transOMIC technologies inc.

shERWOOD UltramiR shRNA Lentiviral Target Gene Set in pZIP

TLVU1401 (1-5 x 10^6 TU/ml), TLVU1402 (1-5 x 10^8 TU/ml)
Format: Lentiviral particle

shERWOOD-UltramiR shRNA

shERWOOD-UltramiR short hairpin RNA (shRNA) 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.

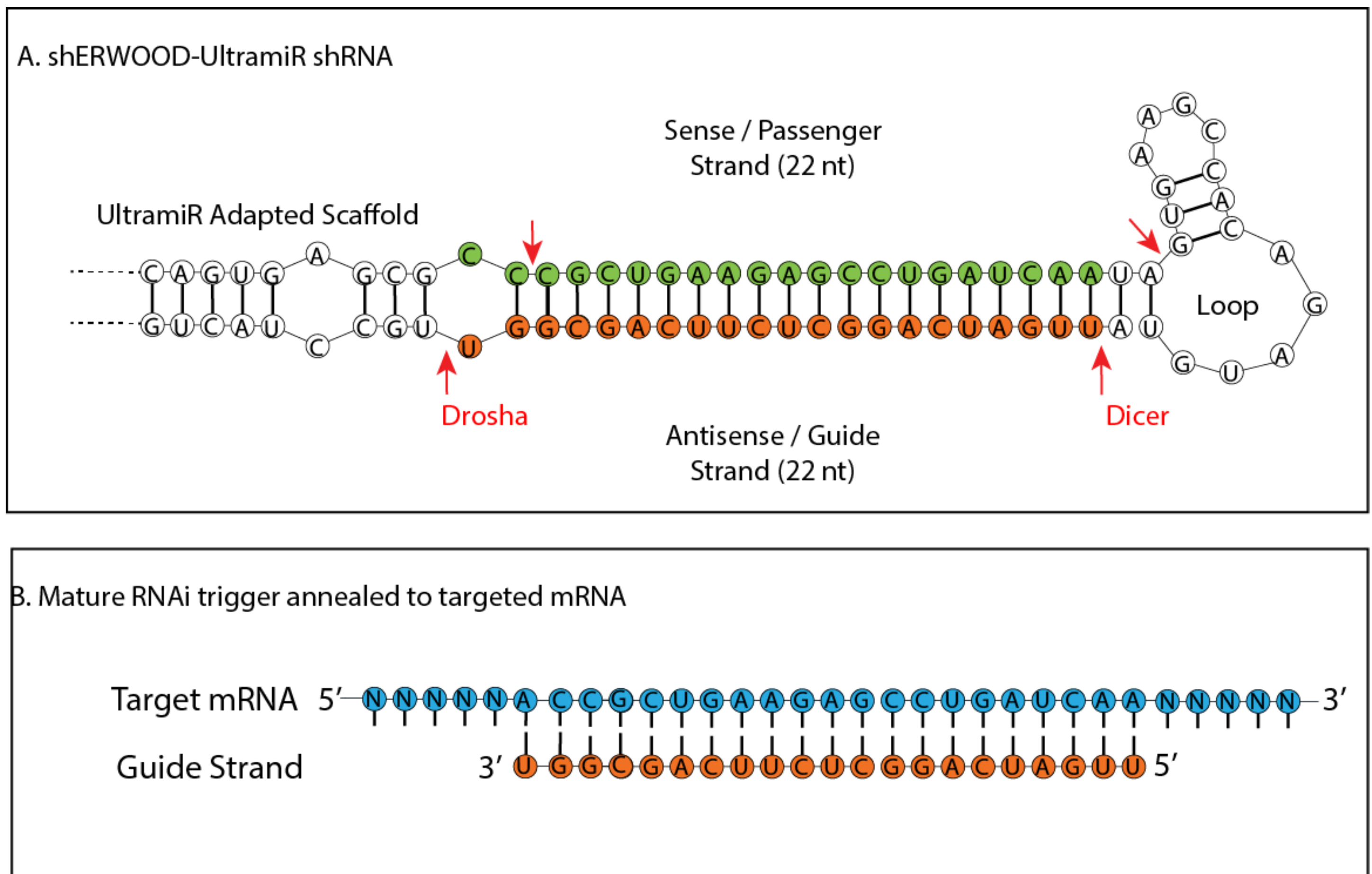


Figure 1. Schematic of shERWOOD-UltramiR shRNA. (A) Passenger (green) and Guide (orange) strand are shown with Dicer and Drosha nuclease cleavage sites are in red. (B) The final step of shRNA processing loads the Guide Strand (orange) into the RISC complex which binds to the target mRNA (blue) in a sequence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

Optimized microRNA scaffold sequence increases small RNA processing

Previous generation microRNA-adapted shRNA libraries have alterations in conserved regions of the flanking sequences that were thought to disrupt processing and reduce knockdown efficiency. The miR-30 scaffold for shERWOOD-UltramiR designs have been optimized based on knowledge of key microRNA determinants for optimal primary microRNA processing (Auyeung *et al.*, 2013).

