shERWOOD-Ultramir Lentiviral shRNA Pooled Libraries

Format: Plasmid DNA

I. Introduction

shERWOOD-Ultramir Lentiviral shRNA Pooled Libraries from transOMIC technologies are pooled lentiviral constructs expressing shRNA targeting human or mouse genes to enable highly multiplexed, RNAi mediated gene knockdown. Screening libraries are available as pooled plasmid DNA or pooled high titer lentiviral particles and target gene families and pathways, whole genome or any custom gene list.

shERWOOD-UltramiR shRNA screening libraries can be used to screen hundreds to thousands of genes in a high-throughput, multiplexed assay using a standard tissue culture hood without the aid of costly automation and liquid handling required for traditional arrayed screens. Lentiviral vectors allow the user to select from the broadest range of cell lines for functional screens including primary, stem and non-dividing cells.

All shERWOOD-UltramiR shRNA (Figure 1) constructs in the pooled screening libraries are vector-based RNAi triggers with a new generation shRNA-specific design and an optimized microRNA scaffold "UltramiR" which has been shown to produce more potent and consistent knockdown performance than existing shRNA reagents. The UltramiR scaffold has been optimized for efficient primary microRNA processing (Auyeung *et al*., 2013) and shRNA designs are predicted using the proprietary shERWOOD algorithm developed in Dr. Gregory Hannon's laboratory at Cold Spring Harbor Laboratory. Based on the functional testing of 270,000 shRNA sequences using a high-throughput sensor assay (Knott *et al*., 2014), the shERWOOD algorithm has been trained to select the rare shRNA-mir designs that are consistently potent even at single copy representation in the genome which make them ideal for pooled screens and greatly simplifies downstream analysis by decreasing both false positive and false negative results.

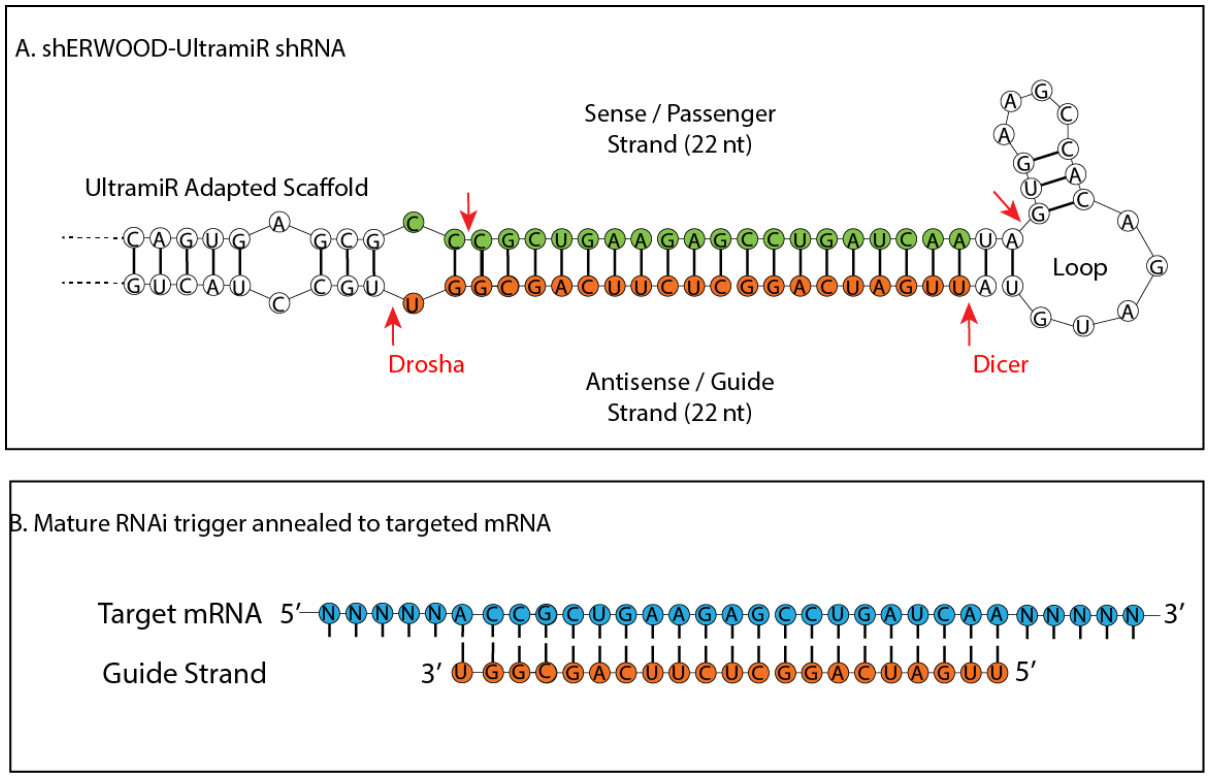


Figure 1. Schematic of shERWOOD-UltramiR shRNA. (A) Passenger (green) and Guide (orange) strand are shown with Dicer and Drosha nuclease cleavage sites are in red. (B) The final step of shRNA processing loads the Guide Strand (orange) into the RISC complex which binds to the target mRNA (blue) in a sequence specific manner.

Lentiviral pooled shRNA screening libraries are provided standard in the ZIP-mCMV vector. They are also available in ZIP-hCMV, ZIP-hEF1-alpha, ZIP-mEF1-alpha and ZIP-SFFV vectors. **No changes to the protocols are required for use with the alternate ZIP vectors. Detailed vector graphics are provided in Appendix D.**

Figure 2. Schematic of ZIP lentiviral shRNA vectors. All elements are identical with the exception of the promoter (LTRs are not shown). The ZIP vectors constitutively express the shRNA, fluorescent marker and puromycin selection marker from a single transcript driven by a viral promoter. This allows for direct visual confirmation of shRNA-mir expression. A promoter selection kit (TLN0005) is available for quickly determining the optimal promoter for a given cell line.

II. Safety

See Appendix A for safety considerations.

Pooled shRNA Screening Strategies

Pooled shRNA libraries are typically used to perform enrichment (positive) or dropout (negative) selection screens. Both strategies begin by transduction of cells with viral particles expressing a complex pool of shRNA such that each transduced cell expresses a single shRNA. Next, a selective pressure is applied to the heterogeneous population of cells to elicit a desired phenotypic change (e.g. survival screen as shown in 3). The shRNA is integrated into the cellular genome and its representation in the culture increases or decreases as the cells are selected for or against, respectively. Changes in the shRNA representation can be determined through sequence analysis of the shRNA sequence.

