shERWOOD UltramiR shRNA Lentiviral Target Gene Set in pZIP

TLHSU1400 (human), TLMSU1400 (mouse), TLRSU1400 (rat)
Format: Bacterial glycerol stock

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 designs that are consistently potent even at single copy representation in the genome.

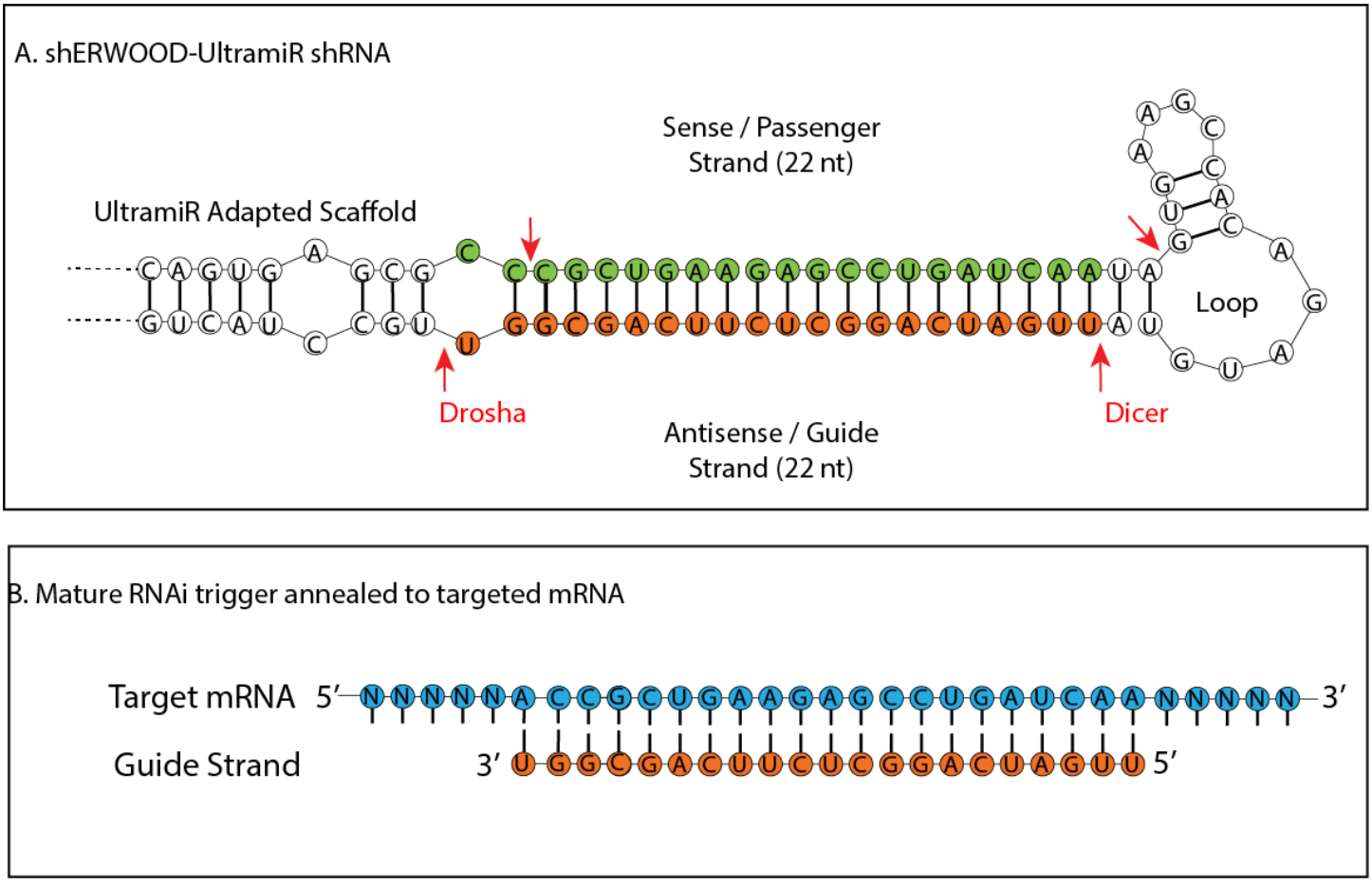


Figure 1. Schematic of shERWOOD-UltramiR shRNA. (A) Passenger (green) and Guide (orange) strand are shown with Dicer and Drosha nuclease cleavage sites are in red. (B) The final step of shRNA processing loads the Guide Strand (orange) into the RISC complex which associates with the target mRNA (blue)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