This new scaffold increases small RNA levels presumably by improving maturation through the microRNA biogenesis pathway. When shRNA were placed into the UltramiR scaffold, mature small RNA levels were increased roughly two fold relative to levels observed using the standard miR-30 scaffold (Knott *et al.,* 2014).

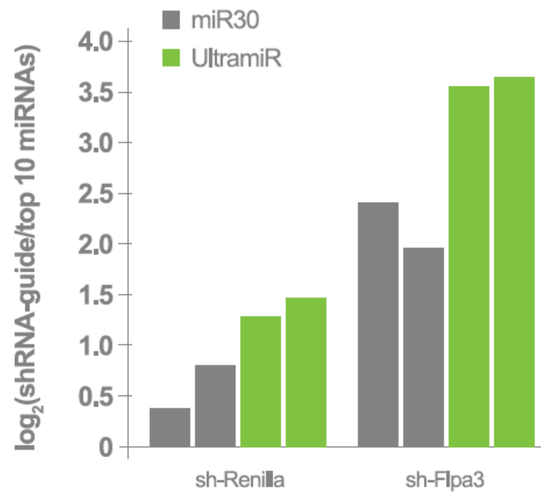


Figure 2. Relative abundances of processed guide sequences for two shRNA as determined by small RNA cloning and NGS analysis when cloned into traditional miR-30 and UltramiR scaffolds. Values represent log-fold enrichment of shRNA guides with respect to sequences corresponding to the top 10 most highly expressed endogenous microRNA.

Guaranteed knockdown

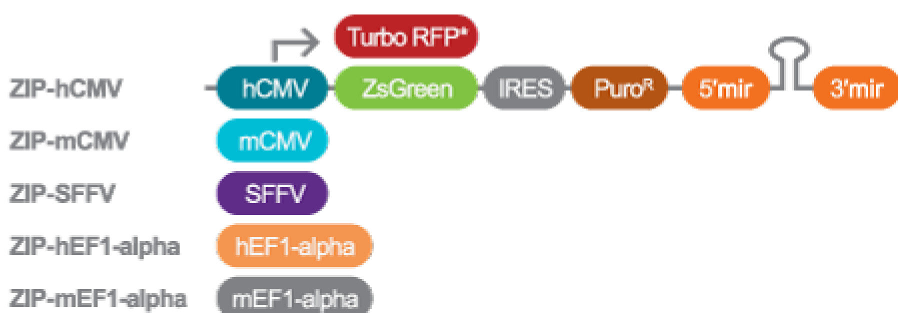
All shRNA constructs in a target gene set are guaranteed to knock down mRNA expression by >70%.
Cell line of choice should demonstrate expression of the target gene using the non-targeting controls and should demonstrate gene knockdown using a positive control shRNA.

I. Introduction

This manual provides information for transduction with the ZIP lentiviral vector. Appendix 2 contains basic safety information for production and handling of lentiviral particles. Review local safety guidelines for complete regulations.

Lentiviral shRNA are provided in the ZIP-mCMV, ZIP-hCMV, ZIP-hEF1-alpha, ZIP-mEF1-alpha and ZIP-SFFV vectors. The protocols refer to pZIP as a general reference to all. **No changes to the protocols are required for use with either of the alternate ZIP vectors. Detailed vector graphics are provided in Appendix 1.**

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. In addition, the hybrid 5′ LTR packages efficiently without expression of Tat making it compatible with second, third and fourth generation packaging systems.



Function	Element
Promoter choice	Human CMV (hCMV), human EF1-alpha (hEF1-alpha), mouse CMV (mCMV), mouse EF1-alpha (hEF1-alpha), or Spleen Focus Forming Virus (SFFV)
Fluorescent marker	ZsGreen - excitation maximum = 496 nm; emission maximum = 506 nm TurboRFP - excitation maximum = 553 nm; emission maximum = 574 nm
Mammalian selection marker	Puromycin resistance gene (PuroR)
shRNA	shERWOOD-UltramiR shRNA showing 5' and 3' flanking UltramiR sequences

Figure 3. Schematic of ZIP lentiviral shRNA vectors. All elements are identical with the exception of the promoter and fluorescent marker. (LTRs are not shown.)