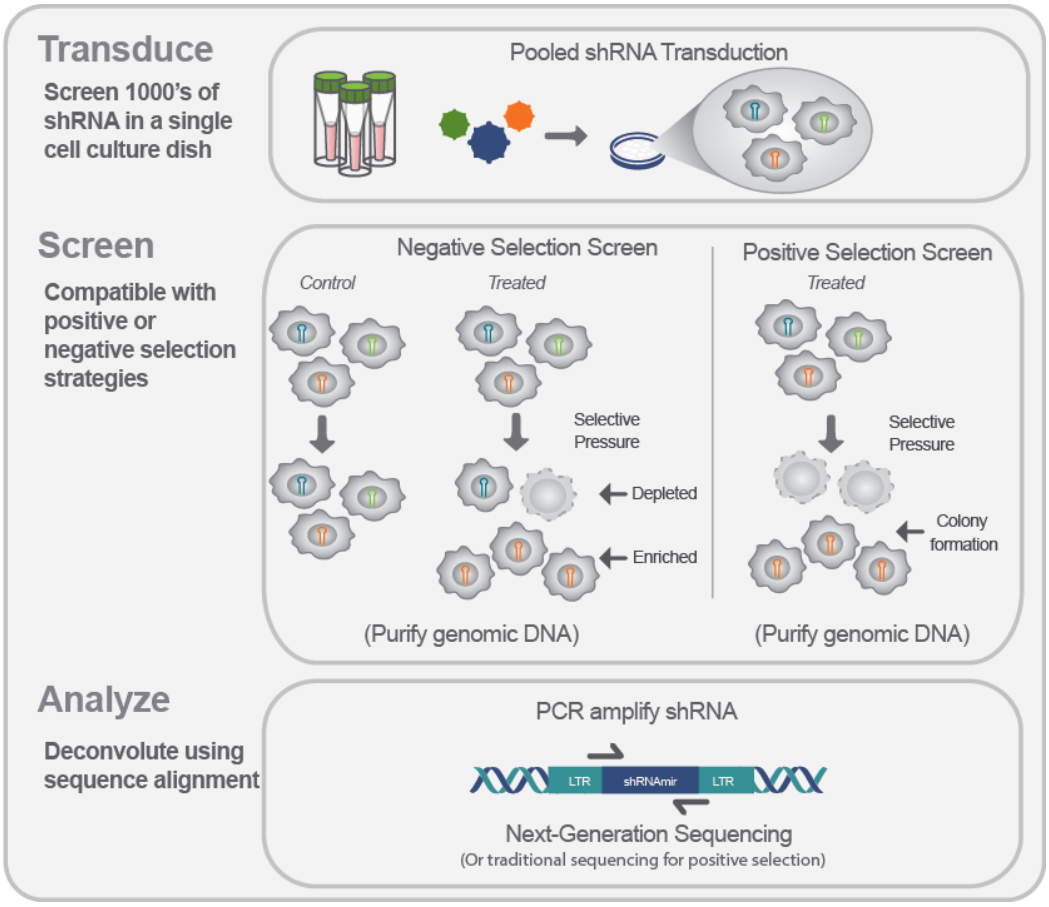


Figure 3. Schematic showing the three basic phases of the screening process: transduction, screening and analysis.

III. Product specifications

Shipping and storage

Components are shipped on wet ice and should be stored at -20°C upon receipt.

Plasmid DNA library components

Table1. Components provided

Component	Quantity	Amount
Pooled Screening library - plasmid DNA	1	50 µg
Non-targeting Control plasmid DNA	1	25 µg
Primary PCR Forward Primer	2	400 µl at 10 µM
Primary PCR Reverse Primer	2	400 µl at 10 µM

Equipment and reagents to be supplied by user

For packaging: *Note: Pools can also be packaged with other systems.*

- FuGENE6 (Promega, Cat. # E2691)
- Complete culture medium (DMEM + 10% FBS, 1X Pen/Strep, and 1X L-glutamine)
- OPTI-MEM I + GlutaMAX-I (Gibco, Cat. # 51985-034)
- Transfer vector dilute plasmid(s) to 0.2 µg/µl
- Lentiviral packaging mix (0.5 µg/ µl):
  - 100 µl pCMV-dR8.2 (0.5 µg/µl) - (Addgene)
  - 50 µl pCMV-VSV-G (0.5 µg/µl) - (Addgene)
- 10 cm tissue culture plates
- Filter, 0.45 µm made of cellulose acetate, or polysulfonate (low protein binding)
- Automatic pipetter/Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

For titering:

- 1.5 ml microcentrifuge tubes
- HEK293T cells (NIH-3T3 may be used as an alternative)
- Complete media for HEK293T cells and experimental cell line
- Serum free media for each cell line
- 24-well tissue culture plate

For Puromycin kill curve:

- Complete media experimental cell line
- Puromycin (1.25 µg/µl stock solution)
- 24-well tissue culture plate

For Transduction:

- Polybrene
- Puromycin

For PCR:

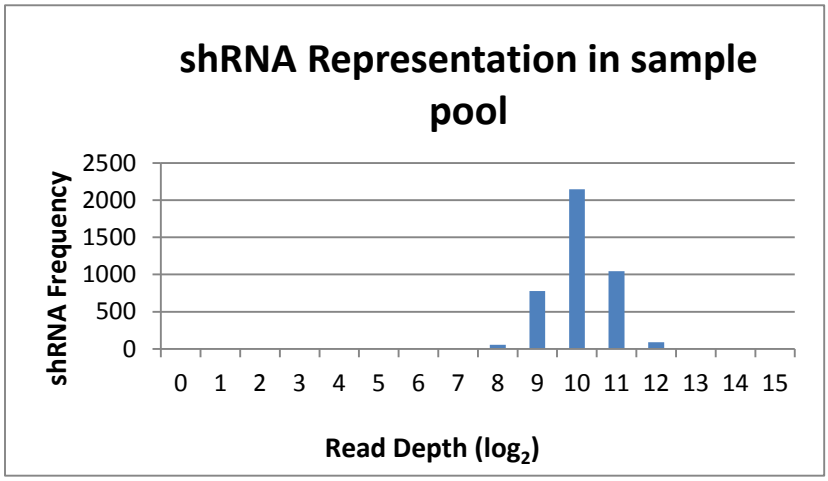
- KOD Hot Start Polymerase (Novagen Cat# 71086-4)
- 96 well PCR plates
- Betaine
- Agarose gel
- PCR purification column (*QIAquick PCR Purification Kit, Qiagen Cat# 28104*)
- DNA gel extraction kit (*QIAquick Gel Extraction, Qiagen Cat# 28104)*
- Multiplex indexed primer kit (12-plex, TRP0001)

Equipment:

- Automatic pipetter/Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Fluorescent microscope with GFP filter (refer to Appendix BB for alternative methods that do not require this)
- Qubit® fluorometric
- Illumina® sequencing platform

shRNA representation in pooled libraries – Quality Control

shRNA pools have been developed using strategies that maintain equimolarity of the pooled shRNA constructs – to ensure that screening results are not artificially biased to any one or some gene/s or construct/s prior to the screen. All shRNA plasmid DNA pools are evaluated by next-generation sequencing (NGS) for distribution and representation analysis. Quality metrics are outlined in the Certificate of Analysis (C of A) provided with each pool. A representative graph of shRNA distribution is shown in Figure 4. For a more detailed vector map and link to vector sequence see Appendix D.



