

## Product Information

### **MISSION® Human Kinase Lentiviral shRNA Pool**

Human Kinase Pooled shRNA Library

Catalog Number **SHK001**

Storage Temperature  $-70\text{ }^{\circ}\text{C}$

## TECHNICAL BULLETIN

### **Product Description**

RNA interference is an effective mechanism for gene silencing, whereby double-stranded RNA triggers the cleavage and subsequent degradation of homologous transcript sequences. In this evolutionarily conserved process, longer double-stranded RNA molecules are processed into shorter sequences (21–23 nucleotide small interfering RNAs or siRNAs) that can bind to the multi-component RNA-induced silencing complex (RISC). Within this complex, the siRNA is unwound and the sense strand is cleaved and dissociated. The antisense strand then remains bound and acts as a guide to target activated RISC to complementary mRNA for cleavage and degradation.

This potent, sequence-specific RNA degradation mechanism was first discovered in plants, where it was termed post-transcriptional gene silencing. It has since been demonstrated in a wide variety of eukaryotic organisms, ranging from fission yeast to humans. These discoveries and subsequent studies into how the RNAi process works have enabled researchers to exploit this pathway and develop tools in order to better elucidate gene function. Plasmid-based expression of gene-specific small hairpin RNAs (shRNA) under the control of RNA polymerase III-dependent promoters is an effective way to trigger this process. With this approach, the shRNAs are processed intracellularly by the enzyme Dicer into siRNAs, which are then able to directly engage RISC.

While individual genes can be efficiently and robustly targeted using arrayed lentiviral libraries, pooled shRNA libraries may be used to rapidly conduct many phenotypic screens. These pooled screens are typically set up such that the majority of cells have been transduced with a single shRNA to aid in downstream deconvolution. Positive selection screens are an example of the type of screen that may be conducted using pooled libraries. Selection of a desired phenotype is the basis of this type of screen and cell viability/survival is a commonly used phenotype. After selection, cells are then isolated by colony cloning or FACS, and the integrated shRNA is identified. The identity of potential "hits" can then be used to develop hypotheses regarding the biological role of the corresponding gene(s). The major steps in this type of screen are outlined in Figure 3. As with all screens, validation of leads by independent methods such as siRNA, small molecule inhibition, or gene knockout will be required.

The protein kinase gene family is among the largest and most well studied of the gene families. Protein phosphorylation plays an essential role in intercellular communication in eukaryotic organisms by mediating signal transduction during development, transcription, immune response, metabolism, apoptosis, and cell differentiation. Aberrant regulation of kinases plays a causal role in many diseases, and the study of these proteins and their functions will contribute to the discovery and development of new therapeutics.

The MISSION® Human Kinase Lentiviral shRNA Pool consists of plasmid-based shRNA constructs targeting a wide variety of human kinase genes. The shRNA sequences (TRC1-2 clones) are designed using a proprietary algorithm developed by the Broad Institute of MIT and Harvard. On average, there are over 12 shRNA designs for each gene target in the kinase pool. The shRNA plasmids are further processed into lentiviral particles to facilitate stable gene silencing in both dividing and quiescent cells.

The MISSION Human Kinase Lentiviral shRNA Pool (Catalog Number SHK001) is a lentiviral pool produced using a proprietary process. Assay for representation of each individual shRNA ensures robust library coverage. The Pool is provided in ready-to-use lentiviral format at titers of at least  $5 \times 10^8$  TU/ml via p24 assay. See Table 1 for approximate number of shRNA constructs.

**Table 1.**

Clone and Gene Coverage by Human Kinase Pooled shRNA Library

Product	Approximate Total Clone Coverage	Approximate Number of Genes
SHK001	9,400+	745+

The MISSION Human Kinase Lentiviral shRNA Pool contains an average of 12.6 clones per gene to increase the statistical strength of the hit analysis; numerous built-in enrichment (PTEN and NF1) and depletion (ANAPC2, ANAPC4, and ESR1) controls to confidently gauge the success of the pooled screening experiments; and over 50 non-target controls as normalization standards (GFP, RFP, LacZ, luciferase, scrambled, etc).

#### Quality Control

Each MISSION Human Kinase Lentiviral shRNA Pool has been tested for representation of each individual shRNA through an internal QC process. The pool has also been tested via p24 assay and has demonstrated a titer of at least  $5 \times 10^8$  TU/ml.

#### **Components**

The MISSION Human Kinase Lentiviral shRNA Pool includes four aliquots - enough virus to perform multiple screens. Each aliquot is provided at  $5 \times 10^8$  TU/ml or higher in a volume of 25  $\mu$ l. Please refer to the Certificate of Analysis for the exact titer.

Additionally, each Kinase Pool kit will include a USB Flash Drive containing the MISSION Human Kinase pool platemap file and the complete list of shRNA clone information.

Note: Primers for amplification and sequencing of MISSION shRNA are not included, but sequence information is provided. See Table 2 for details.

#### **Materials And Reagents Required But Not Provided** For Transduction of MISSION Human Kinase Lentiviral shRNA Pool into Target Cells

- Hexadimethrine bromide (chemical equivalent of Polybrene, Catalog Number H9268)
- Tissue Culture Plates and Related Tissue Culture Supplies
- Puromycin dihydrochloride (Catalog Number P9620)

#### For Purification of Genomic DNA

- GenElute™ Mammalian Genomic DNA Miniprep Kit (70 preps, Catalog Number G1N70)

#### For PCR Amplification of shRNA Inserts

- JumpStart™ Taq ReadyMix™ (Catalog Number P2893)
- Magnesium chloride solution (Catalog Number M8787)
- 1% 1× TAE Agarose gel
- DirectLoad™ PCR 100 bp Low Ladder (Catalog Number D3687)
- Thermal Cycler

#### For Purification of Amplified shRNA Inserts

- GenElute PCR Clean-Up Kit (Catalog Number NA1020)