II. Safety

See Appendix 2 for safety considerations.

III. Overview of Transduction and Titer

Transduction by lentiviral vectors is the process by which the viral particle attaches to the cell surface, enters into the cell and uncoats into a pre-integration complex, reverse transcribes its single-stranded RNA genome into a double-stranded DNA provirus, followed by translocation into the cell's nucleus and stable integration of the provirus into the host cell genome. The number of viral particles used and the transduction efficiency will determine the average number of lentiviral integrations into the target cell genome.

Various factors affect the transduction efficiency in any given cell line. The titer, or number of viral particles in a particular volume, has the most influence on the number of cells transduced. Viral titers can be measured in a number of ways. One approach is simply to measure the number of viral particles, usually by ELISA, which detects and quantifies a component of the viral particle. In the case of HIV-based lentiviral vectors a p24 ELISA is used to measure the amount of HIV capsid protein in the sample. This is referred to as p24 titer or particle titer. Because p24 ELISAs detect capsid antigen present on both functional and non-functional viral particles, p24 titers overestimates the true viral titer, as most lentiviral vector viral particles are non-functional (missing one or more required vector components). Another approach is to use RT-PCR to quantify the number of viral genomic RNAs packaged into viral particles. Titers determined by this method are usually provided as copies per ml, and like p24 titer it too is a measure of the number of viral particles, not a transduction unit, and overestimates true titer (~100-fold). A more meaningful measurement is referred to as functional titer, which measures the number of cells that are transduced and express a marker gene that is encoded within the viral vector. Functional titers are given as transduction units per milliliter (TU/ml) and are determined by detecting expression of either a fluorescent protein or selectable antibiotic resistance gene. Thus functional titering is a more accurate measurement because it not only takes into account the number of functional viral particles and the efficiency of transduction into the cell, but also the activity of the promoter to drive expression of the transgene.

Cells differ with regards to transduction efficiency and promoter activity. It is therefore very important that the functional titer be determined using your experimental cell line to ensure an optimal transduction. This manual includes the protocol for functional titering using fluorescent or selection markers thereby providing the most accurate measure of titer for your experiment.

transOMIC technologies provides functional titers (TU/ml) for the lentiviral shRNA vectors using HEK293T cells. This is calculated by counting the number of ZsGreen-positive cells/colonies for a given volume of viral particles and is provided in the Certificate of Analysis (C of A).

1. Puromycin Selection (puromycin kill curve)

The optimal puromycin concentration should be determined for a cell line prior to transduction. Since cell lines differ in their sensitivity to puromycin, the optimal concentration of puromycin (pre-transduction) should be determined. 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 1. Use a puromycin stock solution of 1.25 µg/µl stock solution.

Table 1. 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.*

2. Relative transduction efficiency, functional titer and transduction optimization

Transduction efficiency of lentiviral particles is influenced by cell type and transduction conditions. It is therefore important to determine the impact of your cell type and experimental conditions. The relative transduction efficiency can be calculated by comparing the titer provided in the C of A (calculated in HEK293T cells) to the titer in the target cell line. By calculating this for the non-targeting control the expected titer in the targeted cell line can be predicted for the viral particles from the targeting constructs.

Increasing transduction efficiency:

Optimizing transduction conditions can extend the utility of viral particles and limit cell toxicity. 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 experiment.

- 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.
- Transduction volume should be kept to a minimum. Media should barely cover cells.
- 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.
- 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 with minimal cell toxicity.
- 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 and Relative Transduction Efficiency

Functional titer must be determined using the experimental cell line to ensure optimal transduction. 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. It should therefore be calculated for every batch of virus produced and every 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 known to influence transduction efficiency.

- The following protocol evaluates titer by manually counting ZsGreen positive colonies. Alternate methods for determining titer are provided in Appendix 3.
- Transduction optimization should be done with the with Non-Targeting Control viral particles.
- If the packaging protocol was followed for viral particle production, use the titering aliquots made in the virus production section above to determine the titer.
- HEK293T cells are readily transduced under standard conditions and are included in the protocol as a positive control for transduction.