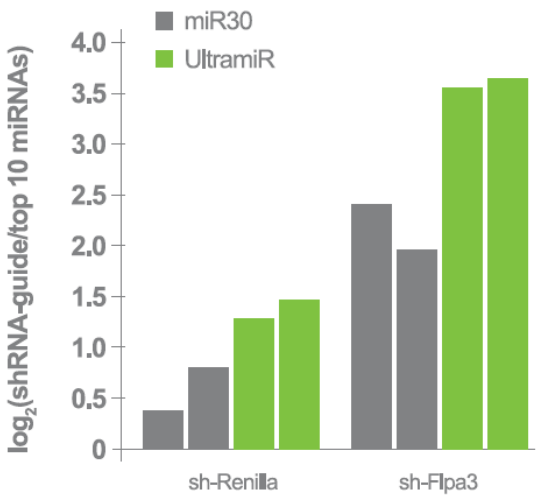


Figure 2. Relative abundances of processed guide sequences for two shRNA as determined by small RNA cloning and NGS analysis when cloned into traditional miR-30 and UltramiR scaffolds. Values represent log-fold enrichment of shRNA guides with respect to sequences corresponding to the top 10 most highly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

I. Safety

See Appendix 2 for safety considerations.

II. 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.

1. Replication protocols for glycerol stocks

Materials for individual and plate replication

LB-Lennox Broth (low salt)	VWR EM1.00547.0500
Glycerol	VWR EM-4760
Carbenicillin	VWR 97063-144

Propagate culture for storage

pZIP lentiviral shRNA cultures should be propagated in LB broth with ampicillin or carbenicillin (100 µg/ml) at 30°C for 30 hours or until the culture appears turbid. 2-10 ml starter cultures can be inoculated using 2 to 10 µl of the glycerol stock provided. Once turbid, place 920 µl of culture into a polypropylene tube and add 80 µl sterile glycerol (8% glycerol). Mix well and store at –80˚C. Glycerol stocks kept at -80°C are stable indefinitely as long as freeze/thaw cycles are minimized.

2. Plasmid preparation

For transfection and transduction experiments the pZIP plasmid DNA will first have to be extracted from the bacterial cells. Cultures should be grown in LB broth with ampicillin or carbenicillin (100 µg/ml) at 30°C for 30 hours or until the culture appears turbid. 2-10 ml starter cultures can be inoculated using 2 to 10 µl of the glycerol stock provided. Either a standard plasmid mini-preparation or one that yields endotoxin free DNA can be used. When isolating plasmid DNA for virus production using endotoxin free kit will generally yield higher viral titers.

3. Puromycin selection (puromycin kill curve)

The optimal puromycin concentration should be determined for a cell line prior to transduction or transfection. The pZIP lentiviral vector has a puromycin resistance marker for selection in mammalian cells. To establish stable cell lines, once transfection/transduction has occurred, the cells can be placed on puromycin to select for stable integrants. Since cell lines differ in their sensitivity to puromycin, the optimal concentration of puromycin (pre-transfection/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.

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.

5. Transfection

Use the following procedure to transfect plasmid DNA into mammalian cells in a 24-well format. For other plate formats, scale up or down the amounts of DNA and OMNIfect reagent proportionally to the total transfection volume (Table 2).

Adherent cells: One day prior to transfection, plate cells in 500 µl of growth medium without antibiotics so that cells will be 70–95% confluent at the time of transfection. The number of cells to plate will vary based on the double time.

Suspension cells: On the same day of transfection just prior to preparing transfection complex plate 160,000/well cells in 500 µl of growth medium without antibiotics.

Transfection complex preparation (Figure 4):

Volumes and amounts are for each well to be transfected.

1. Plasmid DNA preparation: Dilute 0.5 µg of plasmid DNA in a microfuge tube containing Opti-MEM® I Reduced Serum Media*** up to a total volume of 25 µl.
2. OMNIfect reagent preparation: In a separate microfuge tube, add 1 µL of OMNIfect into 24 µl Opti-MEM® I Reduced Serum Media*** for a total volume of 25 µl.
3. Final transfection complex: Transfer the diluted DNA solution to the diluted OMNIfect reagent (total volume = 50 µl). Mix gently and incubate at room temperature for 10 minutes.

Adding transfection complex to wells:

1. Add the 50 µl of transfection complex to each well containing cells and medium.
2. Incubate cells at 37°C in a CO_2 incubator for 24-48 hours.
3. After 24-48 hours of incubation, assay cells for gene activity.