Pool Screening Metrics	
Total shRNA in Pool	4185
shRNA Detected (by NGS)	4161
% Recovery	99.4%
Mean Read Depth (MRD)	1246
# ± 5X MRD	4107
% ± 5X MRD	98.7%

Figure 4. Example histogram showing distribution of shRNA from a plasmid DNA pool as seen by NGS analysis (left). Summary statistics of QC analysis (right). The summary includes the total shRNA constructs pooled to create the library and the number detected by NGS.

IV. Pooled shRNA Screen – Preparation and optimization

The following User's Guide provides detailed protocols for each step performed during pooled shRNA screening. Please review Appendix A for all safety considerations prior to beginning these protocols.

1. Lentivirus production

The following protocol is recommended for packaging the plasmid DNA pool into lentiviral particles.

Considerations before packaging the pZIP (mCMV) shERWOOD-UltramiR shRNA lentiviral vector:

Non-Targeting Control DNA (provided) should be used to determine the packaging and transduction efficiency of the target cell used in the screen prior to packaging the pool DNA. This ensures optimal conditions and sufficient viral particle production for the screen. If required, additional control virus (catalog # TLNVU4420) can be purchased from transOMIC technologies.

Required Materials

Note: All plasmids are purified using endotoxin-free purification. All plasmids and siRNA are resuspended in dH_2O

- FuGENE6 (Promega, Cat. # E2691)
- Complete culture medium (DMEM + 10% FBS, 1X Pen/Strep, and 1X L-glutamine)
- OPTI-MEM I + GlutaMAX-I (Gibco, Cat. # 51985-034)
- Transfer vector dilute plasmid(s) to 0.2 μg/μl
- Lentiviral packaging mix
  - pCMV-dR8.2 - (Addgene)
  - pCMV-VSV-G - (Addgene)
- 10 cm tissue culture plates
- Filter, 0.45 μm made of cellulose acetate, or polysulfonate (low protein binding).
  - *Note: The filter used should not be nitrocellulose. Nitrocellulose binds proteins present in the membrane of lentivirus and destroys the viral particles.*

Equipment

- Automatic pipetter/Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

Protocol

1. On the day prior to transfection, seed 5-6 x 10^6 cells into a 100 mm plate in a total volume of 12 ml complete culture medium (DMEM + 10% FBS, 1X Pen/Strep, and 1X L-glutamine). It is recommended that the cells be 70-80% confluent at the time of transfection.
2. Two hours prior to transfection remove the culture media and replace with 12 ml of fresh culture media again containing no antibiotics.
3. Allow FuGENE6 (Promega, Cat. # E2691) and OPTI-MEM I + GlutaMAX-I (Gibco, Cat. # 51985-034) to come to room temperature.
4. Preparation of transfer vector, lentiviral vector packaging mix, and siRNA targeting Pasha/DGCR8 (optional) for transfection:
   Note: All plasmids and siRNA are resuspended in dH2O.
   a. Transfer vector – dilute plasmid(s) to 0.2 µg/µl
   b. Lentiviral packaging mix (0.5 µg/ µl):
      i. 100 µl pCMV-dR8.2 (0.5 µg/µl)
      ii. 50 µl pCMV-VSV-G (0.5 µg/µl)
   c. DGCR8 siRNA (0.3 µg/µl)
5. Mix FuGENE6 by inverting or vortexing briefly. In one well of a polystyrene round-bottom plate add the following:
   a. 807 µl of OPTI-MEM (795.6 µl if co-transfecting DGCR8 siRNA)
   b. 45 µl of FuGENE6 (50.4 µl if co-transfecting DGCR8 siRNA)
6. Incubate FuGENE6/OPTI-MEM mixture for 5 minutes at room temperature.
7. Add 30 µl transfer vector, 18 µl Lenti packaging mix, and 6 µl DGCR8 siRNA (optional) to the FuGENE6/ OPTI-MEM mixture. Mix immediately and incubate an additional 15 min at room temperature.
8. Add entire transfection mix dropwise to cells/culture. Swirl gently.
9. Return to incubator with 5% CO_2 at 37°C.
10. Collect viral particles (supernatant) 48 hours post-transfection.
11. Centrifuge the media briefly (800 x g for 10 min) or filter through a 0.45 µm filter to remove cellular debris.
    Note: The filter used should be made of cellulose acetate, or polysulfonate (low protein binding), instead of nitrocellulose. Nitrocellulose binds proteins present in the membrane of viral particles and destroys them.
12. Aliquot supernatant into sterile cryovials and store at -80°C.
    a. Make 2 x 50 µl aliquots for titering and divide the rest into screening aliquots.

Note: The 50 µl aliquots will be used in the functional titering protocol. They should be stored at -80°C overnight prior to titering to reflect any loss of function due to freeze/thaw cycle that will occur for the screening aliquots. Freshly harvested viral particles from well-transfected cells should have a titer of approximately 1-5 x 10^6 TU/ml when measured on HEK293T cells.

2. Functional titer and transduction optimization

The transduction step determines the fold representation of shRNA in the pool and the number of viral particles transducing a cell. Both of these parameters are integral to a successful screening process. The following protocol is designed to evaluate functional titer of the virus produced in the previous section for optimal transductions during the screen. Following transduction, puromycin will be used to remove non-transduced cells. The cell line's natural resistance to puromycin will be evaluated using an antibiotic titrating assay to determine the optimal concentration for selection during the screen.

Increase screening quality and capacity by increasing transduction efficiency:
Optimized transduction allows for maximal shRNA fold representation and biological replicates and thus more effective screen performance. A number of variables influence transduction efficiency including components of the media, duration of transduction, cell type, cell health and plating density. It is possible to optimize many of these variables prior to the screen.

- Serum is a known inhibitor of transduction and should be minimized (0 - 2%) in transduction media. For cells sensitive to low serum conditions either reduce the transduction time in low serum media or increase the transduction time in complete media.
- Hexadimethrine bromide (Polybrene) is a cationic lipid known to enhance viral particle binding to the surface of many cells types. A range of concentration (0 - 10 µg/ml) should be tested to determine the highest transduction efficiency that can be achieved without cell toxicity.
- Extending transduction incubation times may increase efficiency. However, it may be necessary to increase the volume of media applied to the cells for transduction to limit the effects of evaporation.
- Cell density may influence transduction efficiency. Plate cells at a range of densities to determine its effect on your cell line. Rapidly dividing cells are often transduced more efficiently.

Determining Functional Titer

Functional titer must be determined using the experimental cell line to ensure optimal transduction during the screen. The functional titer is the number of viral particles, or transducing units (TU), able to transduce the target cell line per volume and is measured in TU/ml. Cell type, media components and viral production efficiency influence functional titer, and it should therefore be calculated for every batch of virus produced or when using a different cell line.

Once a baseline titer is known, this protocol can be used to further optimize transduction efficiency. To do so, follow this procedure and alter variables know to influence transduction efficiency.

- The following protocol evaluates titer by manually counting ZsGreen positive colonies. Alternate methods for determining titer are provided in Appendix BB.
- Transduction optimization should be done with the with Non-Targeting Control viral particles prior to titering the shRNA pool to ensure optimal conditions and sufficient titers for the screen. If extensive optimization is required additional non-targeting control may be purchased as viral particles (TLNVU4420).
- Use the titering aliquots made in the virus production section above to determine the titer.