Required Materials

- Experimental cells and HEK293T cells
- Complete media for HEK293T cells and experimental cell line
- Serum free media for each cell line
- 24-well tissue culture plate
- Lentiviral particles
- Sterile Microcentrifuge tubes
- Polybrene

Equipment

- Automatic pipettor /Pipette-aid (for tissue culture)
- Pipettor (for dilutions and handling of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO2 cell culture incubator at 37°C
- Fluorescent microscope with ZsGreen filter (refer to Appendix 3 for alternative methods that do not require this)

Functional Titering Protocol

The following protocol represents the standard procedure followed for determining functional titers in your target cell lines and HEK293T (positive control) cells. Optimal cell numbers, serum concentration, polybrene concentrations, times, and culture conditions are likely to be different for the experimental cell line.

1. Plate your target cells and HEK293T cells 18-24 hours prior to transduction in a 24 well plate. Plate at a density of 7 x 10^4 cells per well in 14 wells with complete media. Incubate overnight with 5% CO_2 at 37°C. It is important to seed enough cells so that the cell confluency ranges between 40 and 50% at the time of transduction.
2. Prepare a serial dilution series with media and viral particles (see Figure 4 below):
   a. Set up 7 sterile microcentrifuge tubes. Number tubes 1 to 7.
   b. Add 100 µl of medium containing 1% serum and 6 µg/ml Polybrene.
   c. Add 25 µl of viral stock to the first microcentrifuge tube.
   d. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
   e. Transfer 25 µl from the first microcentrifuge tube to the second tube. Mix well and discard the tip.
   f. Serially dilute the viral particles by repeating the procedure for the remaining tubes.
   g. Allow viral dilutions with Polybrene to incubate for 10 minutes at room temperature.
3. While viral particles are incubating, remove media from cells in each well.
4. Add to each well 200 µl of 1% serum media containing NO Polybrene.

5. **TLVU1401 (10^6 TU/ml)**
   a. After the 10-minute incubation, transfer 25 µl of viral dilution from tubes 1-5 to two corresponding wells (225 µl final volume) for a total of 10 wells for each virus stock. The remaining wells (without viral particles) should be evaluated as negative controls.

5. **TLVU1402 (10^8 TU/ml)**
   b. After the 10-minute incubation, transfer 25 µl of viral dilution from tubes 3-7 to two corresponding wells (225 µl final volume) for a total of 10 wells for each virus stock. The remaining wells (without viral particles) should be evaluated as negative controls.

6. Rock plate gently a few times to distribute the viral particles across the well.
7. Incubate overnight with 5% CO_2 at 37°C (12-24 hours).
8. Replace the viral supernatant with complete media and allow cells to grow for 48-72 hours.
9. Count the number of colonies expressing ZsGreen. A colony consisting of multiple cells should be counted as a single transduction event.
   Note: Choose wells that have approximately 50-100 ZsGreen positive colonies for more accurate counting.
10. Use the calculation below and Table 2 below to determine functional titer and relative transduction efficiency. See the following page for an example calculation.

Once the titer is established in your cell line the relative transduction efficiency can be calculated by comparing your titer to the titer provided in the C of A. This can be used to calculate the expected titer in your cell line for the rest of virus provided.

Titering Calculations:

Functional titer of non-targeting control (NTC) viral particles in experimental cell line:

$$(Number\ of\ colonies\ ) \times (Dilution\ factor) \div (volume\ added\ to\ cells\ (ml)) = \frac{TU}{ml}\ Functional\ titer$$

Relative transduction efficiency of experimental cell line:

(NTC titer in HEK293T cells) ÷ (functional titer of NTC in experimental cell line)
=Relative transduction efficiency

Functional titer of viral particles in experimental cell line:

(titer in HEK293T cells (provided in C of A)) ÷ (Relative transduction efficiency)