**** serum-free DMEM medium can also be used.*

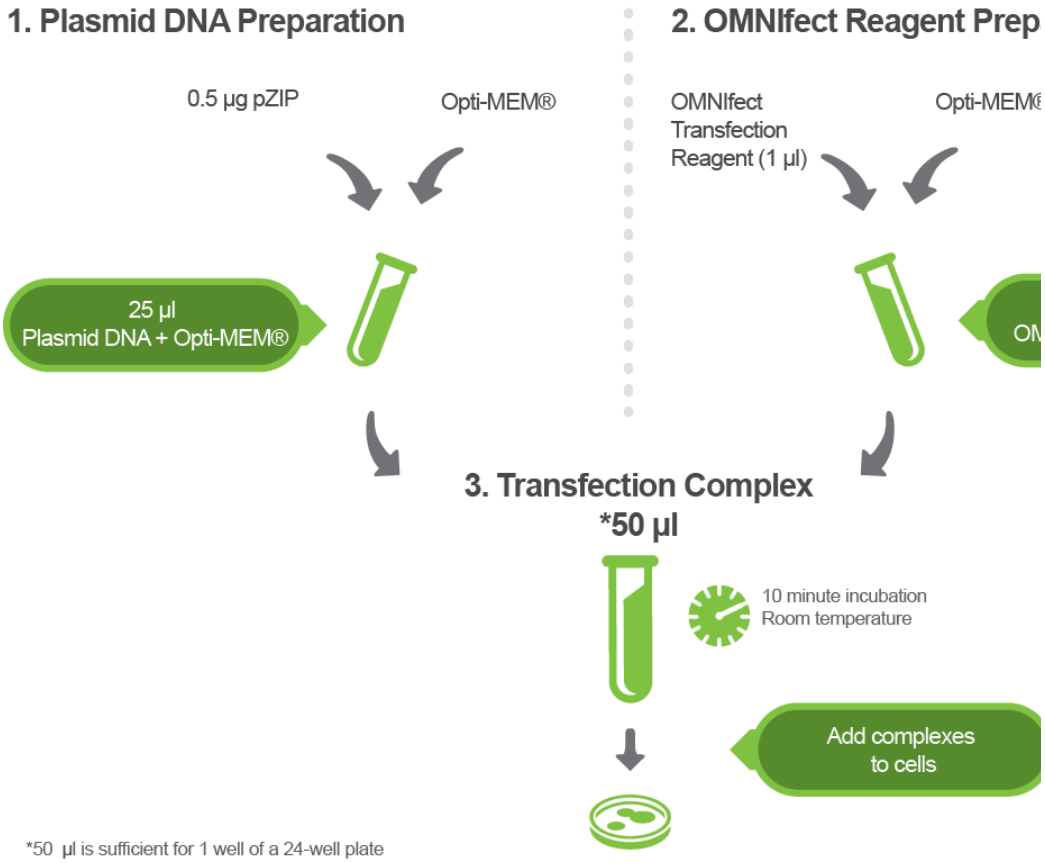


Figure 4. Transfection protocol for 24 well plates (volumes indicated are per well). To transfect the entire plate multiply all volumes and DNA amount by 24.

Table 2: Suggested amounts of DNA, medium and OMNIfect for transfection of plasmid DNA into adherent and suspension cells.

Tissue Culture Plates	Surface Area per Well (cm^2)	Plating Medium per Well (µl)	Plasmid DNA per Well (µg)†	OMNIfect per Well (µl)†	Transfection Complex per Well†† (µl)
6- well	9	2000	2 (100 µl Opti-MEM® I)	4 (100 µl Opti-MEM® I)	200
12-well	4	1000	1 (50 µl Opti-MEM® I)	2 (50 µl Opti-MEM® I)	100
24-well	2	500	0.5 (25µl Opti-MEM® I)	1 (25µl Opti-MEM® I)	50
96-well	0.3	200	0.1 (10µl Opti-MEM® I)	0.2 (10µl Opti-MEM® I)	10-20

†*Volume of* Opti-MEM® I shown for suspension of plasmid DNA and OMNIfect.
†† *Total volume of the transfection complex is made up of equal parts of DNA solution and OMNIfect solution.*

Transfection Optimization:

It is important to optimize transfection conditions to obtain the highest transfection efficiency with lowest toxicity for various cell types. The optimal ratio of OMNIfect to DNA is relatively consistent across many cell types. For further optimization try the following steps in order.

1. Use the recommended ratio of DNA:transfection reagent (at 1 µg DNA:2 µl OMNIfect), but vary the volume.
    a. Start with a range of volumes that cover +20% to -20%.
    For example, in a 24-well plate a range of 40 µl to 60 µl of transfection complex would be added to the well. (The plating media would remain the same.)
2. If further optimization is needed, transfection efficiency and cytotoxicity may be altered by adjusting the ratio of DNA (µg) to OMNIfect reagent (µl). A range of ratios from 1:1.5 to 1:2.5 is recommended.