Required materials

- HEK293T cells (NIH-3T3 may be used as an alternative)
- Complete media for HEK293T cells and experimental cell line
- Serum free media for each cell line
- 24-well tissue culture plate
- Lentiviral particles (Harvested or purchased)
- Microcentrifuge tubes

Equipment

- Automatic pipetter /Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Fluorescent microscope with GFP filter (refer to Appendix BB for alternative methods that do not require this)

Protocol

1. Plate cells (HEK293T and experimental cell line) 24 hours prior to transduction in a 24 well plate. Plate at a density of 5-8 x 10^4 cells per well in 12 wells with complete media. Well should be seeded at the appropriate density in order for the confluency of the cells to be approximately 40% the following day. Incubate for overnight with 5% CO_2 at 37°C.
2. The following day, thaw the tube of viral supernatant in 37°C water bath and immediately place on ice. Prepare a serial dilution series with serum free media and viral supernatant as shown in Table 2 and Figure .
    a. Set up 5 sterile microcentrifuge tubes.
    b. Add 160 µl of serum free medium to each tube.
    c. Add 40 µl of viral stock to the first microfuge tube.
    d. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
    e. Transfer 40 µl from the first microcentrifuge tube to the second tube. Mix well and discard the tip.
    f. Repeat the procedure for the 4 remaining tubes.
3. Remove media from each well.
4. Add 225 µl of serum free media to each well containing cells.
5. Add 25 µl from each viral dilution to two wells (250 µl final volume) for a total of 10 wells per cell line. The remaining two wells (without viral particles) should be evaluated as negative controls.
6. Rock plate gently a few times to mix.
7. Incubate overnight with 5% CO_2 at 37°C.
8. Replace the viral supernatant with complete media and allow cells to grow for 48 hours.
9. Count the number of colonies expressing ZsGreen. A colony consisting of multiple cells should be counted as a single transduction event.
   Note: Counting 50-200 colonies is sufficient to provide accurate titers. Only analyze wells that have < 20% of cells transduced to ensure none of the cells have been transduced with more than one viral particle.
10. Use the calculation below and Table 2 to determine functional titer.

$$(Number\ of\ colonies\ ) \times (dilution\ factor) \div 0.025\ ml = \frac{TU}{ml}\ functional\ titer$$

For Example:
If the average number of colonies counted in well A4 and B4 is 70 the titer is calculated as follows:
70 colonies X 625 ÷ 0.025 ml = 1.75 X 10^6 TU/ml

Dilution table and schematic for titration protocol

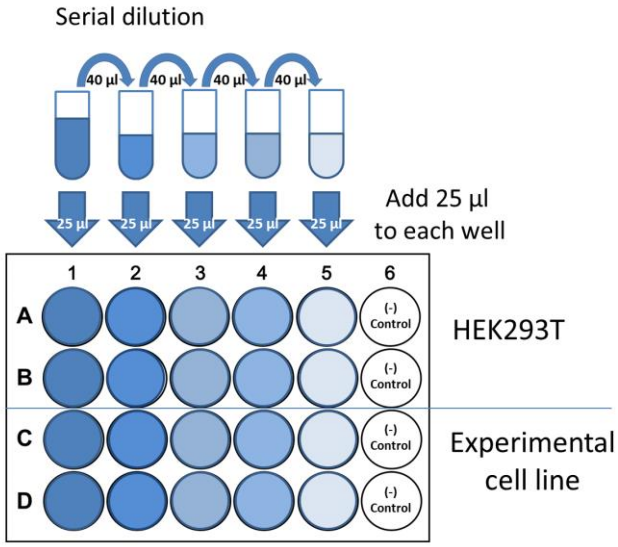


Figure 5. Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

Table 2. Dilution factors for calculating viral titer

Tube	Viral particles	Dilution medium	Dilution factor
1	40 µl (from titer aliquot)	160 µl	5
2	40 µl (from Tube 1)	160 µl	25
3	40 µl (from Tube 2)	160 µl	125
4	40 µl (from Tube 3)	160 µl	625
5	40 µl (from Tube 4)	160 µl	3125
6	0 µl		n/a

Example:
Typical virus production will yield 1-5 x 10^6. The expected number of fluorescent colonies for a viral titer of 1 x 10^6 would yield the following number of fluorescent colonies in titering assay:

Tube	1	2	3	4	5	6
Dilution	1/5	1/5	1/5	1/5	1/5	n/a
Diluted titer TU/ml	200,000	40,000	8,000	1,600	320	0
ml transduced cells	0.025	0.025	0.025	0.025	0.025	0
Fluorescent colonies expected	5,000	1,000	200	40	8	0

Counting 50-200 colonies is sufficient for an accurate measure of titer.

3. Puromycin selection (puromycin kill curve)

During the screen cells are transduced at an MOI of 0.3 leaving 70% of the cells untransduced. Puromycin selection should be applied to remove the untransduced cells. In the following protocol the lowest concentration of puromycin that provides adequate selection is determined for the experimental cell line.

Required materials

- Complete media experimental cell line
- Puromycin (1.25 µg/µl stock solution)
- 24-well tissue culture plate

Equipment

- Automatic pipetter /Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

Protocol

1. Plate 5 x 10^4 cells per well in 11 wells of a 24-well tissue culture plate using media without puromycin.
2. Prepare puromycin dilutions in culture media for antibiotic titration as shown in Table 3. Use a puromycin stock solution of 1.25 µg/µl stock solution.

Table 3. Dilutions and volumes required for establishing optimal puromycin concentration

Volume of Puromycin Stock Solution Added (µl)	Total Volume of Media plus Antibiotic per 24 Well (µl)	Final Concentration (µg/ml)
0	500	0
0.2	500	0.5
0.4	500	1
0.6	500	1.5
0.8	500	2
1	500	2.5
1.2	500	3
1.6	500	4
2	500	5
3	500	7.5
4	500	10

3. Begin antibiotic selection the following day by replacing antibiotic free media with media containing the appropriate concentrations of puromycin.
4. Incubate cells with 5% CO_2 at 37°C, or use conditions normal for your target cells.
5. Check cells daily to estimate rate of cell death.
6. Replenish the media containing the appropriate concentrations of puromycin every 2 days for 6 days.
   Note: The optimal puromycin concentration will kill the cells rapidly (2 - 4 days). This is particularly important for screens involving essential genes that may be selected against prior to the experiment.

V. Primary Selection Screen: Recommendations & Considerations

Once functional titer, transduction efficiency and puromycin concentration have been evaluated in the experimental cell line, the primary selection screen can be performed. The following protocol provides recommendations and guidelines to perform the selection screen.

Assay optimization

The objective of a pooled screen is to selectively enrich or deplete cells in a population based on phenotypic change. Biological assays used to evaluate phenotypic changes in the pooled screen may take different forms including survival, behavior (e.g. migration or adhesion), changes in reporter expression or changes in surface marker expression. Each will have variables inherent to the assay that should be optimized such as sensitivity of the assay, concentration of selective agent, duration of treatment and method of selection.

