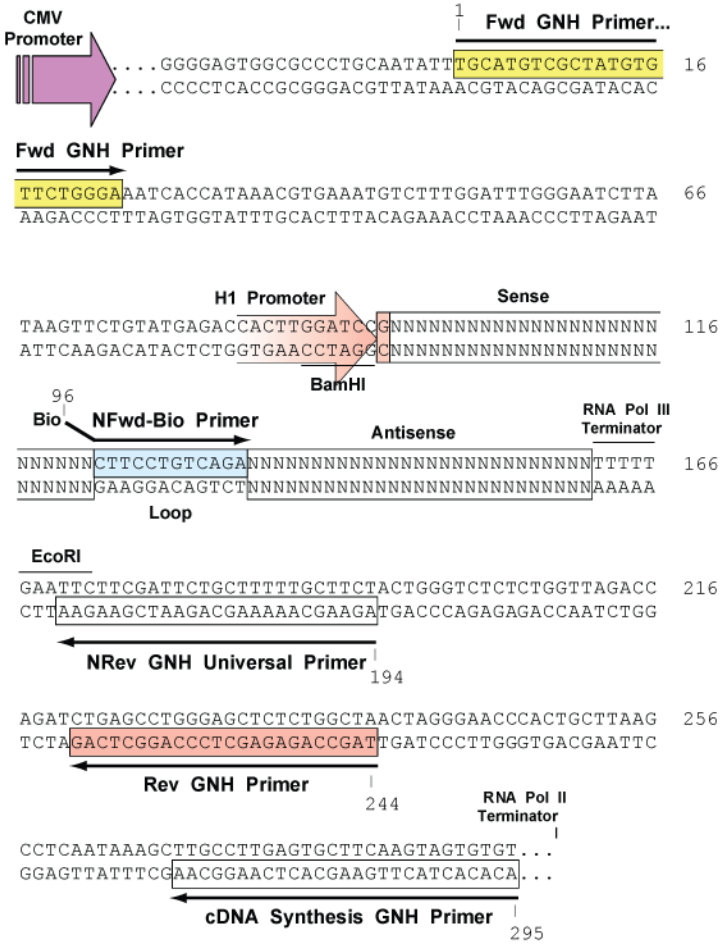
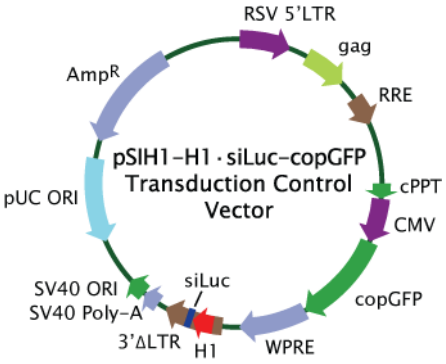


Fig. 12. Rev GNH and Fwd GNH RT-PCR Primers are designed to amplify the siRNA cassette from cDNA generated by reverse transcription of CMV-driven transcripts with the cDNA Synthesis GNH Primer. In order to generate labeled samples for microarray hybridization with Affymetrix GeneChip® Arrays without a stem-loop structure, we designed a second set of nested primers with one containing a biotinylated loop primer (NFwd-Bio) and NRev GNH Universal Primer.

D. Maps and Features of the copGFP Transduction Control Vectors

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



Feature	Location*	Function
RSV/5'LTR	7-414	Hybrid RSV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
gag	567-919	Packaging signal
RRE	1076-1309	Rev response element binds gag and involved in packaging of viral transcripts
cPPT	1798-1916	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
CMV promoter	1922-2271	Human cytomegalovirus (CMV)--constitutive promoter for transcription of copGFP
copGFP	2279-3037	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/transduced cells
WPRE	3044-3584	Woodchuck hepatitis virus posttranscriptional regulatory element--enhances the stability of the viral transcripts
3' ΔLTR (ΔU3)	3723-4263	Required for viral reverse transcription; self-inactivating 3' LTR with deletion in U3 region prevents formation of replication-competent viral particles after integration into genomic DNA
H1 RNA promoter	3761-3977	RNA polymerase III promoter for expression of siRNA insert
siLuc	3979-4053	shRNA targeting Firefly Luciferase
SV40 Poly-A	4335-4466	Transcription termination and polyadenylation
SV40 Ori	4475-4621	Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	4991-5664 (C)	Allows for high-copy replication in *E. coli*
AmpR	5809-6669 (C)	Ampicillin resistant gene for selection of the plasmid in *E. coli*

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

E. Protocol for Amplification of siRNA Targets from Genomic DNA (Alternative to Sections II.D and II.E).

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

1. Purify Genomic DNA

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

2. Amplify siRNA Targets

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

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

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

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

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

e. Commence thermal cycling using the following program:

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

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

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

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

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

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

j. Commence thermal cycling using the following program:

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

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

sec, 68°C for 1 min). Alternatively you can repeat the second round PCR starting from a 5 μl aliquot from step 2.f.

