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Product Information

MISSION® LentiPlex™ Human and Mouse Pooled shRNA Libraries

Catalog Number	Product Description	Catalog Number	Product Description
SHPH01	Human TRC 1.0 Pooled shRNA Library	SHPM01	Mouse TRC 1.0 Pooled shRNA Library
SHPH15	Human TRC 1.5 Pooled shRNA Library	SHPM15	Mouse TRC 1.5 Pooled shRNA Library
SHPH2	Human Unique & Validated TRC 2.0 Pooled shRNA Library	SHPM2	Mouse Unique & Validated TRC 2.0 Pooled shRNA Library
SHPHLIBR	Human TRC 1.0, 1.5, & Unique & Validated 2.0 Pooled shRNA Library	SHPMLIBR	Mouse TRC 1.0, 1.5, & Unique & Validated 2.0 Pooled shRNA Library

Storage Temperature –70 °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.

The MISSION TRC shRNA libraries consist of plasmid-based shRNA constructs targeting a wide variety of human and mouse genes. The shRNA sequences are designed using a proprietary algorithm developed by the Broad Institute of MIT and Harvard. On average, there are five shRNA designs for each gene target. The shRNA plasmids are further processed into lentiviral particles to facilitate stable gene silencing in both dividing and quiescent cells.

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 2. As with all screens, validation of leads by independent methods such as siRNA, small molecule inhibition, or gene knockout will be required.

The MISSION® LentiPlex™ Human Pooled shRNA Libraries (Catalog Numbers SHPH01, SHPH15, SHPH2, and SHPHLIBR) and the MISSION LentiPlex Mouse Pooled shRNA Libraries (Catalog Numbers SHPM01, SHPM15, SHPM2, and SHPMLIBR) are genome-wide lentiviral pools produced using a proprietary process. Representation of individual shRNAs from each library is tested before product release to ensure robust library coverage. Each library is provided in ready-to-use lentiviral format at titers of at least 5×10^8 TU/ml via p24 assay and is pre-divided into subpools. See Table 1 for approximate number of shRNA constructs in each subpool. Amplification and sequencing primers are also provided for downstream hit identification.

Table 1.
Clone Coverage by Library

Product	Approximate Total Clone Coverage	Number of Subpools	Approx # Clones/ Subpool
SHPH01	80,000 +	10	8,000
SHPH15	25,000 +	3	8,000
SHPH2	20,000 +	3	6,000
SHPHLIBR	125,000 +	16	6,000 or 8,000
SHPM01	75,000 +	10	8,000
SHPM15	10,000 +	2	5,500
SHPM2	25,000 +	3	8,000
SHPMLIBR	110,000 +	15	5,500 or 8,000

Components
Each LentiPlex kit includes two aliquots per subpool - enough virus to perform approximately six total screens at 100× representation. Each aliquot is provided at 5×10^8 TU/ml or higher in a volume of 25 µl. Please refer to the Certificate of Analysis for exact titers of each subpool. Primers for amplification and sequencing of MISSION shRNA are also provided. See Tables 2 and 3 for component details.

Additionally, each LentiPlex kit will include a USB Flash Drive which contains the following: LentiPlex Search Database Software for shRNA hit identification and MISSION® LentiPlex pool files which contain the complete list of shRNA clone information.

Table 2.
Component List – Lentiviral Pools

Catalog Number	Component Description	Quantity	Minimum Volume	Minimum Titer
SHPH01	Hu TRC 1.0 Subpools 1–10	2 each	25 µl	5×10^8 TU/ml
SHPH15	Hu TRC 1.5 Subpools 1–3	2 each	25 µl	5×10^8 TU/ml
SHPH2	Hu TRC 2.0 Subpools 1–3	2 each	25 µl	5×10^8 TU/ml
SHPHLIBR	Hu TRC 1.0 Subpools 1–10 Hu TRC 1.5 Subpools 1–3 Hu TRC 2.0 Subpools 1–3	2 each	25 µl	5×10^8 TU/ml
SHPM01	Ms TRC 1.0 Subpools 1–10	2 each	25 µl	5×10^8 TU/ml
SHPM15	Ms TRC 1.5 Subpools 1–2	2 each	25 µl	5×10^8 TU/ml
SHPM2	Ms TRC 2.0 Subpools 1–3	2 each	25 µl	5×10^8 TU/ml
SHPMLIBR	Ms TRC 1.0 Subpools 1–10 Ms TRC 1.5 Subpools 1–2 Ms TRC 2.0 Subpools 1–3	2 each	25 µl	5×10^8 TU/ml