= Functional titer of viral particles in experimental cell line

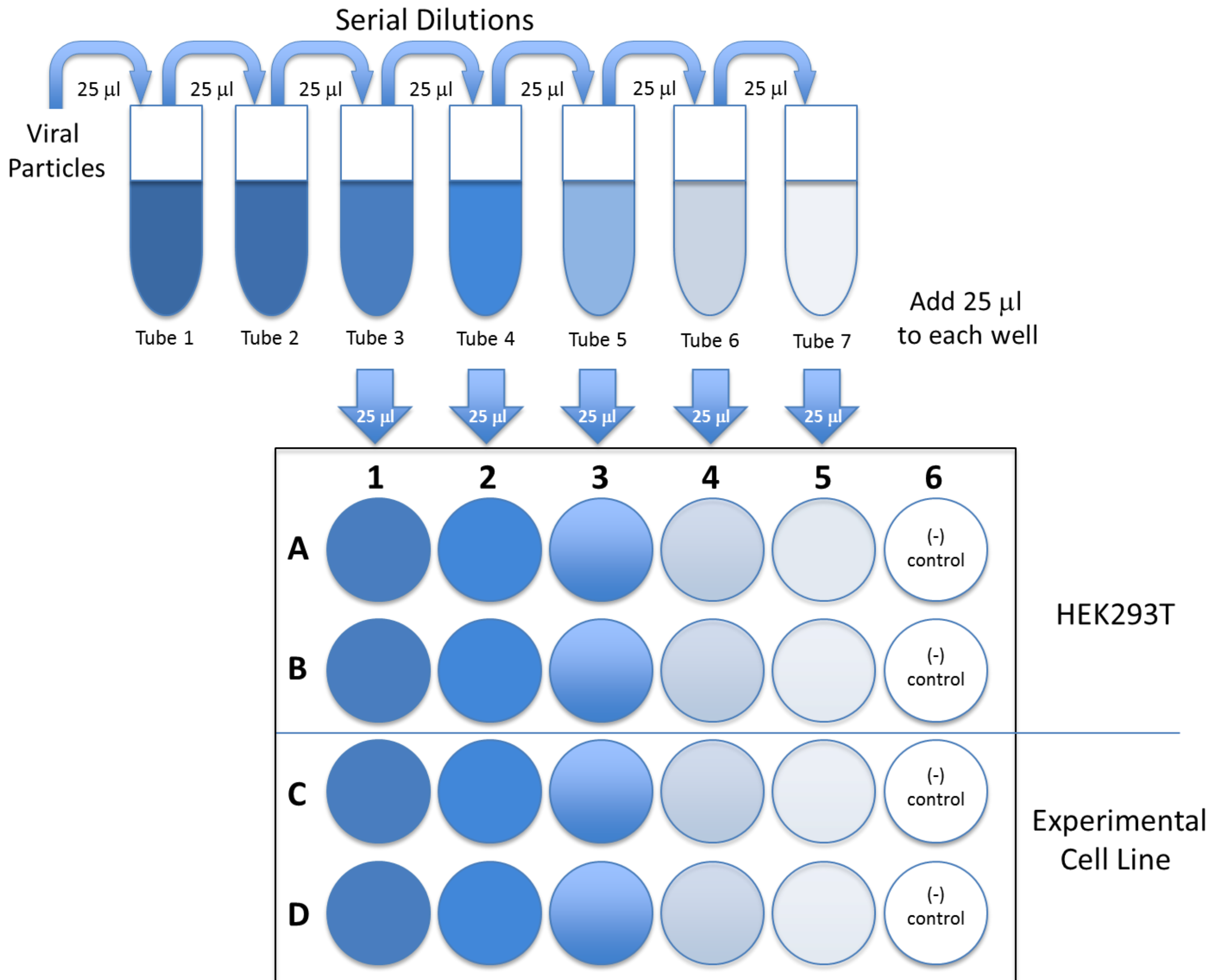


Figure 4. Schematic of serial dilutions for viral particle titering. (-) indicates untransduced control.

Table 2.

Tube	Viral Particles	Dilution Medium	Dilution Factor
1	25 µl (from NTC control)	100 µl	5
2	25 µl (from Tube 1)	100 µl	25
3	25 µl (from Tube 2)	100 µl	125
4	25 µl (from Tube 3)	100 µl	625
5	25 µl (from Tube 4)	100 µl	3125
6	25 µl (from Tube 5)	100 µl	15,625
7	25 µl (from Tube 6)	100 µl	78,125

Example for titering and relative transduction calculations

If the control virus has a titer of 1 x 10^8 TU/ml (Refer to the C of A for the exact titer of your lot of viral particles.), then the expected number of fluorescent colonies in the titering assay using HEK293T would be:

Tube	2	3	4	5	6	7
Dilution	1/5	1/5	1/5	1/5	1/5	1/5
Diluted titer TU/ml	4,000,000	80,000	160,000	32,000	6,400	1280
Volume used to transduce cells (ml)	0.025	0.025	0.025	0.025	0.025	0.025
Fluorescent colonies expected	100,000	20,000	4,000	800	160	32

If the experimental cell line has an average of 100 colonies in well C3 and D3 (tube 5) then the functional titer for the experimental cell line is calculated as follows:

100 colonies X 3,125 ÷ 0.025 ml = 1.25 X 10^7 TU/ml

Since the functional titer of the control in HEK293T cells = 1 x 10^8 (provided on the C of A) the experimental cell line is transduced 8 times less efficiently. The relative transduction efficiency is calculated as follows:

1 x 10^8 TU/ml (functional titer in HEK293T) ÷ 1.25 X 10^7 TU/ml (functional titer in experimental cell line) = 8

Once the relative transduction efficiency is established the functional titer of other viral particles can be estimated using the titer provided in the C of A. If the functional titer (provided in the C of A) in HEK293T cells = 4 x 10^8 then the function titer in the experimental cell line is calculated as follows:

4 x 10^8 TU/ml (functional titer in HEK293T) ÷ 8 (Relative transduction efficiency)
= 5 x 10^7 TU/ml (functional titer in experimental cell line)

Transduction with Lentiviral shRNA Vector Particles

The following should be optimized prior to transduction:

- Transduction media: % Serum, Polybrene µg/ml
- Time exposed to transduction media: hours or overnight
- Selection media: µg/ml puromycin

Required materials

- Complete media for experimental cell line
- Selection media: complete media for experimental cell line supplemented with puromycin
- Transduction media containing viral particles (optimized for serum and Polybrene concentration)

Equipment

- Automatic pipetter /Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Assay specific equipment (e.g. fluorescent microscope)

Protocol:

Prepare cells

1. Plate cells such that they are actively dividing and 40 - 50% confluent at the time of transduction.
2. Feed cells with complete media 3 - 4 hours prior to transduction.
3. Make transduction media just prior to transduction.

Transduce cells

4. Exchange media with transduction media. Transduce cells at the appropriate MOI.
5. Incubate cells 12 - 24 hours in transduction media.
6. Replace transduction media with complete media (no puromycin).

Puromycin selection

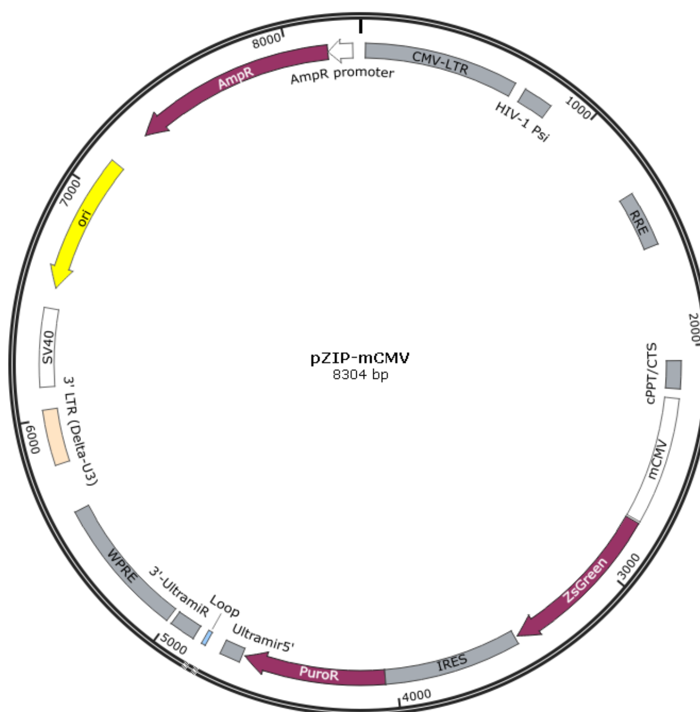
7. Allow cells to culture for 48 hours.
8. Replace media with selection media. (Use information from the puromycin kill curve concentration of puromycin that is determined)
9. Continue feeding cells selection media until untransduced cells have been removed.

Analysis

10. Analyze cellular phenotype or harvest cell for gene expression analysis according to your experimental design.

I. Appendix

Appendix 1a – pZIP-mCMV vector information



Element	Start	Stop
CMV-LTR	23	667
Psi	715	840
RRE	1337	1570
cPPT/CTS	2062	2178
mCMV	2219	2755
ZsGreen	2764	3459
IRES	3471	4047
PuroR	4048	4650
Ultramir5'	4663	4748
3'-UltramiR	4875	4986
WPRE	5009	5597
3' LTR	5805	6038
SV40	6134	6463
Ori	6552	7137
AmpR	7308	8168

Figure 3a: Detailed map of the pZIP-mCMV vector and vector element table.
The full sequence is available here.