Note: If transfection conditions result in unacceptable cytotoxicity in a particular cell line the following modifications are recommended:

1. Decrease the volume of transfection complex that is added to each well.
2. Higher transfection efficiencies are normally achieved if the transfection medium is not removed. However, if toxicity is a problem, aspirate the transfection complex after 6 hours of transfection and replace with fresh growth medium.
3. Increase the cell density in your transfection.
4. Assay cells for gene activity 24 hours following the addition of transfection complex to cells.

Puromycin selection of transfected cells

If less than 90% of all cells are green use puromycin selection to reduce background from untransfected cells. Refer to the protocol for the puromycin kill curve in section 3 to determine the optimal concentration for each cell line.

1. After incubating for 24-72 hours, examine the cells microscopically for ZsGreen expression.
2. Begin the antibiotic selection by replacing the medium with complete medium supplemented puromycin.
3. Replace the selective media every 2-3 days. Monitor the cells daily and observe the percentage of surviving cells.
    a. All untransfected cells should be removed within 3-5 days.
4. Collect samples for assay.

If selecting stably transfected cells, continue to replace the media containing puromycin. Observe the cells for approximately 7 days until you see single colonies surviving the selection. Colonies can be isolated and expanded for analysis.

6. Packaging lentiviral particles

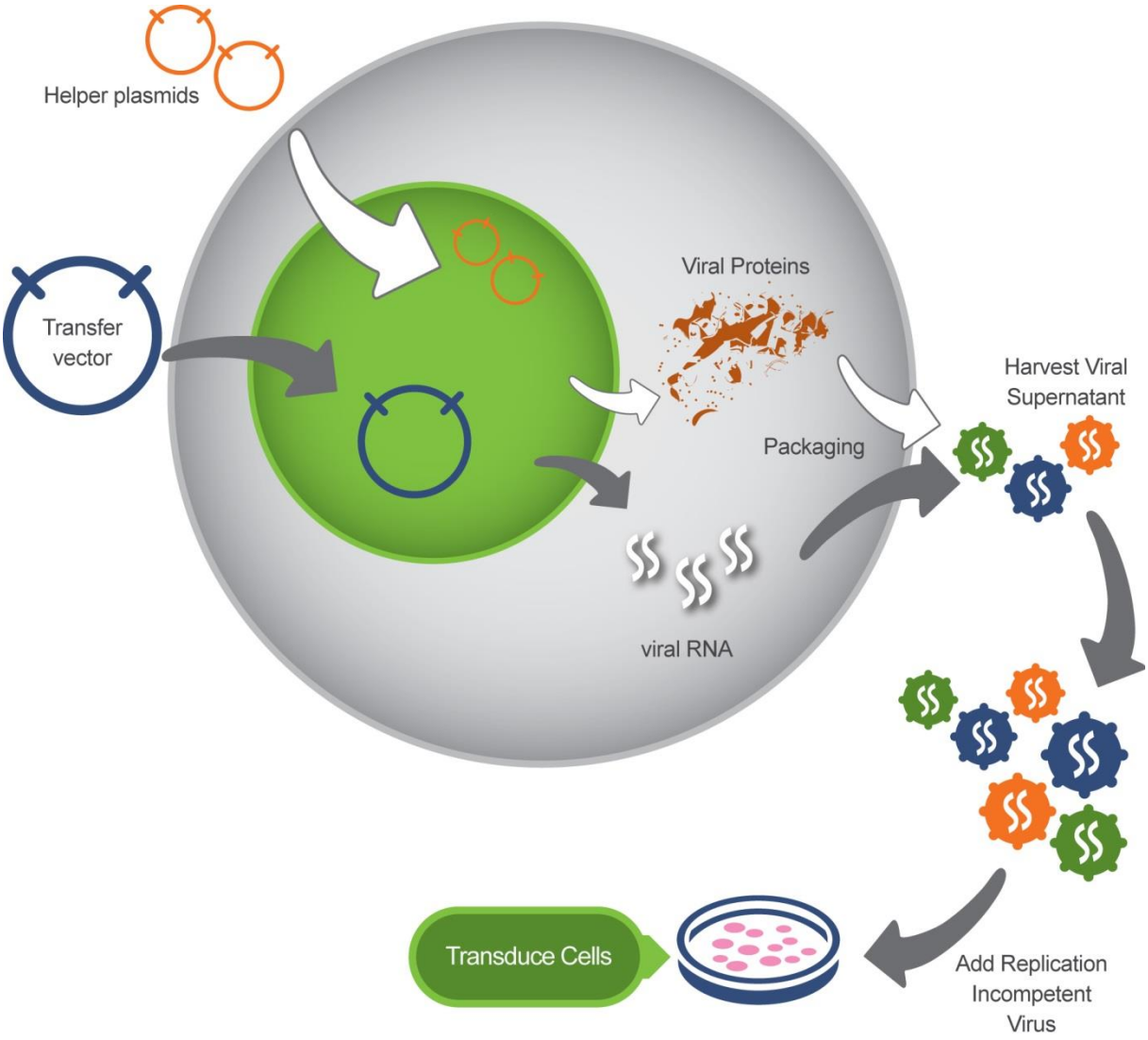


Figure 5. Schematic depicting lentiviral packaging of pZIP lentiviral vectors

When packaging lentivirus, the genetic elements required for assembly of replication incompetent viral particles are transfected into the cell in trans. The lentiviral transfer vector (pZIP) is co-transfected with the desired packaging vectors encoding the *env, gag* and *pol* protein into a packaging cell line. *gag*, *pol* and *env* provide the proteins necessary for viral assembly and maturation. The transfer vector contains sequences that will be packaged as the viral genome and code for the shRNA and selection cassette that will integrate into the target cell's genome. Viral particles are released from the packaging cell and can be harvested from the supernatant of the packaging cell. The resulting viral supernatant can be concentrated or applied directly to the targeted cell line.

Considerations before packaging the pZIP shRNA lentiviral vector:

While the enhanced scaffold processing provides more consistent knockdown in the target cell it can decrease packaging efficiency and lower viral production. During packaging the RNA genome of the lentiviral particle is produced and assembled into viral particles that can be harvested and used to transduce target cells. The packaged RNA must be intact to produce functional viral particles. However, the UltramiR scaffold is included in the transcript which targets it for cleavage by the small RNA processing machinery. Only transcripts that escape processing can be packaged.

siRNA targeting the nuclease Pasha/DGCR8 have been shown to increase virus production. This manual includes recommendations for the addition of Pasha/DGCR8 siRNA to the packaging mix. The method of transfection used for packaging must be able to deliver plasmid DNA and siRNA at the same time.