#### **Additional Suggested Materials**

##### For Screen Controls And Transduction Efficiency Optimization

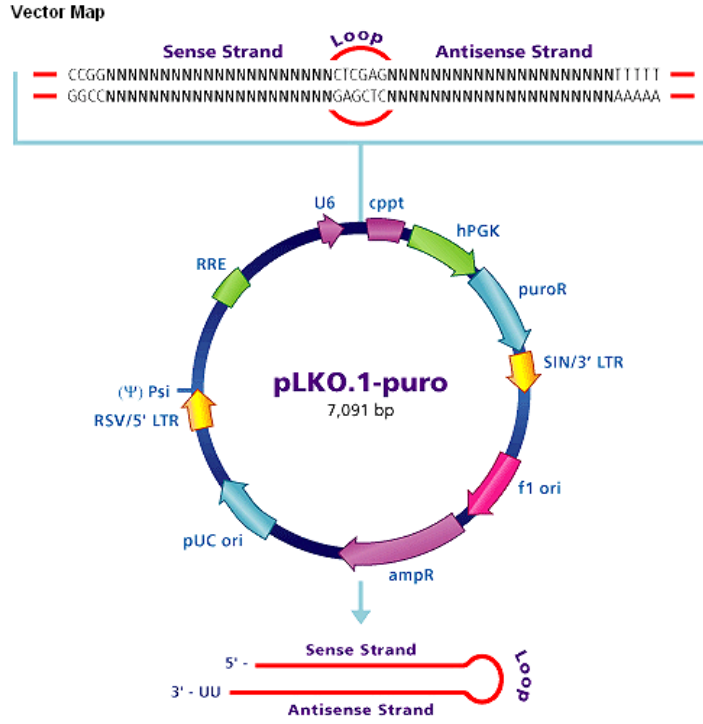
- MISSION TurboGFP™ Control, High Titer (Catalog Number SHC003H):  
This positive control can be used for measuring transduction efficiency and optimizing shRNA delivery in cell lines that do not survive any puromycin exposure. The TurboGFP Control contains a gene encoding TurboGFP driven by the CMV promoter. This control provides fast visual confirmation of successful transduction. It should be noted that the CMV promoter does not work well in some cell lines, resulting in decreased GFP expression. A literature search on the efficacy of CMV-driven expression in the cell line of interest is recommended.

- MISSION pLKO.1-puro Control, High Titer (Catalog Number SHC001H):  
The empty vector high titer control is a useful negative control that will not activate the RNAi pathway because it does not contain an shRNA insert. It will allow for observation of potential cellular effects due to the transduction process.
- MISSION pLKO.1-puro ARHGDI<sup>A</sup> shRNA Positive Control Transduction Particles (Catalog Number SHC009):  
This control is intended for use as a positive control to ensure proper PCR reaction and cycling conditions.
- MISSION Non-Mammalian shRNA Control Transduction Particles, High Titer (Catalog Number SHC002H) OR MISSION Non-Target shRNA Control Transduction Particles, High Titer (Catalog Number SHC016H):  
These non-targeting shRNA controls are a useful negative control that will activate RISC and the RNAi pathway, but do not target any known human or mouse genes. The short hairpin sequence contains 5 base pair mismatches to any known human or mouse gene. This allows for examination of the effects of shRNA transduction/transfection on gene expression. Cells transduced with the non-target shRNA will also provide useful reference for interpretation of knockdown.  
Note: SHC002H Non-Mammalian shRNA high titer control does target and knockdown GFP expression. If a cell line expresses GFP, the use of SHC016H is recommended.

**Table 2.**  
Suggested Materials (**Not Included**) – Primers and Positive Control

Description	Sequence	Minimum Volume	Approximate Concentration
Amplification Primer 1 (Forward)	CGATACAAGGCTGTTAGAGAGATA	750 $\mu$ l	20 $\mu$ M
Amplification Primer 2 (Reverse)	CAAAGTGGATCTCTGCTGTCC	750 $\mu$ l	20 $\mu$ M
Sequencing Primer	ATACAAGGCTGTTAGAGAGA	750 $\mu$ l	5 $\mu$ M
MISSION pLKO.1-puro ARHGDI <sup>A</sup> shRNA Positive Control Plasmid (Catalog Number SHC009)	<b>CCGGCAAGATTGACAAGACTGA</b> <b>CTACTCGAGTAGTCAGTCTTGTC</b> <b>AATCTTGTTTT</b>	20 $\mu$ l	500 ng/ $\mu$ l

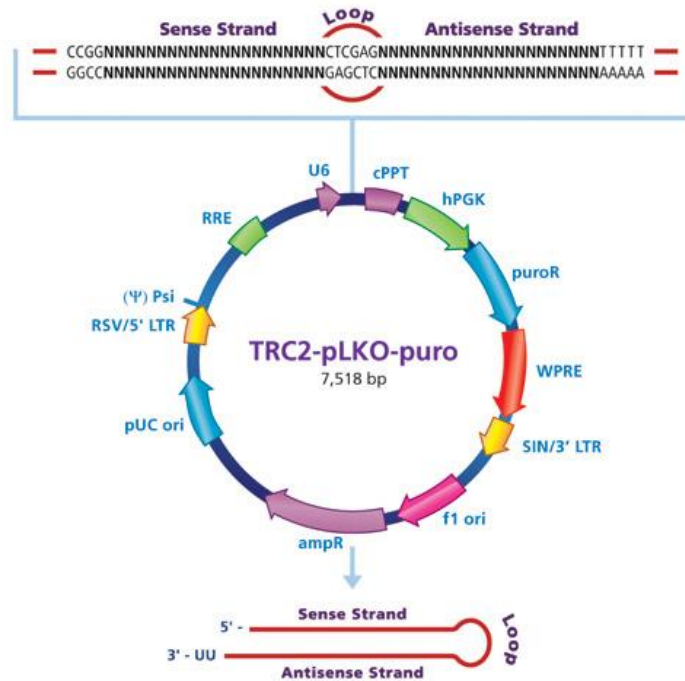
**Figure 1.**  
**TRC1.0 & 1.5 pLKO.1 Vector Map and Features**



**TRC1.0 & 1.5 Vector Description and Features**

Name	Description
cppt	Central polypurine tract
hPGK	Human phosphoglycerate kinase eukaryotic promoter
puroR	Puromycin resistance gene for mammalian selection
SIN/LTR	3' self inactivating long terminal repeat
f1 ori	f1 origin of replication
ampR	Ampicillin resistance gene for bacterial selection
pUC ori	pUC origin of replication
5' LTR	5' long terminal repeat
Psi	RNA packaging signal
RRE	Rev response element