Quality Control
Each MISSION LentiPlex pooled shRNA library has been tested for representation through an internal QC process. Each viral subpool has also been tested via p24 assay and has demonstrated a titer of at least 5×10^8 TU/ml.

Table 3.
Component List – Primers and Positive Control

Catalog Number	Component Description	Quantity	Minimum Volume	Approximate Concentration
SHPH01 SHPH15 SHPH2 SHPHLIBR SHPM01 SHPM15 SHPM2 SHPMLIBR	LentiPlex Amplification Primer 1	1	750 µl	20 µM
	LentiPlex Amplification Primer 2	1	750 µl	20 µM
	LentiPlex Sequencing Primer	1	750 µl	5 µM
	shRNA Human Positive Control Vector #2 Purified DNA (Catalog Number SHC009)	1	20 µl	500 ng/µl

Materials And Reagents Required But Not Provided

For Transduction Of MISSION LentiPlex Pooled shRNA Library 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

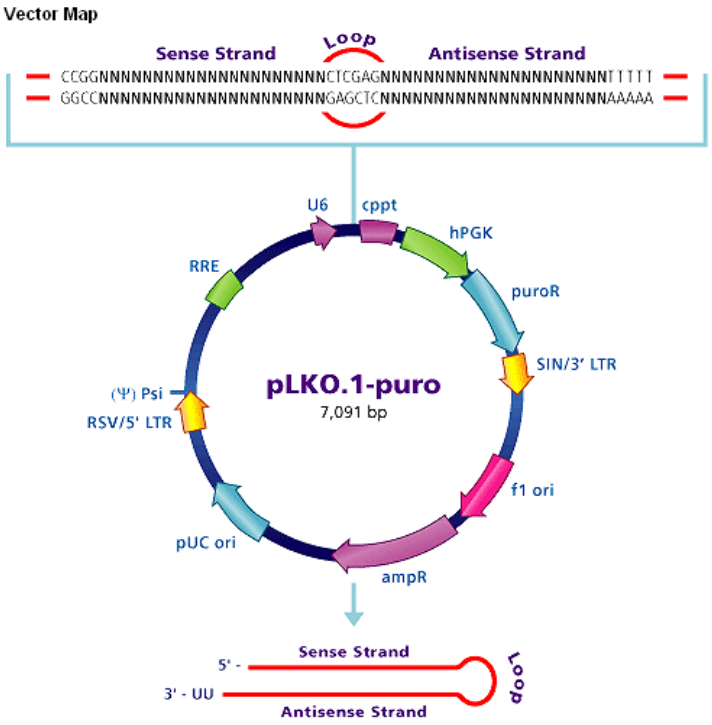
- GenElute PCR Clean-Up Kit (Catalog Number NA1020)

Additional Suggested Materials

For Screen Controls And Transduction Efficiency Optimization

- MISSION 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 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 we recommend the use of SHC016H.
- 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. We recommend a literature search on the efficacy of CMV-driven expression in your cell line.