- Using an siRNA targeting DGCR8 (Pasha) can increase titers. Appendix 4 has instructions for ordering.
- The Non-Targeting Control should be used to determine the packaging and transduction efficiency of the target cell used.

Required Materials

Note: All plasmids are purified using endotoxin-free purification. All plasmids 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)
- Pasha siRNA (0.3 μg/μl) – *See Appendix 4 for ordering instructions*
- 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

Note: For smaller scale production, plate cells in one well of a 6-well plate and use 1/6th of the reagents.

1. On the day prior to transfection, seed 4-5 x 10^6 HEK293Tcells 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 10 ml of fresh culture media containing no antibiotics.
3. Allow FuGENE6 and OPTI-MEM I + GlutaMAX-I to come to room temperature.
4. Preparation of transfer vector and lentiviral vector packaging mix:
   Note: All plasmids 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. Pasha 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 I
   b. 45 µl of FuGENE6
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 Pasha siRNA 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 transduction 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 transduction 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 NIH-3T3 or HEK293T cells.

7. Functional titer and transduction optimization

The number of viral particles used and the transduction efficiency will determine the average number of lentiviral integrations into the target cell genome. The following protocol is designed to evaluate functional titer of the virus produced in the previous section. Puromycin selection may be used to remove untransduced cells. A kill curve should be performed as described in section 4.

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

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

- 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 (Harvested or purchased)
- Microcentrifuge tubes
- Polybrene

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 Appendix3 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 7 x 10^4 cells per well in 12 wells with complete media. Incubate for 24 hours with 5% CO_2 at 37°C.
2. Prepare a serial dilution series with serum free media and viral supernatant as shown in Table 3 and Figure .
    a. Set up 5 sterile microcentrifuge tubes.
    b. Add 160 µl of serum free medium to each tube containing 5-8 µg/ml Polybrene.
    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.
    g. Incubate at room temperature for 10-15 minutes.
3. Remove media from each well.
4. Add 200 µl of serum free media to each well containing cells.
5. Add 25 µl from each viral dilution to two wells (225 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 in a well is sufficient to provide accurate titers.
10. Use the calculation below and Table 3 to determine functional titer.
    (Alternate methods for calculating are described in Appendix 3.)

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

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

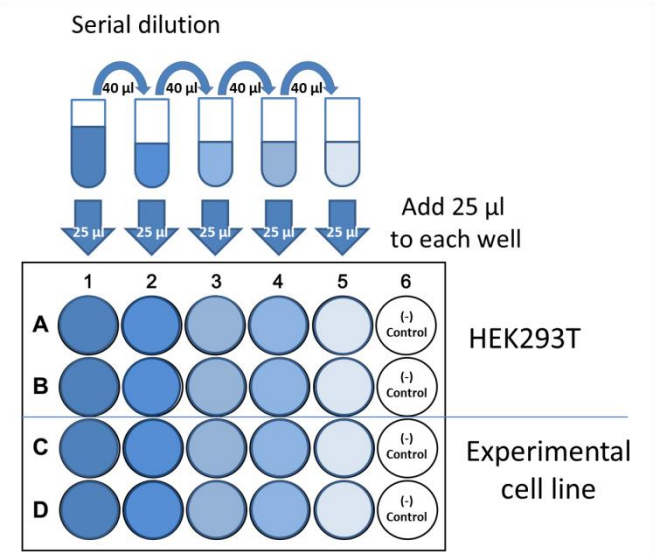


Figure 6 Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

Table 3 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.