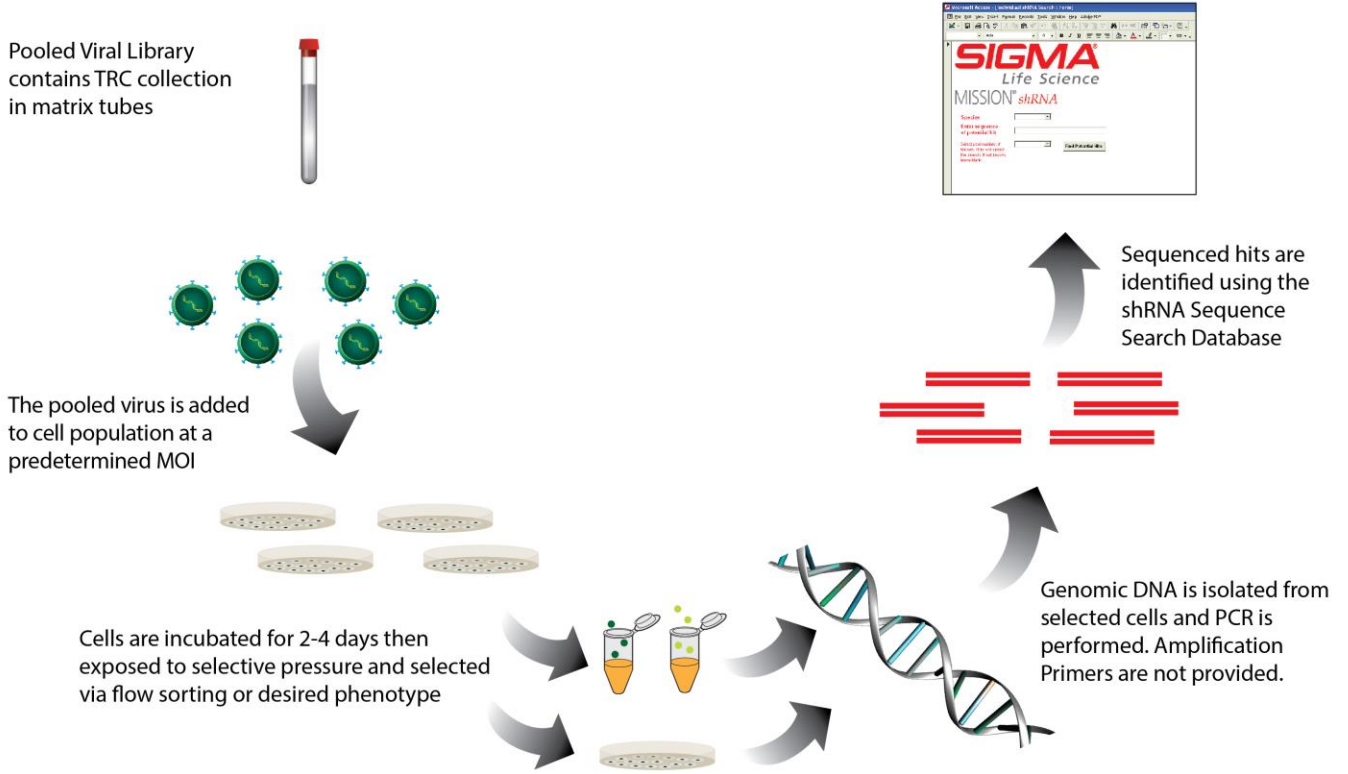
**Figure 2.**  
**TRC 2.0 pLKO.5 Vector Map and Features**



**TRC 2.0 Vector Description and Features**

Name	Description
cppt	Central polypurine tract
hPGK	Human phosphoglycerate kinase eukaryotic promoter
puroR	Puromycin resistance gene for mammalian selection
WPRE	Woodchuck Hepatitis Post-Transcriptional Regulatory Element
SIN/LTR	3' self inactivating long terminal repeat
f1 ori	f1 origin of replication
ampR	Ampicillin resistance gene for bacterial selection
pUC ori	pUC origin of replication
5' LTR	5' long terminal repeat
Psi	RNA packaging signal
RRE	Rev response element

Figure 3.  
Schematic of Workflow



## Precautions And Disclaimers

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Though the lentiviral transduction particles produced are replication incompetent, it is recommended they be treated as Risk Group Level 2 (RGL-2) organisms for laboratory handling. Follow all published RGL-2 guidelines for laboratory handling and waste decontamination. Also, use extra caution because the pooled library will contain lentiviral particles that express shRNAs targeting both cell cycle control and tumor suppressor genes.

## Storage and Stability

All components are stable for at least 6 months after receipt when stored at  $-70^{\circ}\text{C}$ . Avoid repeated freeze/thaw cycles, which will severely reduce transduction efficiency.

## Procedures

### Overview of Pooled Library Screen

There are five primary steps required for utilizing the Human Kinase Lentiviral Library. The following is a brief description of each step and its importance. Detailed protocols follow.

- A. Optimization of Puromycin Selection Conditions:  
To effectively eliminate colonies with no shRNA insert, optimization of puromycin selection conditions is suggested for each new cell line tested. This can be accomplished by performing an antibiotic kill curve to determine the optimal concentration of puromycin needed to eliminate non-transduced cells. The lowest concentration of puromycin that kills all non-transduced cells should be utilized for subsequent experiments. Utilizing higher concentrations of puromycin may lead to unacceptably high cytotoxicity and potential off-target effects.
- B. Optimization of Transduction Efficiency:  
To successfully identify shRNA sequences of interest, it is critical to set up the screen such that each cell receives only one shRNA construct. By using a low MOI (multiplicity of infection) the probability of multiple integrants per cell is greatly decreased. However, transduction efficiencies and, therefore, desired MOIs, depend strongly on the target cell type. Therefore, it is imperative that determination of the optimal MOI is carried out before starting the screen in a new cell type.  
  