For Example:
Selection screens identify genes affecting cell viability. If a targeted gene decreases growth by 10%, these cells will show a 40% depletion in the population on day 5, but an 80% depletion on day 15. So, it is possible to detect more subtle changes simply by extending time points. This may be of particular relevance for RNAi screens where target gene expression is reduced but not eliminated leaving the possibility of a hypomorphic phenotype (Acta Biochim Biophys Sin (2012) 44 (2): 103-112).

It is also critical to understand shRNA fold representation within the assay. The average number of cells independently transduced with constructs expressing the same shRNA is described as shRNA fold representation. Transductions with high (e.g. 500X – 1000X) and uniform representation for every shRNA provide the optimal starting point for a screen. Higher representation maintains the uniformity across the pool and increases reproducibility between biological replicates. At least two biological replicates are recommended per screen.

Multiplicity of Infection (MOI)

In a pooled screen it is critical that transduction is optimized to ensure that each transduced cell has a single genomic integration from the shRNA-expressing viral particle. This enables one to clearly see phenotypes associated with the knockdown of the single gene. The number of integrations correlates with multiplicity of infection (MOI) calculated as the ratio of transducing units to cells. At an MOI of 0.3 or less >95% of cells are predicted to have a single integration and is therefore recommended for pooled screening. The average number of integrations is estimated by the Poisson distribution (Figure). The number of cells needed at transduction is determined using the number of cells with viral integrants and the desired MOI (see calculation below).

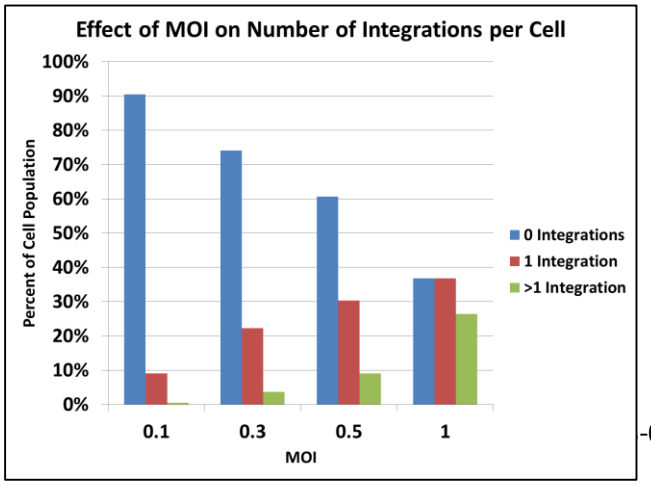


Figure 6. Higher MOIs result in a higher number of cells with multiple integrations. An MOI of 0.3 is recommended for pooled screening to limit the number of cells with multiple integrations (4%) and limit the number of cells required for transduction.

Calculations for MOI

Number of viral integrants needed:

Number of shRNA in the pool x Fold representation = Number of integrants needed

Number of cells needed at transduction:

Number of integrants needed ÷ MOI = Number of cells needed at transduction

For Example:
In a pooled screen, cells should be transduced at an MOI of 0.3 to maximize the number of cells with a single integration and limit the number of cells needed at transduction. Transducing a pool of 500 shRNA at 1000-fold representation will require $5x10^5$ transduction units (TU) and approximately 1.5×10^6 cells to achieve an MOI of 0.3.
Calculate as follows:

500 shRNA x 1000 fold representation = 5×10^5 TU
5×10^5 TU / 0.3 MOI = 1.5×10^6

Viral particles in transduction

Prior to your screen, confirm that there is sufficient volume of viral particles for the biological replicates and representation needed for your experimental design. The total number of TU needed for the experiment and titer will be required to calculate the volume needed for the experiment. Refer to the titer calculated in previous sections.

Number of transducing units:

Representation x shRNA per pool x Biological replicates = Total TU needed for experiment

Total volume of virus needed for experiment:

TU for experiment ÷ Functional Titer in Experimental Cell Line (TU/ml) = Volume (ml) of virus needed for the experiment

VI. Primary Selection Screen: Guidelines & Protocols

The primary selection screen protocol provides a basic outline of the transduction process. The following should be optimized before proceeding to the primary selection screen:

- Transduction media: % Serum, Polybrene µg/ml
- Time exposed to transduction media: hours or overnight
- Selection media: µg/ml puromycin
- Number of live cells that should be plated when splitting
- Number of cells that should be harvested for each sample

Required materials

- Complete media for experimental cell line
- Complete media for experimental cell line with puromycin
- Pooled screening library as viral particles
- Assay specific controls and reagents
- Qubit® dsDNA BR Assay or Quant-iT™ PicoGreen ® dsDNA Kits (or other fluorometric system specific for quantitation of dsDNA)

Equipment

- Automatic pipetter /Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Assay specific equipment
- Qubit® fluorometer or standard spectroflourometer with fluorescein excitation and emission wavelengths

Protocol:

Prepare cells

1. Plate cells such that they are actively dividing and 40 - 60% confluent at the time of transduction.
2. Feed cells with complete media 3 - 4 hours prior to transduction.

Transduce cells

3. Exchange media with transduction media.
   Note: media should be serum free for maximum transduction efficiency.
4. Incubate cells 12 - 24 hours in transduction media.
5. Replace transduction media with complete media (no puromycin).

Puromycin selection

6. Allow cells to grow for 48 hours.
7. Replace media with selection media.
8. Continue feeding cells selection media until untransduced cells have been removed.

Sampling and passaging of cells during screen

9. Split the culture into a control sample and an experimental sample. Each sample should be derived from enough cells to maintain representation of the pool (500-1000 times the number of shRNA in the pool).
10. Start assay selection and take samples as needed. Each sample should include enough cells to maintain representation of the pool (500-1000 times the number of shRNA in the pool).

Genomic DNA (gDNA) extraction

11. Extract gDNA from samples. Ensure that gDNA is extracted from enough cells to maintain representation of the pool (500-1000 times the number of shRNA in the pool).

    Note: Subsequent primary PCR should be performed on all extracted gDNA. To ensure that representation is not lost during the genomic DNA purification step the maximum column capacity should not be exceeded as this can decrease yields. In addition, a second elution from the column preparation may be necessary to maximize yields.

12. Quantitate gDNA isolated from each sample and ensure that 260/280 ratios are greater than or equal to 1.8.

For example:
A pool containing 1500 shRNA used for screening with coverage of 1000 viral integrants per shRNA would require a total of 1.5 x 10^6 transduced cells. To maintain the 1000-fold coverage of shRNA used during the screen, at least 1.5 x 10^6 cells are needed for gDNA isolation from each sample.

VII. shRNA Amplification and Next Generation Sequencing

The representation of each shRNA is detected by next generation sequencing (NGS). The shRNA sequence integrated into the targeted cells genome can be amplified using common sequences flanking the hairpin. Indexes are then added to each sample using the indexed primer kit (TRP0002) and the pooled PCR product from all samples may be analyzed in parallel using NGS analysis. Index primer kits are available from transOMIC technologies.