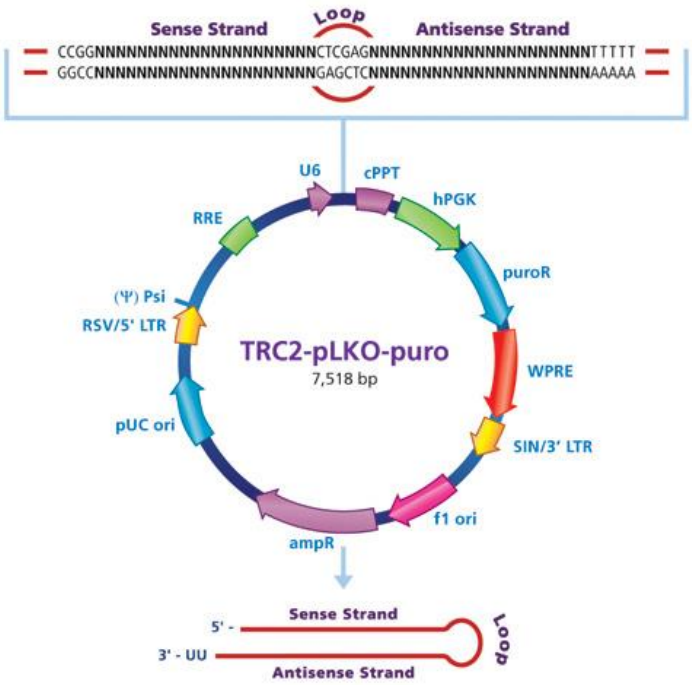
Figure 1.
TRC1.0 & 1.5 pLKO.1 Vector Map and Features



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

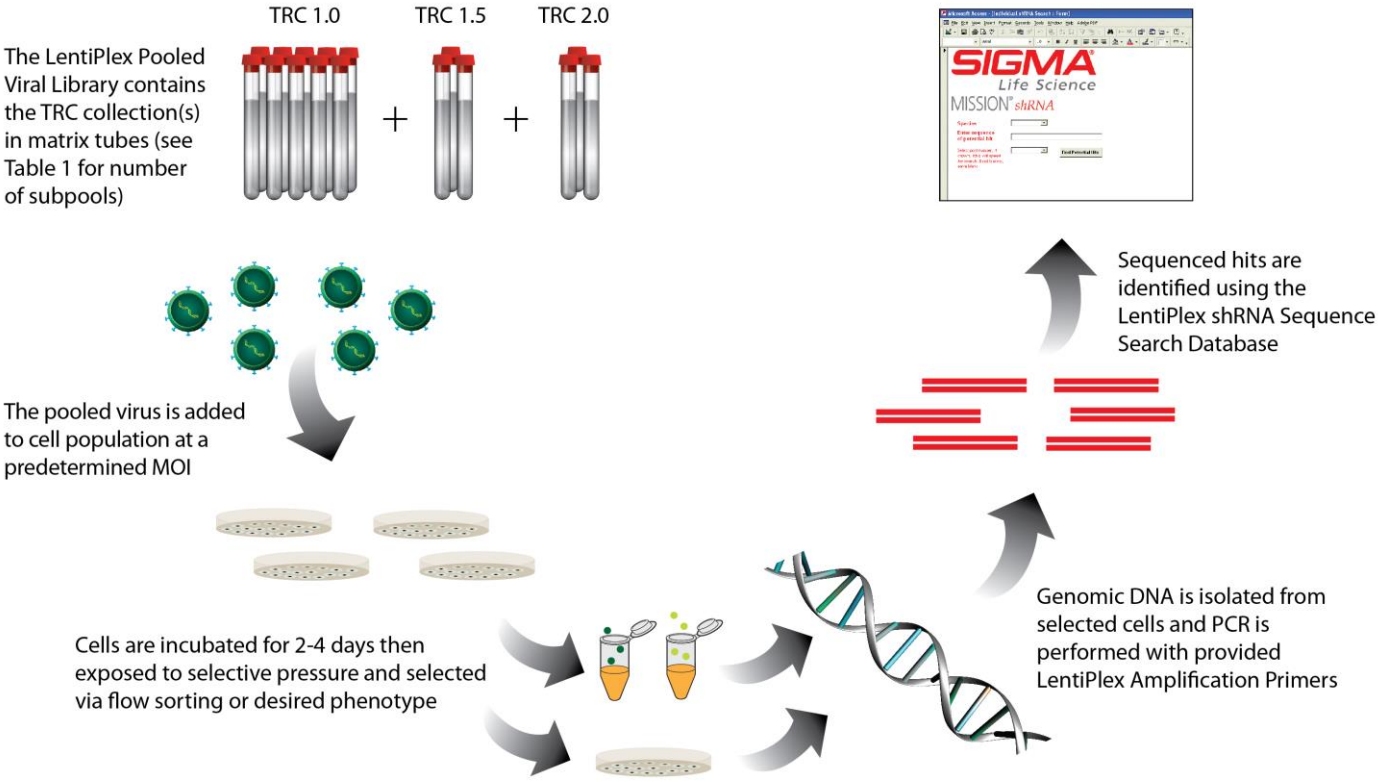
Figure 2.
TRC 2.0 pLKO.5 Vector Map and Features