The optimal MOI can be determined by testing a range of MOIs using either MISSION pLKO.1-Puro (Catalog Number SHC001H), MISSION pLKO.1-Puro Non-Mammalian shRNA (Catalog Numbers SHC002H or SHC016H), or MISSION TurboGFP Control Transduction Particles (Catalog Number SHC003H) on a fixed cell density. Detailed protocols are provided.
- C. Transduction and Selection of Target Cells with the MISSION Human Kinase Lentiviral shRNA Pool:  
After the optimal MOI is determined, cells are transduced with the viral library. Transduced cells may be selected using puromycin before the screen is initiated depending upon the type of screen being performed. For screens utilizing a reporter enzyme, the puromycin selection step will eliminate non-transduced cells, minimizing the number of cells that will need to be sorted in the end, thereby, maximizing the dynamic range of the assay. For screens assessing changes in viability, the puromycin selection step may not be required if the selective pressure is sufficient to eliminate non-transduced cells. Protocols provided are written to include the puromycin selection step.  
  
In the provided protocols, several negative controls have been suggested. Positive controls for pooled library screens will be assay and cell-type dependent. While not required, a positive control specifically designed for the assay will aid in data interpretation and downstream troubleshooting.

D. PCR Amplification:

Total genomic DNA is isolated from the selected and expanded cell populations. The shRNA inserts can then be readily amplified using the primer sequences provided. The MISSION pLKO.1-puro ARHGDI<sup>A</sup> shRNA Positive Control Plasmid (Catalog Number SHC009, not included) should be used as template in a separate reaction in order to ensure the PCR reactions are working optimally.

E. Identification of Positive Hits:

Sequence analysis of the PCR amplicons recovered from cells expressing the phenotype of interest can identify hits from the positive selection screen. The primer sequences are provided in Table 2.

**Detailed Protocols**

A. Optimization of Puromycin Selection

To generate a fully transduced population of cells it is important to determine the minimum amount of puromycin required to eliminate non-transduced cells. The appropriate concentration of puromycin for each cell type will vary. If the concentration for the desired cell type is unknown, a titration experiment is required to determine the lowest concentration of puromycin needed to efficiently select transduced cells. Typically, 1–10  $\mu\text{g}/\text{ml}$  is sufficient to kill most non-transduced mammalian cell types. Higher concentrations of puromycin are undesirable as they can lead to toxicity even in successfully transduced populations.

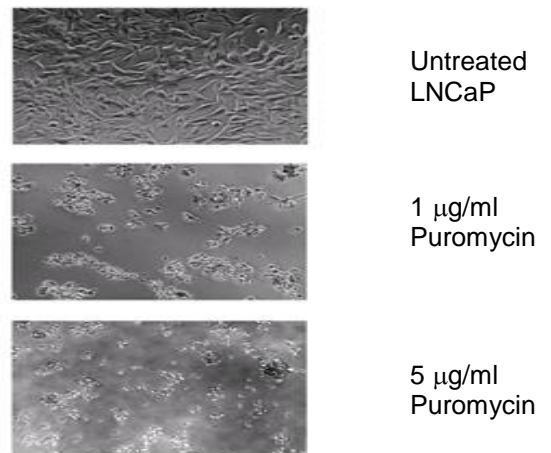
1. Plate non-transduced cells at the same density per well (~30% confluency) in 6 or 24 well plates. Utilize the same number of wells as the planned number of concentrations to test, plus 1 control well that will be maintained in puromycin-free conditions.
2. Allow cells to adhere by incubating overnight at 37 °C in a humidified incubator in an atmosphere of 5% CO<sub>2</sub>.

3. The next day substitute the culture medium with medium containing varying concentrations of puromycin (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10  $\mu\text{g}/\text{ml}$ ). For a control, always maintain one well of cells without puromycin.
4. Replenish the selective medium containing puromycin every 2–3 days and observe the percentage of surviving cells.
5. The minimum concentration of puromycin that causes complete cell death after 7–10 days should be used in subsequent selection steps for that particular cell type.  
Helpful Tip: If cells require a higher concentration of puromycin than the recommended 1–10  $\mu\text{g}/\text{ml}$ , it is recommended to check the expiration date of the puromycin used. Also avoid multiple freeze-thaw cycles of the prepared puromycin stock.

For suggested puromycin concentrations for your cell line of choice, please refer to this table: <http://www.sigmaaldrich.com/life-science/functional-genomics-and-mnai/shrna/learning-center/lentivirus-cell-line.html>

**Figure 4.**

LNCaP cells treated with 0, 1, and 5  $\mu\text{g}/\text{ml}$  of puromycin for five days





## B. Optimization of Transduction Efficiency

MOI refers to the number of transducing lentiviral particles per cell. Because the relative transduction efficiency between cell types may vary widely, it is highly recommended to determine the appropriate MOI for each individual cell type that will be transduced. This will establish the optimal amount of lentiviral supernatant needed for subsequent experiments. There are two commonly used methods to determine the most efficient MOI for a particular cell line prior to starting a screen:

Option 1 – Titering of lentiviral particles on the target cells and subsequent scoring for puromycin resistance.

Option 2 – Titering of MISSION TurboGFP Control transduction particles and assessment of GFP expression.

Option 1 Procedure – Titering by Transfer of Puromycin Resistance (Protocol For Adherent Cells, recommended format is a 6 well cell culture plate)  
Either of the following shRNA lentiviral particles controls can be used: MISSION pLKO.1-puro Control (Catalog Number SHC001H), MISSION Non-Mammalian shRNA Control (Catalog Number SHC002H), or MISSION Non-Target shRNA Control (Catalog Number SHC016H).

Day 1 – Plate the cells.

1. Plate the appropriate number of wells with enough cells to obtain ~50% confluency on the following day.
2. Allow cells to adhere by incubating overnight at 37 °C in a humidified incubator in an atmosphere of 5% CO<sub>2</sub>.

Day 2 – Transduce the cells with viral particles.

1. Thaw the lentiviral particles at room temperature and immediately place on ice when thawed. Mix by gently tapping the tube several times with finger. Store the lentiviral stock on ice.
2. Prepare medium containing hexadimethrine bromide with a final concentration of 8 µg/ml for the cell type(s) of interest.
3. Take 15 ml conical vials and prepare 5 10-fold dilutions with a final volume in each tube of 2 ml (10-fold serial dilutions over a range of  $1 \times 10^{-2}$  to  $1 \times 10^{-6}$ ) with the lentiviral stock. After each dilution, mix gently, by inverting the tubes five to ten times.