Individual shRNA are amplified from gDNA with two rounds of PCR. The primary PCR amplifies the shRNA and the flanking region. The secondary PCR uses nested primers to enrich for the primary PCR amplicons using modified primers adapted for NGS on an Illumina® sequencer.

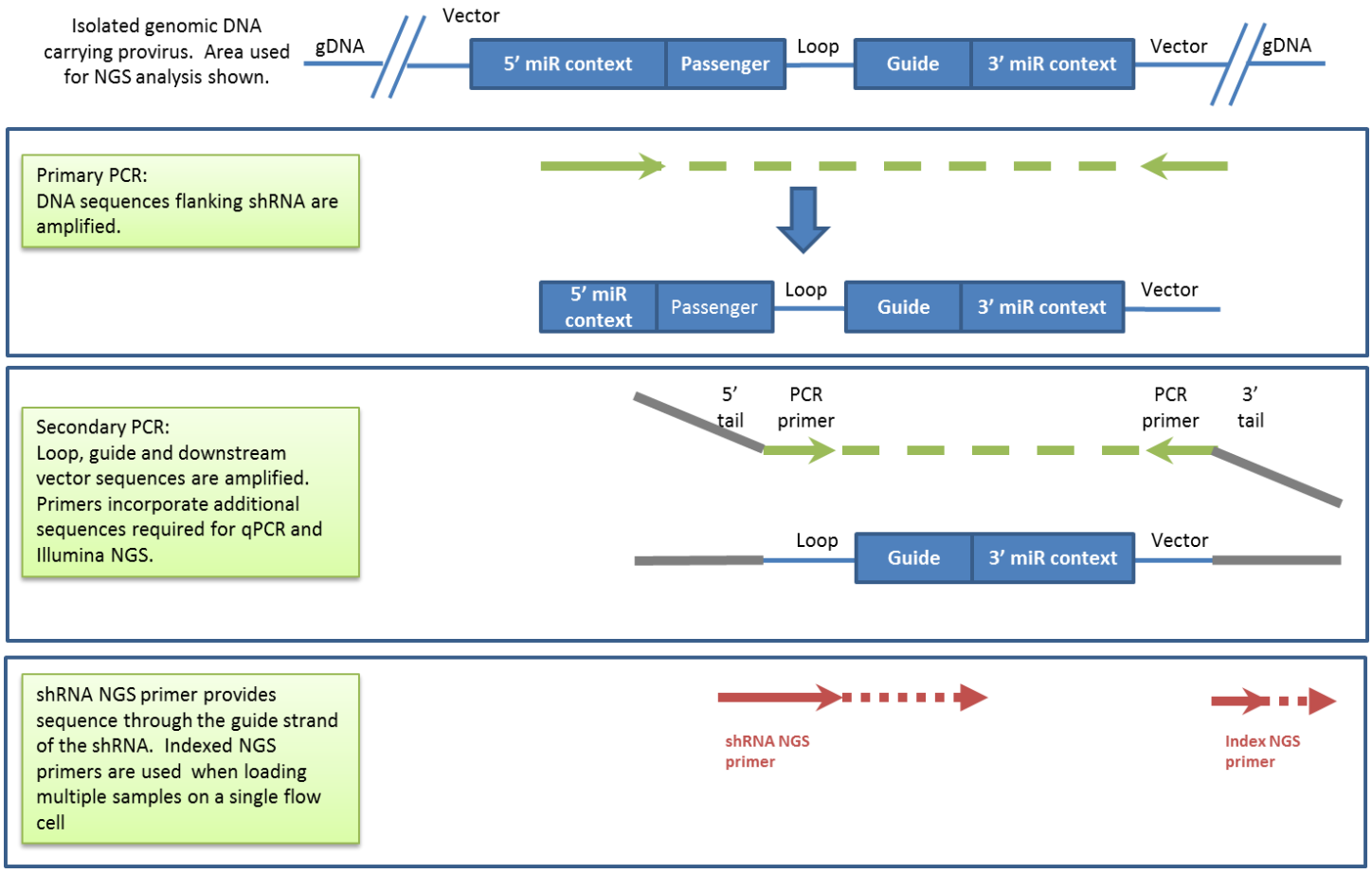


Figure 7. Schematic of PCR primers and sequencing primers as aligned with viral insert.

1. Primary PCR

Primary PCR reactions should be carried out with no more than ~850 ng of gDNA per 50 µl reaction; therefore, multiple PCR reactions will be required per sample. The combined PCR reactions for each sample should include all extracted gDNA to maintain representative of shRNA coverage used during the screen of the pool (500-1000 times the number of shRNA in the pool). See the example below for calculations.

For example:
A pool containing 1500 shRNA used for screening with coverage of 1000 viral integrants per shRNA would require 9.9 µg of gDNA per sample be used for amplification.

- 1500(shRNA) x 1000(coverage) x 6.6^{-12} (g/diploid genome)= 9.9^{-6} grams gDNA (or 9.9µg)

Each 50 µl PCR reaction will contain 850 ng of gDNA.

- 9.9 µg (or 9900 ng) / 850 ng DNA = 11.6 reactions

Twelve PCR reactions per sample will be required for gDNA amplification from this pool.

Required materials

- KOD Hot Start Polymerase (Novagen Cat# 71086-4)
- 96 well PCR plates
- Agarose gel
- Qubit® dsDNA BR Assay or Quant-iT™ PicoGreen ® dsDNA Kits (recommended for their specificity to quantitate dsDNA in solution)
- PCR purification column (*QIAquick PCR Purification Kit, Qiagen Cat# 28104*)
- DNA gel extraction kit (*QIAquick Gel Extraction, Qiagen Cat# 28104)*
- Multiplex indexed primer kit (12-plex, TRP0002)
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Required equipment

- Qubit® fluorometer or standard spectroflourometer with fluorescein excitation and emission wavelengths
- NGS platform

Primary PCR Protocol

Note: KOD Hot Start Polymerase, Novagen Cat# 71086-4 is recommended for PCR.

1. Based on the gDNA quantitation determine the total number of reactions required to amplify gDNA using no more than 850 ng of gDNA per 50 µl PCR reaction.
2. Set up PCR reactions using components and volumes outlined below for each sample. A 96-well PCR plate is recommended.

A master mix of all components (excluding gDNA) should be made and then added to wells containing the same volume and concentration of gDNA.

Components	µl per reaction
10X KOD Buffer	5
dNTP Mix (2 mM each)	5
$MgSO_4$ (25 mM)	4
Betaine (5 M)	5
Primary PCR Forward (10 µM)	1.7
Primary PCR Reverse (10 µM)	1.7
KOD Hot Start Polymerase	1.5
Genomic DNA (850 ng)	--
Nuclease free water	--
Total Volume	50

3. Set up the following PCR cycling program on a thermal cycler with a heated lid.

Cycles	Temperature (°C)	Time
1	98	5 min
25	95	30 sec
	57	30 sec
	72	30 sec
1	72	5 min
1	4	Hold

4. Place samples in the thermal cycler with heated lid on and run the PCR program outlined above.
5. After cycling is complete, briefly centrifuge the 96 well plate containing PCR reactions.
6. Select at least 4 PCR reactions per sample and run 10 µl from each on an agarose gel to verify the 370 bp amplicon.
7. Pool PCR reactions from each sample and purify following manufacturer's protocol ensuring that column capacity is not exceeded (it may be necessary to use multiple columns per sample for purification). *QIAquick PCR Purification Kit, Qiagen Cat# 28104 is recommended.*
8. Quantitate purified PCR reactions using a fluorometric assay specific for double stranded DNA (dsDNA).