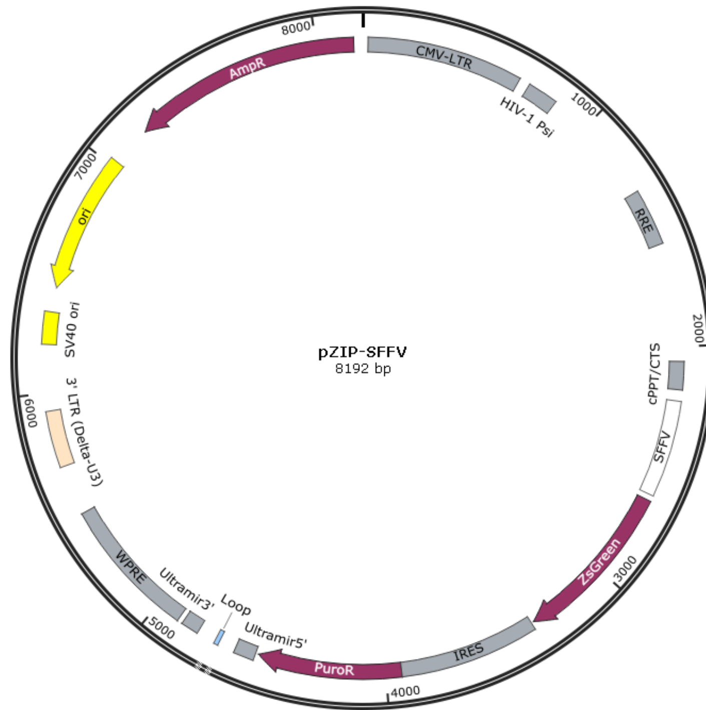
Appendix 1b – pZIP-hCMV vector information

Element	Start	Stop
CMV-LTR	23	667
Psi	715	840
RRE	1337	1570
cPPT/CTS	2062	2178
CMV enhancer	2235	2538
CMV promoter	2539	2742
ZsGreen	2833	3528
IRES	3540	4116
PuroR	4117	4719
5'-UltramiR	4737	4831
Loop	4887	4904
3'-UltramiR	4944	5055
WPRE	5078	5666
3' LTR	5874	6107
SV40	6203	6532
Ori	6621	7206
AmpR	7377	8237

Figure 3b: Detailed map of the pZIP-hCMV vector and vector element table.

The full sequence is available here.

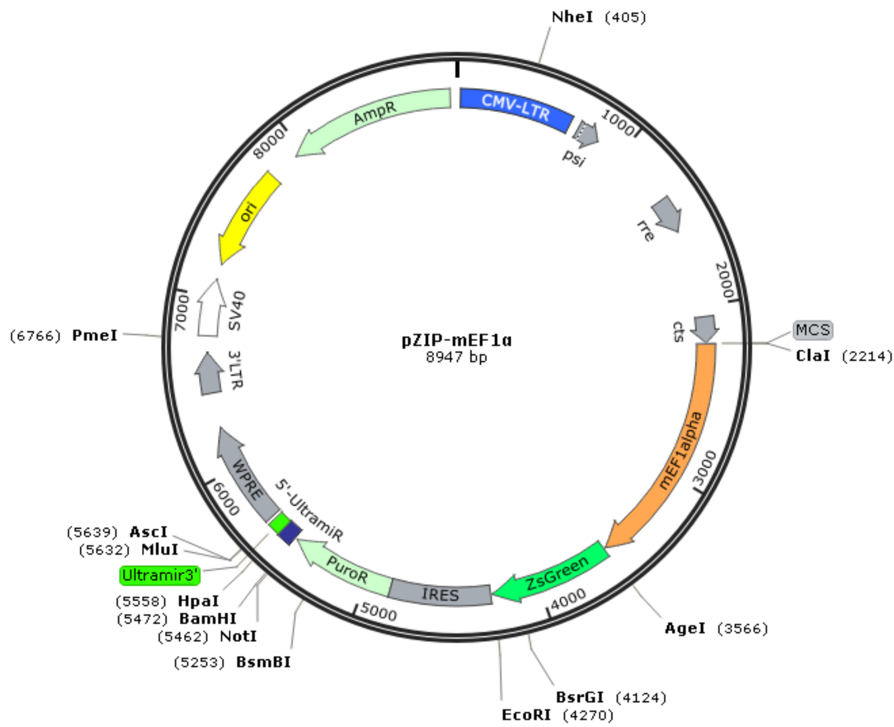
Appendix 1c – pZIP-SFFV vector information



Element	Start	Stop
CMV-LTR	23	667
Psi	715	840
RRE	1337	1570
cPPT/CTS	2062	2178
SFFV	2225	2625
ZsGreen	2652	3347
IRES	3359	3935
PuroR	3936	4538
Ultramir5'	4551	4636
shRNA	4666	4762
Ultramir3'	4804	4877
WPRE	4897	5485
3' LTR	5693	5926
SV40 Ori	6202	6337
Ori	6440	7025
AmpR	7196	8155

Figure 3c: Detailed map of the pZIP-SFFV vector and vector element table.
The full sequence is available here.