4. Add 1 ml of medium containing hexadimethrine bromide to one well of the 6 well plate as a control well. Starting with the  $10^{-6}$  dilution and working back to the  $10^{-2}$  dilution, add 1ml of each of the lentiviral dilutions to the remaining wells of the plate.
5. Incubate 18–20 hours at 37 °C in a humidified incubator in an atmosphere of 5% CO<sub>2</sub>.

Day 3 – Change the medium.

1. Remove the virus-containing medium from the wells.
2. Add fresh complete medium.
3. Return cells to incubator in order to allow cells time to recover after transduction for 24 hours.

Day 4 – Puromycin selection of transduced cells.

After the 24 hour recovery time, change medium to medium supplemented with puromycin

Note: The puromycin concentration for a particular cell line should be determined experimentally by performing an antibiotic kill curve assay prior to starting the limiting dilution experiment, previously described.

Days 5 through 14 – Removal of non-transduced cells.

Replace the puromycin-containing medium as necessary during the selection process, for up to 7–10 days.

Day 15 – Crystal violet staining assay

1. Verify that all cells in the control well are dead. This ensures that any living cells in the transduced wells are alive because of the active puromycin resistance from the integrated virus.
2. Remove medium and gently wash each well with warmed 1× Phosphate Buffered Saline (made from 10× PBS stock, Catalog Number P5493).
3. Add 1 ml of Crystal Violet Solution (Catalog Number HT90132) and incubate 10 minutes at room temperature.
4. Remove crystal violet solution.
5. Wash with 3 ml of PBS and swirl gently.
6. Repeat steps 4 and 5 until PBS is clear. Minimum of 2 PBS washes is recommended after crystal violet staining.
7. Count the purple-stained colonies using a microscope at a magnification of 40×.

Helpful tips for Option 1 Procedure:

1. Multiple freeze-thaw cycles and prolonged exposure to ambient temperatures will decrease lentiviral functional titer. It is recommended to aliquot leftover virus after the first thaw and store aliquots at  $-80\text{ }^{\circ}\text{C}$ .
2. If cells exhibit sensitivity to hexadimethrine bromide, omit the reagent from the protocol. The cells will still be transduced but with a lower efficiency.
3. It is important to remove virus-containing medium from the wells after 24 hours and to add fresh complete medium in order to avoid toxicity.

Option 2 Procedure – Titering using TurboGFP Particles (Recommended format is a 6 or a 24 well cell culture plate)

MISSION TurboGFP control particles enable fast determination of the appropriate MOI for efficient transduction of cells with lentiviral particles.

Note: The CMV promoter is known to function poorly in stem cells, lymphocytes, and primary neurons. For these cell types, observed GFP signal is not always concordant with positively transduced cells, often leading to an underestimation of transduction efficiency. For these cell types, utilize Option 1 Procedure – Titering by Transfer of Puromycin Resistance.

Day 1 – Plate the cells.

1. Plate the appropriate number of wells with enough cells to obtain ~50% confluency on the following day. The required number of wells to be seeded depends on the range of MOIs to be tested.
2. Allow cells to adhere by incubating overnight at  $37\text{ }^{\circ}\text{C}$  in a humidified incubator in an atmosphere of 5%  $\text{CO}_2$ .

Day 2 – Transduce the cells with viral particles.

1. Thaw the lentiviral particles at room temperature. Mix by gently tapping the tube several times with finger. Store the lentiviral stock on ice.
2. Add fresh medium containing hexadimethrine bromide at a final concentration of  $8\text{ }\mu\text{g/ml}$  to the cells. Meanwhile, prepare 10-fold serial dilutions of the TurboGFP viral preparation and add to the designated wells.
3. Incubate 18–20 hours at  $37\text{ }^{\circ}\text{C}$  in a humidified incubator in an atmosphere of 5%  $\text{CO}_2$ .

Day 3 – Change the medium.

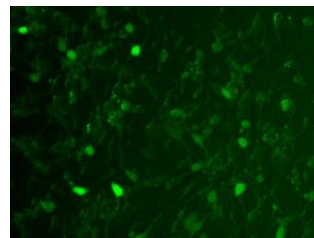
Remove the viral-containing medium from wells and add fresh medium.

Day 4 – Examine the TurboGFP expression.

1. Incubate cells for an additional 24–48 hours to allow for GFP expression. Ensure adequate time has been allowed for protein expression. Robust expression of TurboGFP is typically observed 72 hours post-transduction.
2. Observe the cells using a fluorescent microscope. For the majority of pooled screens, the amount of virus that leads to 30–50% of the cells being transduced is preferable, as the majority of cells will, therefore, harbor only a single integrant.

**Figure 5.**

Example: LNCaP cells 7 days post-transduction with TurboGFP at MOI of 1



Helpful tips for Option 2 Procedure:

1. Include a non-transduced negative control well, where no virus is added.
2. Make sure to allow sufficient time for the expression of TurboGFP (usually at 72 hours post transduction).

C. Transduction of Target Cells with the MISSION® Human Kinase Lentiviral shRNA Pool

This protocol provides general guidelines for the transduction of target cells using an MOI of 1. The actual MOI used will vary for different cell types. If replicates are desired then the number of plates must be increased accordingly.

Day 1 (Protocol For Adherent Cells, 100 mm dish format) – Plate the cells.

1. Seed the appropriate number of 100 mm dishes with enough cells to obtain ~50% confluency on the following day.
2. Allow cells to adhere by incubating overnight at 37 °C in a humidified incubator in an atmosphere of 5% CO<sub>2</sub>.
3. (Optional) Include two additional 100 mm dishes for transduction with negative control shRNAs. Label the dishes “Control A” for SHC001H and “Control B” for SHC002H or SHC016H.

Day 2 – Transduce the cells with the Kinase Pool viral particles.

1. Using the p24 titers provided on the Certificate of Analysis, calculate how much of the viral library to add to the cells to get the MOI of 1 across all cell culture dishes.