2. Secondary PCR

Note: Each sample should be amplified using a uniquely indexed (6 base pair index) Secondary PCR Reverse primer if multiple samples are run in parallel on the sequencer. Indexed primer kit is available in 12-plex format (TRP0002) from transOMIC technologies.

Protocol

1. 2.0 µg of primary PCR product should be amplified across 4 reactions (500 ng DNA per 50 µl PCR reaction) for each sample.
2. Set up PCR reactions using components and volumes outlined below for each sample. A 96 well plate is recommended.

   A master mix of all components should be made for each Secondary PCR Reverse- Indexed primer used (excluding DNA) which can then be added to wells containing the same volume and concentration of DNA.

Component	µl per reaction
10X KOD Buffer	5
dNTP Mix	5
$MgSO_4$ (25mM)	4
Betaine (5M)	2
Secondary PCR Forward (10 µM)	7.5
Secondary PCR Reverse(10 µM)- Indexed	7.5
KOD Hot Start Polymerase	1.5
Pooled Primary PCR DNA (500 ng)	--
Nuclease-free water	--
Total Volume	50

3. Set up the following PCR cycling program on a thermal cycler with a heated lid.

Cycles	Temperature (°C)	Time
1	98	5 min
15	94	30 sec
	52	30 sec
	72	30 sec
1	72	5 min
1	4	Hold

4. Place samples in the thermal cycler with heated lid on and run the PCR program outlined above.
5. After cycling is complete, centrifuge the 96 well plate containing PCR reactions.
6. Pool PCR reactions from each sample.
7. Gel purify the 408 bp product following manufacturer's recommendations from the kit used. Elute in 30 µl EB (or molecular-grade water).

   We recommend using QIAquick Gel Extraction, Qiagen Cat# 28104.

3. Quality Control of NGS-adapted shRNA amplicon libraries

After secondary PCR and purification, it is necessary to accurately quantitate each sample prior to NGS analysis.

Protocol

1. Quantitate shRNA amplicon libraries using a fluorometric assay specific for double stranded DNA (dsDNA).
2. Determine molarity of DNA and dilute each sample to 15 nM using the calculations below.
   - *(base pairs in DNA amplicon) X (660 grams/mol/base pair) = molecular weight (MW) of DNA in sample*
   - *(DNA ng/µl) ÷ (MW of amplicons) X 10^6 = DNA nM*
   - *(DNA nM) ÷ (15) = dilution factor for 15 nM final concentration*
   - *(Dilution factor) X (Sample µl) = Total Volume of Sample and dilution buffer*

For example:
A sample with concentration of 140 ng/µl and amplicon size of 380 bp would require the following dilution factor to achieve a 15 nM final concentration:

(380 bp) x (660 grams/mol/bp) = 247000 grams/mol
(140 ng/µl) ÷ (247000 grams/mol) x 10^6 = 566.8 nM
(566.8 nM) ÷ 15 = dilution factor of 37.8
37.8 X (3 µl sample) = 113.4 µl total volume
(3 µl sample) + (110.4 µl EB) = 15 nM sample

3. Samples diluted to 15 nM, should be analyzed by qPCR to verify concentrations of Illumina® adapted amplicons. NGS qPCR kits that are designed for quantitation with Illumina adapted libraries will work on all samples amplified with Secondary PCR primers (typically, NGS core facilities will provide this service prior to sequencing).
4. Dilute shRNA amplicon libraries to 10 nM based on qPCR quantitation.
5. Pool equimolar (uniquely indexed) amplicon libraries. It is not necessary to use the entire volume from each library when pooling, 5-10 µl from each should be sufficient.

4. Sequencing shRNA amplicon libraries

An NGS core facility is recommended if assistance is needed for NGS sequencing and analysis. The instructions below describe changes (to standard Illumina NGS protocols) that are required to prepare and sequence shRNA amplicon libraries.

A 50 base pair, single read sequencing run is sufficient to sequence through the unique 22 bases of the guide/sense and identify shRNA. Follow manufacturer protocols for preparing libraries (including denaturing, diluting and loading) and sequencer runs on an Illumina sequencer. We recommend loading ~12 - 18 pM for the pooled libraries; however, optimization may be necessary to obtain cluster densities that are recommended by the manufacturer.

Note the following when using Illumina kits:

- The custom shRNA Loop Read 1 Primer is provided as 100 µM, dilute accordingly (typically 1:200 in HT1 buffer).
- Illumina® provided Read 1 and Read 2 Primer Mixes should not be used/selected for sequencing shRNA amplicon libraries.
- PhiX control should not be run with shRNA amplicon libraries as the custom shRNA Loop Read 1 primer is not compatible with PhiX control.
- If samples were amplified using Secondary Indexed Reverse Primers, Illumina provided Index Primer Mix should be used/selected for index reads (indexes correspond to Illumina® TruSeq indices 1 - 12)

5. Data Analysis

The custom shRNA Loop Read 1 Primer binds to the loop of the shRNA (as shown in Figure 6) therefore, the first base read is the 5' end of the guide/sense sequence. The 22 bases of the guide/sense sequence can be used to identify shRNA. Multiplexed samples should be separated and analyzed according to their index reads.

Analysis of NGS data from a shRNA screen is complex and can be difficult to evaluate without the proper experience or guidance. There are many web-based tools that can be used for alignment and analysis of NGS data; however, for optimal analysis with viable hit identification, it is recommended to have screen data analyzed by an expert in the field of NGS and bioinformatics. Once data has been analyzed and individual shRNA representation has been identified as statistically significant between control and experimental samples, secondary validation of these hits by shRNA should be performed for confirmation of hits. Individual shRNA constructs can be used for hit validation. In many cases, further validation can be performed from over expression as well.

V. Appendix

Appendix A. *Safety*

Recombinant lentivirus is considered a Biosafety Level 2 organism by the National Institutes of Health and the Center for Disease Control and Prevention. However, local health and safety regulations should be determined for each institution.

For more information on Biosafety Level 2 agents and practices, download Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition (Revised December 2009) published by the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH. The publication can be found here: http://www.cdc.gov/biosafety/publications/bmbl5/.

If additional measures are needed, review biosafety guidance documents such as the NIH's "Biosafety Considerations for Research with Lentiviral Vectors" which refers to "enhanced BL2 containment". More information can be found through the NIH Office of Biotechnology Activities web site (http://oba.od.nih.gov/rdna_rac/rac_guidance_lentivirus.html)

Summary of Biosafety Level 2 Practices

The following is meant to be a summary of Biosafety Level 2 practices and should not be considered comprehensive. A full account of required practices should be determined for each institute and/or department.