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

- For each PCR reaction (test tube), add 6 volumes of PB buffer and bind to a single QIAquick column.
- Perform the wash step two times (instead of one), using 0.5 ml of washing buffer for each wash.
- For maximum PCR product recovery, elute PCR product from the column once with 22 μl of elution buffer, followed by a second elution with 22 μl of elution buffer. Combine all four eluates from each sample into one test tube. The total volume will be about 160 μl. Take a 1 μl sample from each test tube, dilute it in an appropriate volume of TE, and measure the yield of PCR products using a spectrophotometer at 260 nm. The expected yield of PCR products should be approximately 15-25 μg.

3. Lambda Exonuclease Treatment

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

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

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

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

- For each PCR reaction (test tube), add ten volumes of PB buffer (2 ml) and sequentially apply 0.5 ml at a time to two QIAquick columns.
- Perform the wash step two times (instead of one), using 0.5 ml of washing buffer for each wash.
- For maximum PCR product recovery, elute PCR product from each column once with 22 μl of elution buffer, followed by a second elution with 22 μl of elution buffer. Combine all eluates for each sample into one test tube and concentrate by vacuum centrifugation to a 50 μl volume.

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

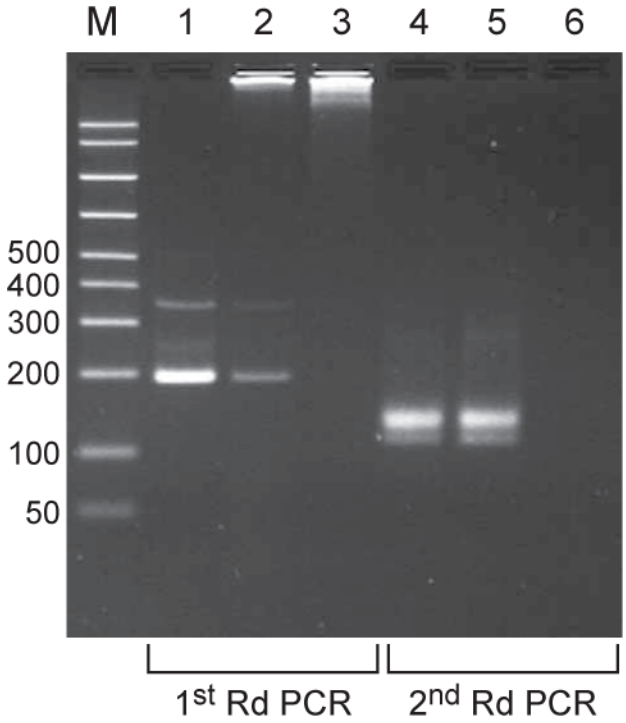


Fig. 13. Analysis of siRNA insert products amplified from genomic DNA. In this experiment, an HIV-based GeneNet™ Human 50K siRNA Library in pSIH1-H1-Puro was used to transduce H1299 cells.

1,4 – Positive Control DNA (plasmid H.50K siRNA library);
2,5 – Genomic DNA from H1299 cells transduced with GeneNet H.50K siRNA library;
3,6 – Genomic DNA from negative control non-infected H1299 cells.

F. Example of a GeneNet™ siRNA Library Screen to Identify Genes Involved in Radiation Resistance

The screening protocol is summarized in the following steps:

- Cells of interest were infected with a stock of GeneNet™ Human 1.5K siRNA library in pFIV-H1-Puro vector, pre-packaged in VSV-G pseudotyped viral particles. The lentiviral constructs integrate into the cellular genome and each cell acquires and expresses one or a few unique siRNA library inserts.
- The library-transduced cells were screened for resistance to radiation. Cells with higher resistance to radiation were expanded (*e.g.* by survival after radiation treatment).
- The siRNA inserts in the selected cells were rescued by RT-PCR.
- The siRNA inserts and corresponding target genes that are involved in specific phenotypic changes were identified by hybridization with a GeneChip® Array.