Example:  $1 \times 10^6$  cells/dish;

Viral titer =  $5 \times 10^8$  TU/ml; a desired MOI of 1

$1 \times 10^6$  cells/dish  $\times$  (MOI of 1) =  $1 \times 10^6$  transducing units (TU) needed

$1 \times 10^6$  TU / ( $5.0 \times 10^8$  TU/ml) from Certificate of Analysis = 2  $\mu$ l of lentiviral stock solution should be added to the appropriate dish. Dilute the calculated amount of the virus in 100–300  $\mu$ l of complete medium in order to ensure better distribution of the virus when added to the cell culture dish.

2. On the day of transduction, thaw the lentiviral particles at room temperature and immediately place on ice when thawed.
3. Transfer the thawed particles to a laminar flow hood and keep on ice if not being used immediately.
4. Remove medium from the seeded cells. Add complete medium containing hexadimethrine bromide solution to each dish. The recommended final concentration of hexadimethrine bromide in medium is 8  $\mu$ g/ml. Use less hexadimethrine bromide if it is toxic to the target cells.
5. Add shRNA lentiviral particles to appropriate dishes in accordance with the predetermined experimental design.
6. Gently swirl the plates to evenly distribute the virus across cells.
7. Incubate 18–20 hours at 37 °C in a humidified incubator in an atmosphere of 5% CO<sub>2</sub>.
8. (Optional) Repeat steps 3-8 at the identical MOI with SHC001H in Control Dish A and SHC002H or SHC016H in Control Dish B. Treat these dishes identically to the experimental dishes until the end of this procedure.

Day 3 – Change the medium.

1. Aspirate virus-containing medium and add fresh complete medium (without hexadimethrine bromide).
2. Incubate the cells at 37 °C overnight.

Day 4 – Puromycin selection of transduced cells

Aspirate medium from wells and add fresh medium containing puromycin at the optimal concentration, determined from the puromycin kill curve assay.

Days 5 and 6 – Removal of non-transduced cells

Replace medium with fresh puromycin-containing medium every 2–4 days until only transduced cells remain.

Day 7 –Perform selection

1. Apply the selective pressure of interest. Duration of selective pressure is highly dependent upon the nature of the screen.
2. (Optional) At the end of selection, inspect Control Dish A and Control Dish B. Dish A and Dish B should each have fewer colonies than at least one of the pooled library dishes.

If there are an unexpectedly high number of colonies in Dish A, the transduction process has affected a pathway related to the desired phenotype or the selective pressure isn't sufficiently strong and re-optimization of the assay may be required.

If Dish B contains too many colonies, then expression of shRNA and engagement of the RNAi pathway may have an effect on the phenotype of interest. If this is the case repeat with SHC001H, to ensure that the effect isn't specific to SHC002H or SHC016H.

D. Individual Colony Isolation

After stringent selective pressure has been applied, colonies representing individual shRNA sequences should become visible.

Picking Colonies – Using sterile technique in a tissue culture hood, pick out individual colonies using a small volume pipettor (10–20  $\mu$ l maximum volume). Use a fresh tip for each colony. Seed colonies into individual wells of a 96 well plate, which contain medium with puromycin. As colonies expand they can be transferred to larger wells.

Genomic DNA Isolation – To obtain enough DNA for downstream analysis steps, culture the cells until they are confluent. The GenElute Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70, 70 preps) can be used for genomic DNA purification procedure. Alternatively, any other genomic purification kits that are currently available on the market can be used for DNA isolation. It is important to follow protocol recommendations for the starting amount of cultured cells.

E. Deep Sequencing and Deconvolution

For a list of pooled screening and deep sequencing protocols please refer to this public TRC Broad Public link:  
<http://www.broadinstitute.org/rnai/public/resources/protocols>

Easily Identify Hits from Pooled shRNA Screens - Sigma provides help with deconvolution of pooled shRNA samples after screening is complete. Sigma's deconvolution service will help you identify TRC clones that are of significance in your screen.

Once you provide us with cell or tumor samples post screening, we will extract genomic DNA (or you provide gDNA initially), conduct our proprietary sequencing reaction and provide data on the presence of each TRC clone in each sample. Using our proprietary sequencing technique, we can decode the kinase library within a single sequencing reaction.

Please contact [MissionRNAi@sial.com](mailto:MissionRNAi@sial.com) or visit <http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/shrna-screening/deconvolution-of-pooled-shrna-screens.html> for more information or to obtain pricing information.

F. Identification of Positive Hits

In order to identify hits, the individual shRNA construct must be amplified from the cells and then sequenced. Once sequenced, the individual shRNA can be identified and re-ordered from the Sigma website to confirm the observed phenotype and further study the target.

PCR Amplification of Targets – The shRNA template recovery procedure enables amplification of the entire pool of shRNA inserts from the selected cell population, or the retrieval of individual shRNA templates from colonies that were individually picked and expanded. After amplification of shRNA inserts from control and selected target cells, the PCR products can be sequenced (protocol follows).

Purification and amplification of genomic DNA

**Note:** The PCR (steps 4–7) was optimized using JumpStart *Taq* ReadyMix (Catalog Number P2893). Other reagents can be used for amplification, but the cycling conditions and/or magnesium concentration may need to be modified for successful amplification.

1. Begin with  $2 \times 10^6$  cultured cells from each colony to be analyzed.
2. Prepare genomic DNA using the GenElute Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70) following the procedure outlined in the technical bulletin of the kit.
3. The concentration and quality of the genomic DNA prepared with the GenElute kit can be determined by spectrophotometric analysis and agarose gel electrophoresis.
4. Use the following reagents to set-up PCR reaction:
  - JumpStart *Taq* ReadyMix (Catalog Number P2893)
  - Magnesium chloride solution Catalog Number M8787)
5. Set up the PCR reaction, see Table 4. Add the components in the order listed. The described amounts are for one 50  $\mu$ l reaction. For more reactions, scale the master mix components by the desired number of reactions to be run and include a 10% overage. The amount of DNA template recommended is 50–100 ng. It is strongly recommended that one reaction template consists of the MISSION pLKO.1-puro ARHGDIa shRNA Positive Control Plasmid (Catalog Number SHC009, not included) diluted to the appropriate concentration. Successful amplification with this template indicates that the PCR assay components and cycling conditions are adequate.

**Note:** To prevent carryover contamination of experimental samples and reagents, it is suggested that the positive control be diluted in a separate location from where subsequent PCR reactions are set up.