Standard microbiological practices

- Limit access to work area
- Post biohazard warning signs
- Minimize production of aerosols
- Decontaminate potentially infectious wastes before disposal
- Use precautions with sharps (e.g., syringes, blades)
- Review biosafety manual defining any needed waste decontamination or medical surveillance policies

Safety equipment

- Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is not recirculated
- Protective personal equipment includes: protective laboratory coats, gloves, face protection if needed

Facilities

- Autoclave available for waste decontamination
- Chemical disinfectants available for spills

Appendix B. *Alternate methods for titering*

1. Puromycin titering by selection and colony counting:
   a. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal puromycin concentration determined in "puromycin kill curve"
   b. Continue feeding and observe the cells for approximately 7 days until you see single colonies surviving the selection. The negative control should have no surviving cells.
   c. Use a microscope to count the number of surviving colonies.
   d. Calculate the functional titer using the number of colonies visible at the largest dilution that has colonies.

$$(Number\ of\ colonies\ ) \times (dilution\ factor) \div 0.025\ ml = \frac{TU}{ml}\ functional\ titer$$

2. ZsGreen titering by FACS analysis
   a. When calculating the percentage of transduced cells **use the number of cells present on the day of transduction as the denominator**.

$$\frac{(Number\ of\ cells\ at\ transduction\ )}{(Number\ of\ ZsGreen\ positive\ cells\ in\ well)} \times (dilution\ factor) \div 0.025\ ml = \frac{TU}{ml}\ functional\ titer$$

Appendix C. *PCR and NGS sequencing primer sequences*

Primary PCR Forward (5'->3')
CAGAATCGTTGCCTGCACATCT TGGAAAC

Primary PCR Reverse (5'->3')
CGTATCCACATAGCGTAAAAGGAGCAAC

Secondary PCR Forward primer (5'->3')
AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGTGAAGCCACAGATGTA
binds to shRNA loop

Secondary PCR Reverse Indexed primer (5'->3')
CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTGCTAAAGCGCATGCTCCAGACTGC
6 bp Index

Secondary PCR Reverse Index	Secondary PCR Primer sequences with 6 base pair index (5'->3')
1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG
2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG
3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG
4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG
5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG
6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG
7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG
8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG
9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG
10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG
11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG
12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGTATCCACATAGCGTAAAAGG

Note: Since the sequences shown in the above table are reverse primers in 5'->3' orientation, the highlighted indexes are shown as reverse complements. Indexes 1-12 above correspond to Illumina TruSeq indexes 1-12.

shRNA Loop Sequencing Primer (5'->3')
ACGACGCTCTTCCGATCTTAGTGAAGCCACAGATGTA

If low level multiplexing is needed (6 samples or less), please refer to *Multiplexing Sample preparation Guide (Illumina® Part# 1005361)* for recommendations on which sets of the indexes above can be used together.

Appendix D. *Vector map*

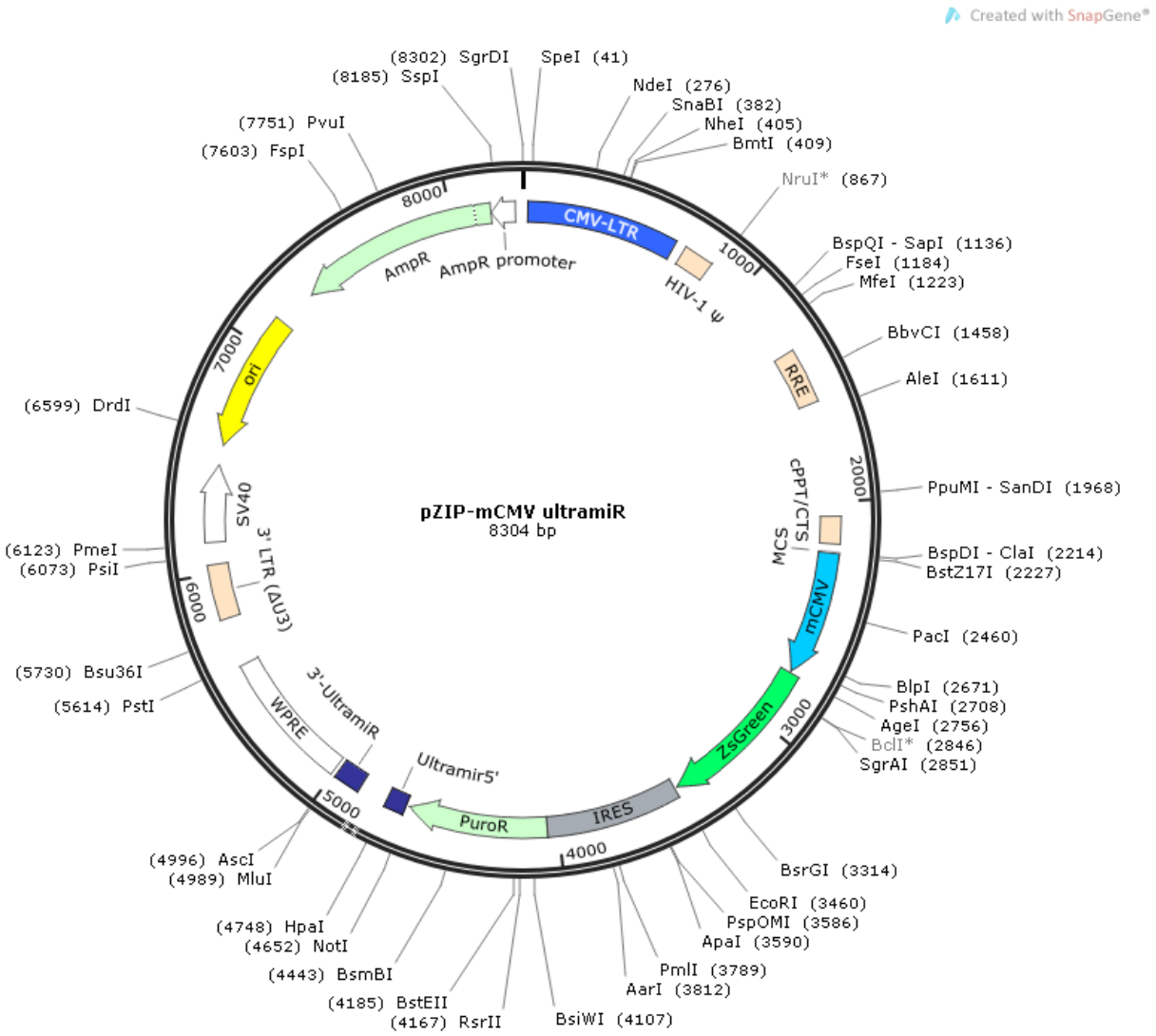

Figure 8. Detailed vector map of pZIP-mCMV

Sequence information and detailed maps for all ZIP vectors can be found on the [Pooled Screening Documents Page](#).

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