V. Transduction Guidelines & Protocols

This protocol provides a basic outline of the transduction process. 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

Protocol:

Prepare cells

1. Plate cells such that they are actively dividing and 60 - 70% 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.
   (Note: media should be serum free for maximum transduction efficiency. Alternatively, see section 7 for information on transduction optimization.)
5. Incubate cells 12 - 24 hours in transduction media.
6. Replace transduction media with complete media (no puromycin).

Puromycin selection

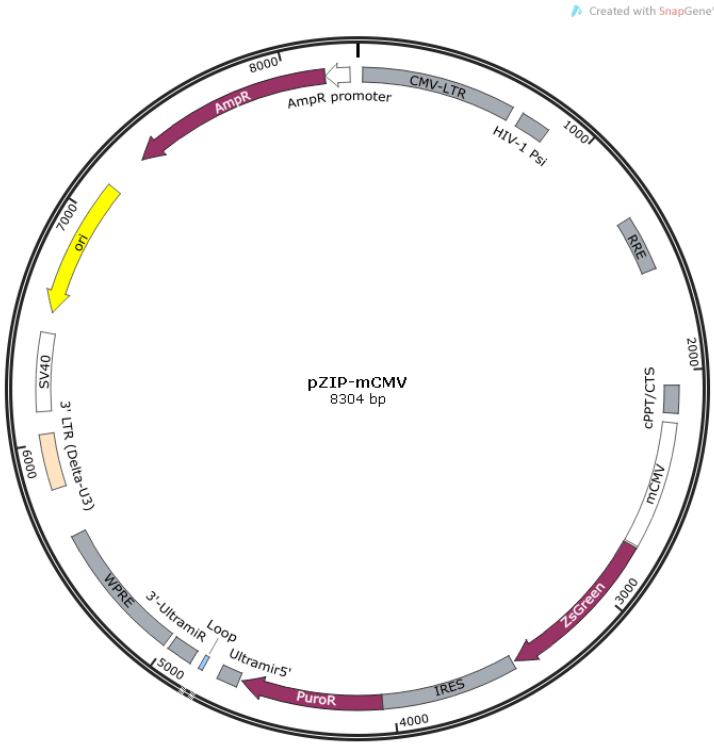
7. Allow cells to grow for 48 hours.
8. Replace media with selection media.
9. Continue feeding cells selection media until untransduced cells have been removed.

Analysis

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

Appendices

Appendix 1a – pZIP-mCMV vector information

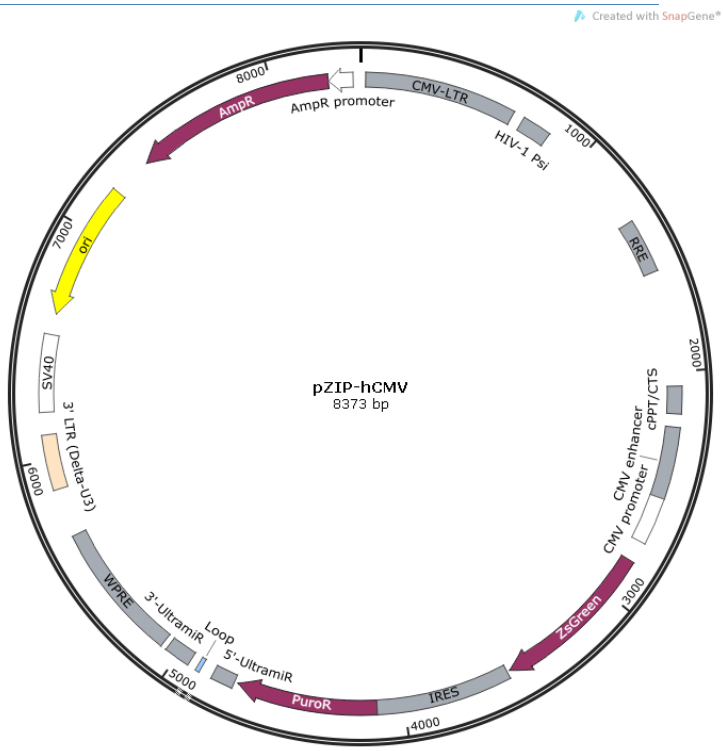


Element	Start	Stop
CMV-LTR	23	667
Psi	715	840
RRE	1337	1570
cPPT/CTS	2062	2178
mCMV	2219	2755
ZsGreen	2764	3459
IRES	3471	4047
PuroR	4048	4650
Ultramir5'	4663	4748
3'-UltramiR	4875	4986
WPRE	5009	5597
3' LTR	5805	6038
SV40	6134	6463
ori	6552	7137
AmpR	7308	8168

Figure 7a: Detailed map of the pZIP-mCMV vector and vector element table.