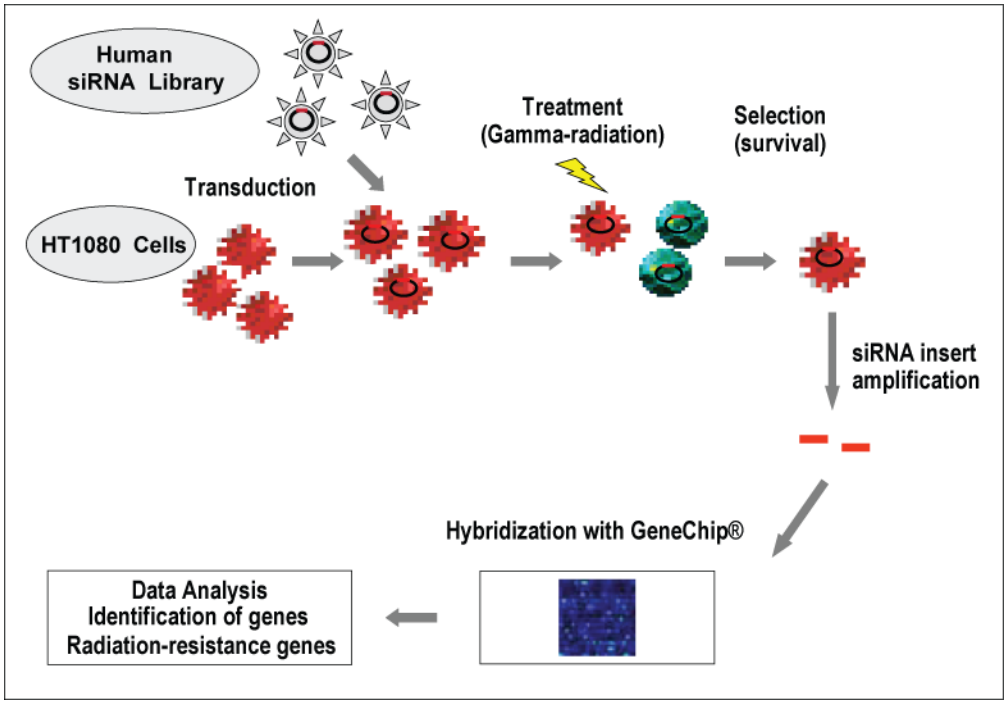


Fig. 14. Outline of the Functional Screening for Genes Involved in Radiation Resistance.

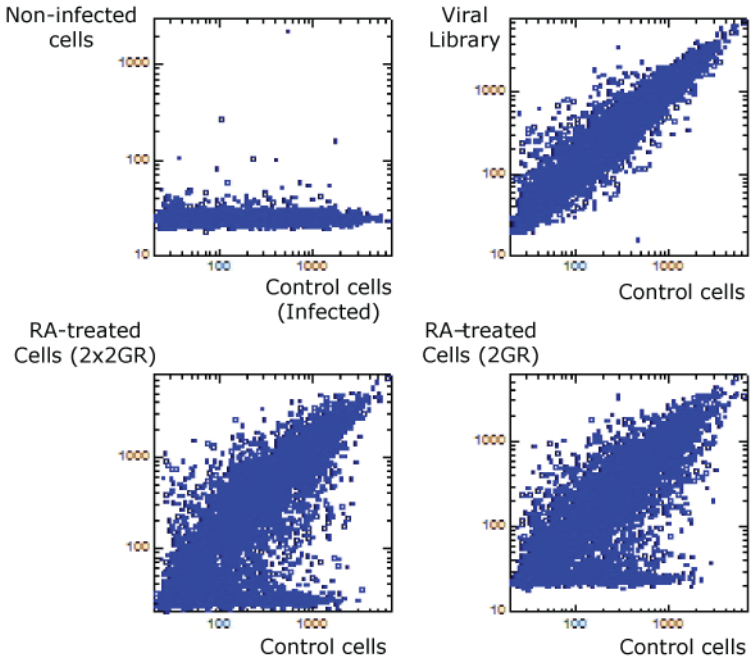


Fig. 15. Scatter Plot Analysis of Genes Involved in Radiation Resistance in HT1080 cells. HT1080 cells (1×10^6 cells) were transduced with the VSV-G pseudotyped packaged GeneNet™ Human 1.5K siRNA Library (5×10^6 infection units) and then subjected to gamma-irradiation (2 grs) 48 hr after infection. Cells were grown for 4 days after this initial irradiation, then a fraction of the cells were treated with an additional dose of gamma-irradiation (2 grs) and grown for an additional four days. To identify siRNA sequences which mediate radiation resistance, we recovered the siRNA templates from total RNA by RT-PCR in each of the cell populations (*i.e.*, non-irradiated control cells, singly-irradiated cells, and double-irradiated cells), and hybridized the amplified biotinylated siRNA targets to the Affymetrix Human Genome Focus Array using the manufacturer's standard protocol.