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 °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 LentiPlex pooled 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 untransduced cells. The lowest concentration of puromycin that kills all untransduced 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 LentiPlex Pooled shRNA Library:
After the optimal MOI is determined, cells are transduced with the viral pools. 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 untransduced 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 untransduced 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 primers provided. The included MISSION shRNA Human Positive Control Vector should be used as template in a separate reaction in order to ensure that 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 sequencing primer is provided in the kit.

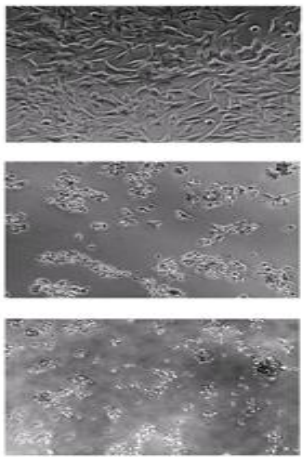
Detailed Protocols

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 μg/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_2.
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 μg/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 μg/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-rnai/shrna/learning-center/lentivirus-cell-line.html

Figure 4.
LNCaP cells treated with 0, 1, and 5 μg/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_2.

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 for your cell type(s) containing hexadimethrine bromide with a final concentration of 8 μg/ml.
3. Take 15 ml conical vials and prepare 7 10-fold dilutions with a final volume in each tube of 2 ml (10-fold serial dilutions over a range of 1×10^{-2} to 1×10^{-8}) 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^{-8} 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_2.

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 your 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 and incubate 10 minutes at room temperature (Catalog Number HT90132).
4. Remove crystal violet solution.
5. Wash with 3 ml of PBS and swirl gently.

6. Repeat steps 4-5 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×.
   Note: You should expect to see a 10-fold change in number of colonies between 2 adjacent dilution factors.
8. Multiply the number of colonies by the dilution factor used for the well to determine functional titer.
9. These functional titer results should be used as the starting point for determining the relationship between p24 titer and functional virus titer in your cell line.

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 °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 °C in a humidified incubator in an atmosphere of 5% 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 μg/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 °C in a humidified incubator in an atmosphere of 5% CO_2.

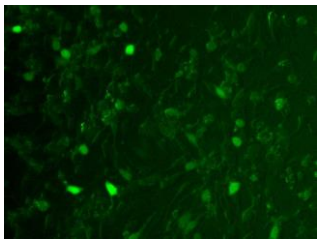
Day 3 – Change the medium.

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.
LNCaP 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 LentiPlex Pooled shRNA Library

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. In addition, typically it will not be advantageous to combine the different subpools. Keeping the subpools separate will aid in the deconvolution process. Ensure proper labeling of each tissue culture dish with the subpool number that is applied during transduction.

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_2.
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 LentiPlex viral particles.

1. Using the p24 titers provided on the Certificate of Analysis, calculate how much of each individual viral subpool to add to the cells to get the MOI of 1 across all cell culture dishes.
   Example: 1×10^6 cells/dish;
   Viral titer = 5×10^8 TU/ml; a desired MOI of 1

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

   1×10^6 TU/(5.0×10^8 TU/ml) from Certificate of Analysis = 2 μl of lentiviral stock solution should be added to the appropriate dish. Dilute the calculated amount of the virus in 100–300 μl 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 μg/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_2.
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 μl 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. 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 2×10^6 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. Amplification primers (20 μM) are included with the LentiPlex kit.