The full sequence is available here.

Appendix 1b – pZIP-hCMV vector information

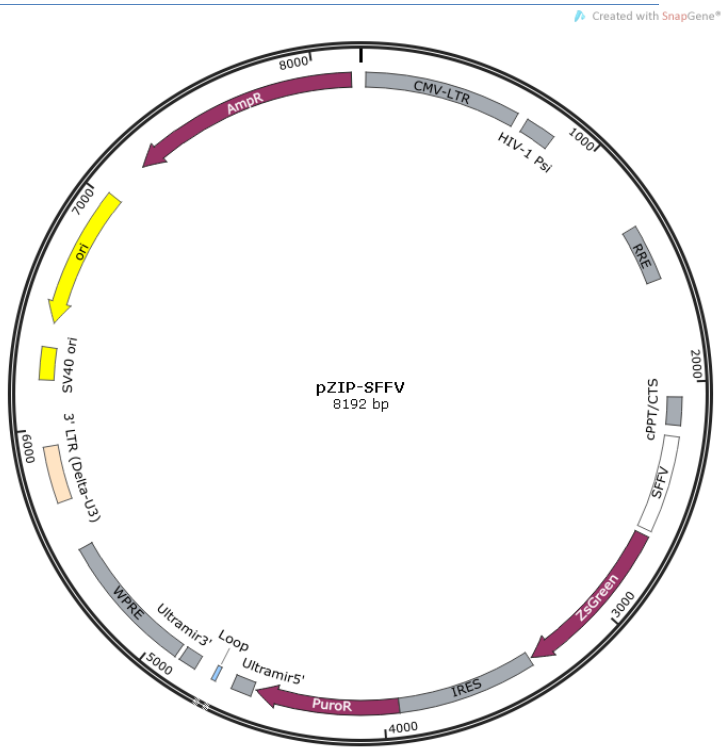


Element	Start	Stop
CMV-LTR	23	667
Psi	715	840
RRE	1337	1570
cPPT/CTS	2062	2178
CMV enhancer	2235	2538
CMV promoter	2539	2742
ZsGreen	2833	3528
IRES	3540	4116
PuroR	4117	4719
5'-UltramiR	4737	4831
Loop	4887	4904
3'-UltramiR	4944	5055
WPRE	5078	5666
3' LTR	5874	6107
SV40	6203	6532
ori	6621	7206
AmpR	7377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

Figure 7b: Detailed map of the pZIP-hCMV vector and vector element table.

The full sequence is available here.