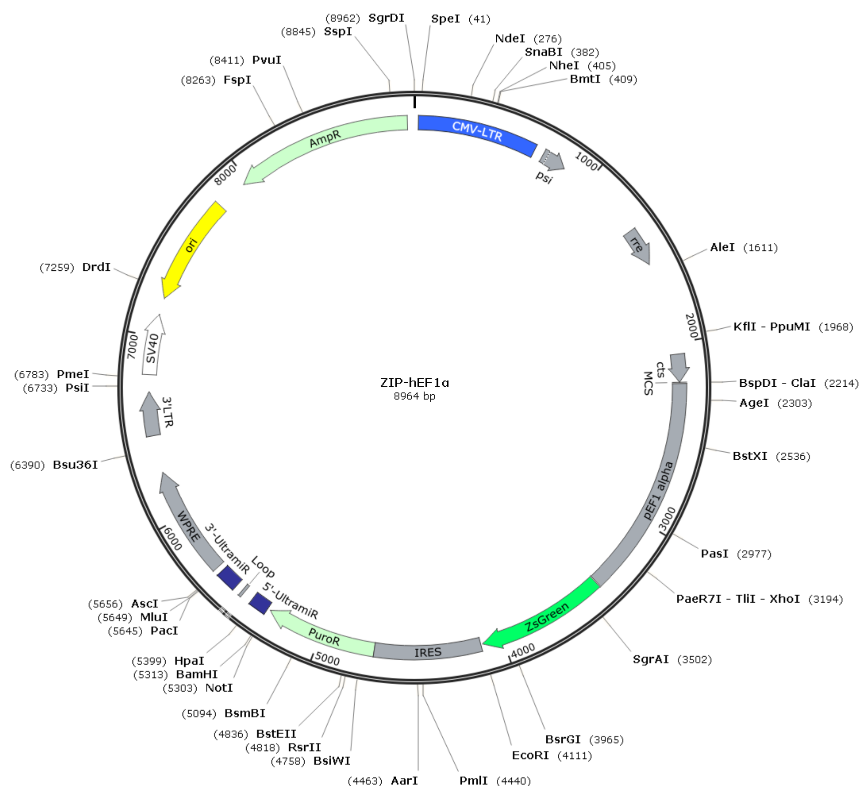
Appendix 1d – pZIP-mEF1-alpha vector information



Element	Start	Stop
CMV-LTR	23	667
Psi	720	856
RRE	1348	1552
CTS	2062	2212
MCS	2213	2214
mEF1alpha	2219	3566
ZsGreen	3574	4269
IRES	4281	4857
PuroR	4858	5460
Ultramir5'	5478	5558
Ultramir3'	5559	5632
WPRE	5652	6243
3' LTR	6448	6683
SV40 Ori	6777	7106
Ori	7195	7780
AmpR	7951	8910

Figure 3d: Detailed map of the pZIP-mEF1a vector and vector element table.
The full sequence is available here.

Appendix 1e – pZIP-hEF1-alpha vector information



Element	Start	Stop
CMV-LTR	23	667
Psi	720	856
RRE	1348	1552
CTS	2062	2212
MCS	2213	2214
pEF1 alpha	2220	3406
ZsGreen	3415	4110
IRES	4122	4698
PuroR	4699	5301
Ultramir5'	5319	5413
shRNA	5414	5530
Ultramir3'	5527	5638
WPRE	5669	6260
3' LTR	6465	6700
SV40 Ori	6794	7109
Ori	7212	7797
AmpR	7968	8927

Figure 3e: Detailed map of the pZIP-hEF1a vector and vector element table.
The full sequence is available here.

Appendix 2 – Safety and handling of lentiviral particles

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 3. 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 4 – References

shRNA–mir and design

Knott et al., A computational algorithm to predict shRNA potency. Molecular Cell (2014), Volume 56, Issue 6, 18 December 2014, Pages 796–807.

Auyeung, V.C., I. Ulitsky, S.E. McGeary, and D.P. Bartel. 2013. Beyond Secondary Structure: Primary-Sequence Determinants License Pri-miRNA Hairpins for Processing. Cell 152:844-858.

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