**Table 4.**  
PCR reaction components

Component	Vol ( $\mu$ l)	Final Concentration
Water (PCR grade)		–
JumpStart <i>Taq</i> ReadyMix (2 $\times$ )	25	1 $\times$ includes 1.5 mM MgCl <sub>2</sub>
Magnesium chloride solution	3	1.5 mM 3 mM total*
Forward Primer (20 $\mu$ M)	1	0.4 $\mu$ M
Reverse Primer (20 $\mu$ M)	1	0.4 $\mu$ M
DNA Template (50–100 ng)		–
Total volume		50 $\mu$ l

\*Final concentration of Mg<sup>2+</sup> in solution will be 3 mM; 1.5 mM is contributed from the *Taq* ReadyMix and 1.5 mM from the Magnesium chloride solution.

6. Save the following program (Table 5) into the thermocycler:

**Table 5.**  
PCR amplification program

PCR Program Positive Selection Screen		
	Temp.	Time
Hot Start Enzyme Activation	94 °C	30 sec
Denaturation	94 °C	15 sec
Annealing	44 °C	30 sec
Extension	72 °C	30 sec
Cycles	40 cycles	
Final Extension Hold	72 °C	1 min
Amplicon Size	459	

7. For each sample, combine 3  $\mu$ l of PCR reaction with 1  $\mu$ l of dye and run along with 5  $\mu$ l of DirectLoad PCR 100 bp Low Ladder (Catalog Number D3687) on a 1% agarose gel to confirm a 459 bp amplicon.
8. Prepare PCR products for sequencing. Purify PCR reactions using the GenElute PCR Clean-Up Kit (Catalog Number NA1020) by following the procedure outlined in the technical bulletin.
9. The concentration and quality of purified DNA is determined by spectrophotometric analysis.

### Sequencing Of Targets

Sequencing the PCR amplicon recovered from the cells expressing the phenotype of interest can identify hits from the positive selection screen. Primer sequences are provided in order to sequence individual shRNA clones. **Note:** Amplified fragments can also be cloned into the plasmid vector of choice for subsequent sequencing steps.

More information on the TRC shRNA sequence(s) identified and the corresponding gene targets can be readily found on Sigma-Aldrich's Batch Search for shRNA: [http://www.sigmaproducts.com/Informatics\\_tools/batch-search.php#shRNA](http://www.sigmaproducts.com/Informatics_tools/batch-search.php#shRNA).

### Target Identification

The shRNA sequence initiates with 5' GAAACACCGG 3'. Enter at least the next 10 but less than 21 nucleotides as the query sequence into the Search box on the enclosed shRNA Sequence Search Access database form. Select the species of the pooled shRNA (human). Now click on "Find Potential Hits" button to perform the search. This should identify the corresponding TRC shRNA sequence(s) that match the sequencing data.

Alternatively, corresponding TRC number(s) generated from shRNA sequence(s) can also be manually entered into the main search tool on Sigma.com to search by gene and associated shRNA clones.

In the event that no sequence is identified using these search functions, please refer to the MISSION Human Kinase Lentiviral shRNA Pool platemap file included on the USB drive which contains shRNA clone information, or contact us at [RNAi@sial.com](mailto:RNAi@sial.com).

### Troubleshooting Guide

For answers to questions that are not addressed here, please email technical support at [RNAi@sial.com](mailto:RNAi@sial.com).

Problem	Cause	Solution
Poor transduction efficiency	Target cell density is too high or too low	The growth rate of different cell types varies greatly. Adjust the number of cells plated to accommodate a confluency of 50% upon transduction.
	Target cell line may be difficult to transduce	Optimize the transduction protocol using TurboGFP Control Lentiviral Particles. Transfection efficiency can often be increased in hard-to-transduce cells by using the ExpressMag <sup>®</sup> Transduction System (Catalog Number SHM01).
	Loss of viral titer during storage	Ensure storage of the MISSION Human Kinase Lentiviral shRNA Pool at -70 °C. Each freeze-thaw cycle may cause reduction of the titer by as much as 20–30%.
Transduction affects target cell viability	Transduction with MISSION Human Kinase Lentiviral shRNA Pool affects target cell growth	Use a shorter transduction time to minimize potential toxic effects on the target cells. Overnight incubation with virus may present a toxicity concern. Cells may be incubated for as little as 4 hours before changing the medium, though the transduction efficiency may be slightly lower.
	Hexadimethrine bromide is toxic to the target cells	Optimize the concentration and exposure time to hexadimethrine bromide during the transduction step. Recommended concentration of hexadimethrine bromide is 8 µg/ml. Include a hexadimethrine bromide control well in the experiment.
	Puromycin selection is started too early	Some cells show decreased viability in the presence of puromycin. Let cells recover for 24–48 hours in complete medium after transduction before the introduction of puromycin.
	Puromycin concentration is not optimal for cells	Use the concentration of puromycin determined by antibiotic kill curve.

## Troubleshooting Guide (continued)

Problem	Cause	Solution
Cannot identify shRNA in hits	No sequencing data	There may have been loss of the PCR product during the clean-up procedure. Repeat PCR amplification step and clean-up product again. Confirm the concentration and quality of purified PCR by spectrophotometric and/or 1% agarose gel analysis.
	No matching sequence identified using search tool	Misreading of nucleotides from the sequence analysis step may prohibit the identification of the proper shRNA sequences. Assess the sequence calls more closely to ensure proper sequence identification.
Non-Specific or No PCR product	Problem with the reaction components, or the thermal cycling conditions	To ensure proper PCR reaction and cycling conditions include the PCR Positive control (a purified plasmid DNA MISSION shRNA Human Positive Control Vector) as the template for one of the reactions. This control will produce a PCR product (~300 bp). If the positive control does not amplify, use fresh aliquots of reagents and repeat the amplification step.
	Contamination from plasmid DNA	Take all necessary precautions (physical separation, dedicated pipettes, barrier tips, etc.) to minimize the risk of contamination from the positive control.
	Carry over contamination	Separate DNA extraction, pre-PCR set up, and post-PCR examination locations. Include a negative (no DNA) control in the DNA extraction and PCR set up. Use filter tips to reduce the risk of transferring DNA between tubes. Use from 1.0–0.01 picograms of Plasmid DNA Positive Control per PCR reaction.