Appendix 1c – pZIP-SFFV vector information

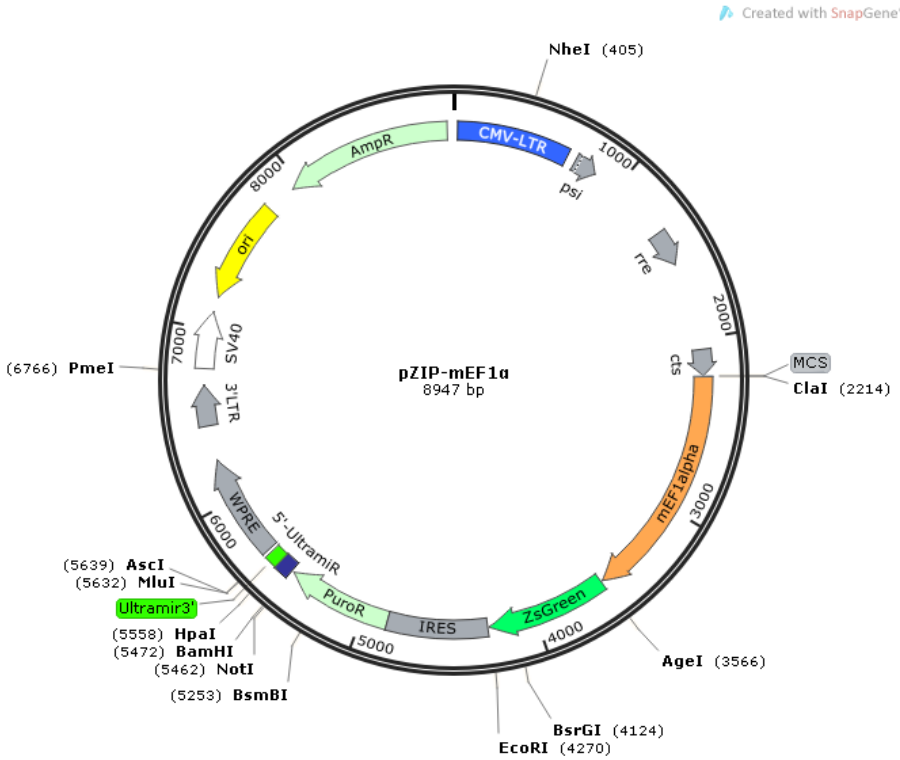


Element	Start	Stop
CMV-LTR	23	667
Psi	715	840
RRE	1337	1570
cPPT/CTS	2062	2178
SFFV	2225	2625
ZsGreen	2652	3347
IRES	3359	3935
PuroR	3936	4538
Ultramir5'	4551	4636
shRNA	4666	4762
Ultramir3'	4804	4877
WPRE	4897	5485
3' LTR	5693	5926
SV40 ori	6202	6337
ori	6440	7025
AmpR	7196	8155

Figure 7c: Detailed map of the pZIP-SFFV vector and vector element table.

The full sequence is available here.

Appendix 1d – pZIP-mEF1-alpha vector information

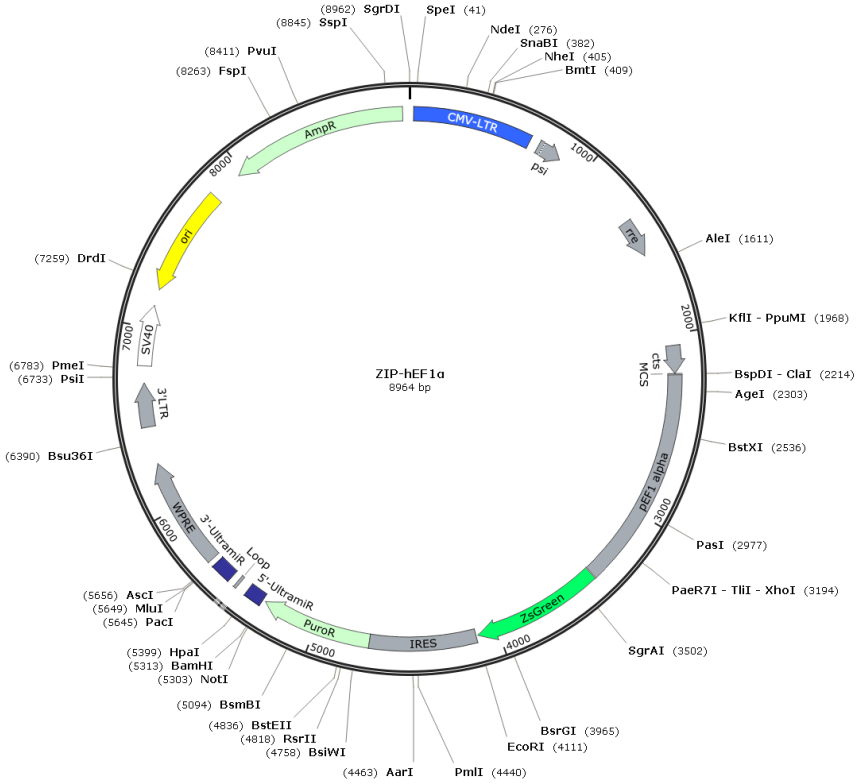


Element	Start	Stop
CMV-LTR	23	667
Psi	720	856
RRE	1348	1552
CTS	2062	2212
MCS	2213	2214
mEF1alpha	2219	3566
ZsGreen	3574	4269
IRES	4281	4857
PuroR	4858	5460
Ultramir5'	5478	5558
Ultramir3'	5559	5632
WPRE	5652	6243
3' LTR	6448	6683
SV40 Ori	6777	7106
Ori	7195	7780
AmpR	7951	8910

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**.
   b. Only analyze wells that have < 20% of cells transduced to ensure none of the cells have been transduced with more than one viral particle.

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

Appendix 4 - siRNA for increased virus production

We recommend using Qiagen to order siRNA targeting Pasha/DGCR8. Please obtain an order form from Qiagen's web site and use the following information to complete the form:

siRNA1	
Choose a name for your siRNA sequence:	**DGCR8**
21 base DNA target sequence:	**5' CGGGTGGATCATGACATTCCA 3'**
Overhang:	**Standard DNA ends**
Amount:	20 nmol (~250 µg, one tube)
Modification (one per duplex)**:**	**3' end, sense strand** **OR** **5' end, sense strand**
	None (default)

Appendix 5 – 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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