The intensities of hybridization signals on the microarray reflect the abundance level of particular siRNAs in the entire pool of amplified inserts. We did not find significant loss in siRNA inserts after infection and integration of pseudoviral constructs into the genomic DNA of target HT1080 cells in comparison with the pool of siRNA inserts in the pseudoviral library (Figure 15). Comparison of signal intensities generated by siRNA inserts from cells infected but not treated with radiation (control) and cells treated by radiation allowed us to identify siRNAs significantly enriched after radiation treatment and growth selection. As shown in Figures 15 and 16, cells treated by radiation lost about 30% (one round) and about 50% (two rounds) of siRNA inserts after radiation treatment. We identified nine genes which are significantly enriched (at least 5-fold) in radiation-treated cells, in duplicate experiments. From these nine genes, three genes (MRE11, DOC1 and AK3) have been described as genes involved in radiation resistance.

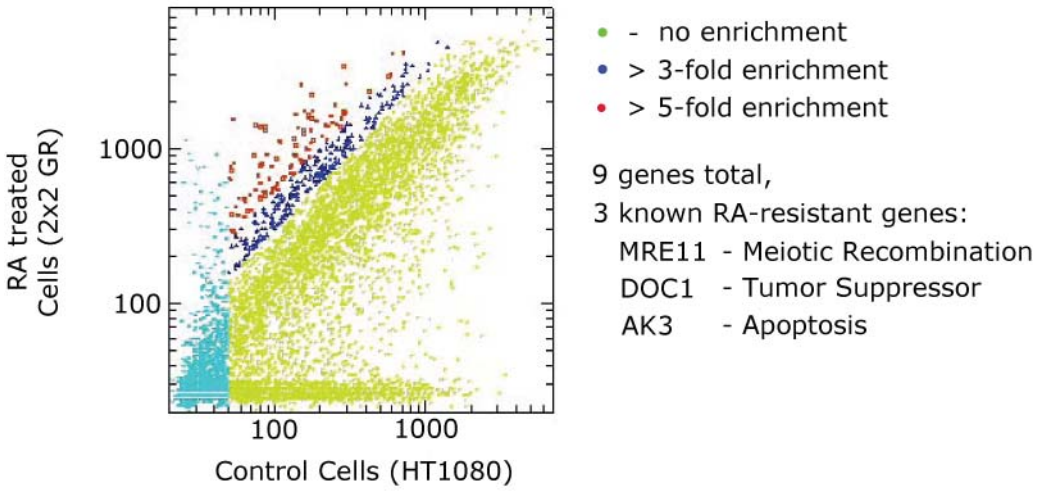


Fig. 16. Identification of genes involved in radiation resistance. Scatter plot analysis of siRNA inserts enriched in HT1080 cells treated by gamma-irradiation.

Pilot studies of the functional performance of GeneNet™ lentiviral siRNA libraries in a radiation resistance cell model demonstrate significant contamination of non-enriched siRNAs in radiation-selected cells due to low efficiency of cell enrichment even after two rounds of selection. Similar low target cell enrichment could be expected for the most commonly used phenotypic assays, based on cell survival or even FACS sorting. These findings demonstrate that, due to the low efficiency of target cell enrichment, hybridization-based analysis of the siRNA population isolated from selected cells is the most practical way to identify weak effectors. As compared with sequencing, microarray technology is a more suitable approach for identification of phenotype-related genes for reasons of cost, ease-of-use, and ease of comparing siRNA representation in several populations. In the case of a more stringent functional selection, resulting in clonal expansion of positive clones, the amplified siRNA inserts (from separate clones) may be identified by sequencing instead of hybridization with GeneChip® Arrays.

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(888) 266-5066 (Toll Free)
Fax: (650) 968-2277
E-mail: tech@systembio.com

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HIV Vector System

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