### Label Licenses:

#### These licenses are relevant for all Mission products:

Use of this product for Commercial Purposes requires a license from Sigma-Aldrich Corporation. The purchase of this product conveys to the buyer the nontransferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party, or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. Commercial Purposes means any activity by a party for consideration, but excludes not-for-profit core facilities providing services within their own research institutions at cost. Core facilities are invited to join Sigma-Aldrich's RNAi Partnership Program. Details of Sigma-Aldrich's RNAi Partnership Program can be found at [www.sigma.com/rpp](http://www.sigma.com/rpp).

This product is licensed under U.S. Pat. Nos. 5,817,491; 5,591,624; 5,716,832; 6,312,682; 6,669,936; 6,235,522; 6,924,123 and foreign equivalents from Oxford BioMedica (UK) Ltd., Oxford, UK, and is provided for use in academic and commercial *in vitro* and *in vivo* research for elucidating gene function, and for validating potential gene products and pathways for drug discovery and development, but excludes any use of LentiVector® technology for: creating transgenic birds for the purpose of producing useful or valuable proteins in the eggs of such transgenic birds, the delivery of gene therapies, and for commercial production of therapeutic, diagnostic or other commercial products not intended for research use where such products do not consist of or incorporate a lentiviral vector. Information about licenses for commercial uses excluded under this license is available from Oxford BioMedica (UK), Ltd., Medawar Center, Oxford Science Park, Oxford OX4 4GA UK [enquiries@oxfordbiomedica.co.uk](mailto:enquiries@oxfordbiomedica.co.uk) or BioMedica Inc., 11622 El Camino Real #100, San Diego CA 92130-2049 USA. LentiVector is a registered US and European Community trademark of Oxford BioMedica plc.

This product (based upon the lentikat system) is sub-licensed from Invitrogen Corporation under U.S. Patent Nos. 5,686,279, 5,834,256, 5,858,740; 5,994,136; 6,013,516; 6,051,427, 6,165,782, and 6,218,187 and corresponding patents and applications in other countries for internal research purposes only. Use of this technology for gene therapy applications or bioprocessing other than for nonhuman research use requires a license from Cell Genesys, Inc. Please contact Cell Genesys, Inc. at 342 Lakeside Drive, Foster City, California 94404. Use of this technology to make or sell products or offer services for consideration in the research market requires a license from Invitrogen Corporation, 1600 Faraday Ave., Carlsbad, CA 92008.

#### These licenses are relevant for all Mission products *but* SHP001:

This product is for non-clinical research use only. It is not to be used for commercial purposes. Use of this product to produce products for sale or for diagnostic, therapeutic or high throughput drug discovery purposes (the screening of more than 10,000 compounds per day) is prohibited. This product is sold under license from Invitrogen Corporation. In order to obtain a license to use this product for these commercial purposes, contact The Regents of the University of California. This product or the use of this product is covered by U.S. Patent No. 5,624,803 owned by The Regents of the University of California.

#### These licenses are relevant for all Mission products *but* SHC001, SHC001H, SHC001V, SHC003, SHC003H, SHC003V, SHP001:

Licensed under Carnegie Institution US Patent 6,506,559 and Massachusetts Institute of Technology and for laboratory and commercial research use only. This product is licensed under agreement with Benitec Australia Ltd.

This Product is covered by US and foreign patent applications or patents and other proprietary intellectual property rights owned by CSHL ("CSHL shRNA IP Rights"), including U.S. Patent Nos. 8,153,776, 8,202,846, 8,383,599, 8,829,264, and EP1546174.

Subject to acceptance and all terms and conditions of this License, sale of the Product to Buyer by Sigma-Aldrich, Co. (acting under its license, an "Authorized Sale") conveys to Buyer only the nonexclusive, nontransferable right (with no right to sublicense) under the shRNA IP Rights to use the Product solely for Customer's internal research purposes, and only at its facility where the Product is delivered by Sigma-Aldrich, Co.

The Product is for research use only and may not be used *in vitro* or *in vivo* for any diagnostic, preventative, therapeutic or vaccine application, or used (directly or indirectly) in humans for any purpose.

**Non-Profit Buyers.** If Buyer is a Non-Profit Entity, then the following additional restrictions will apply:

Customer obtains no right to use, develop or otherwise exploit the product for any commercial purpose.

**Commercial Buyers.** If Buyer is a Commercial Entity, then the following additional restrictions will apply:

A Product sale is an Authorized Sale only if Buyer has already entered into a separate written agreement that has been executed by CSHL or Hairpin Technologies, that covers the CSHL shRNA IP Rights, and that is then currently in effect. Any delivery or transfer of Product to Customer outside of an Authorized Sale is void, conveys no implied or express right under this license and Customer will immediately return Product to Sigma-Aldrich for a refund.

"Commercial Entity" means any entity or organization other than a Non-Profit Entity.

"CSHL" means Cold Spring Harbor Laboratory.

"Hairpin Technologies" means Hairpin Technologies, Inc. located at 2200 Smithtown Avenue, Ronkonkoma, NY 11779, [www.hairpintechnologies.com](http://www.hairpintechnologies.com).

"Non-Profit Entity" means any college, university or governmental entity (including without limitation, governmental and quasi-governmental institutes and research laboratories), or any non-profit scientific, research or educational organization of the type described in section 501(c)(3) of the Internal Revenue Code or qualified under a state non-profit organization statute.

"Product" means a product (including, without limitation, expression vectors encoding a shRNA, the design, manufacture or use of which (in whole or in part) is the subject of the shRNA IP Rights, and is deemed to include all components, progeny, reproductions, modified versions and other derivatives thereof.

This license is subject to a license from CSHL or Hairpin Technologies, and CSHL and Hairpin Technologies reserves all other rights under its license. For information on licensing rights for Commercial Entities, including use of this product for purposes other than research and trial licenses, please contact Hairpin Technologies, Inc. at [info@hairpintechnologies.com](mailto:info@hairpintechnologies.com) or call (631) 881-0844.

ExpressMag and MISSION are registered trademarks and DirectLoad, GenElute, JumpStart, and ReadyMix are trademarks of Sigma-Aldrich Co. LLC. TurbGFP is a trademark of Evrogen Co.

RR,JKR,MAM 08/17-1