6. Set up the PCR reaction, see Table 4. Add the components in the order listed. The described amounts are for one 50 µl 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 shRNA Human Positive Control Vector 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 (µl)	Final Concentration
Water (PCR grade)	–	
JumpStart *Taq* ReadyMix (2×)	25	1× includes 1.5 mM $MgCl_2$
Magnesium chloride solution	3	1.5 mM 3 mM total*
Forward Primer (20 µM)	1	0.4 µM
Reverse Primer (20 µM)	1	0.4 µM
DNA Template (50 ng-100 ng)	–	
Total volume	50 µl	

*Final concentration of Mg^{2+} in solution will be 3 mM; 1.5 mM is contributed from the *Taq* ReadyMix and 1.5 mM from the Magnesium chloride solution.

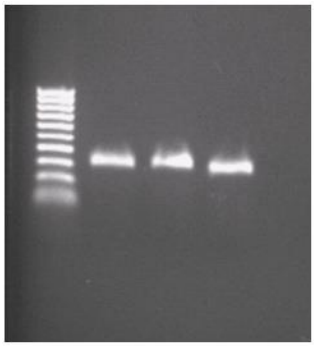
7. Save the following program (Table 5) into the thermocycler:

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	309 bp	

8. For each sample, combine 3 µl of PCR reaction with 1 µl of dye and run along with 5 µl of DirectLoad PCR 100 bp Low Ladder (Catalog Number D3687) on a 1% agarose gel to confirm a 309 bp amplicon.

Figure 6.
PCR amplification of A549 colonies transduced with a single Mission shRNA clone



Lane 1 – PCR Ladder (1,000, 900, 800, 700, 600, 500, 400, 300, 200, 100)
Lane 2 – 3 µl of PCR amplicon from clonal shRNA construct A
Lane 3 – 3 µl of PCR amplicon from clonal shRNA construct B
Lane 4 – 3 µl PCR amplicon from clonal pLKO.1 empty vector control

9. 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.
10. 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. Sequencing primers are provided along with the MISSION LentiPlex kit. Use the provided sequencing primer to sequence individual shRNA clones.

Note: Amplified fragments can also be cloned into the plasmid vector of choice for subsequent sequencing steps.

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. Optionally, select the subpool from which the sequence was found. 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.

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.sigmabioinfo.com/Informatics_tools/batch-search.php#shRNA.

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® LentiPlex pool files included on the USB drive which contain shRNA clone information found in each pool, or contact us at RNAi@sial.com.

Troubleshooting Guide

For answers to questions that are not addressed here, please email technical support at 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® Transduction System (Catalog Number SHM01).
	Loss of viral titer during storage	Ensure storage of the Mission LentiPlex Pooled shRNA Library at –70 °C. Each freeze-thaw cycle may cause reduction of the titer by as much as 20–30%.
	Titer of each subpool was not uniformly standardized prior to transduction	Make sure to calculate how much of each individual viral subpool to add to cells to get the same MOI across the experiment. Be aware that each subpool comes with an individual viral titer.
Transduction affects target cell viability	Transduction with Mission shRNA library 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.
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.

Troubleshooting Guide (continued)

Problem	Cause	Solution
No PCR product	There is a problem with the reaction components, or the thermal cycling conditions	To insure 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 the identical size of PCR product (309 bp) as other MISSION shRNA amplified inserts. 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.
	Cross contamination of genomic DNA with plasmid DNA positive control.	When identifying deconvoluted shRNA inserts, make sure the presence of a shRNA clone for ARHGDIA in the final results is not due to cross contamination of genomic DNA with plasmid DNA positive control. The specific clone number for the ARHGDIA control is TRNC0000008004 and was chosen from the available complete target set (NM_004309). The corresponding sequence of the shRNA clone: CCGG**CAAGATTGACAAGACTGACTA**CTCGAG**TAGTCAGTCTTGTCAATCTTG**TTTTT
	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.

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