shERWOOD UltramiR shRNA Lentiviral Target Gene Set

Format: Glycerol stock or Lentiviral particles

This manual provides information for the propagation, transfection, viral packaging, and transduction of **shERWOOD UltramiR shRNA Target Gene Sets** in pZIP vectors. Appendix 1 contains information regarding how to locate the specific vector map for your shRNA constructs and Appendix 2 contains basic safety information for production and handling of lentiviral particles. Review local safety guidelines for complete regulations.

Section 1: Introduction-shERWOOD Design

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 (**Figure 1**). Based on the functional testing of 250,000 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.

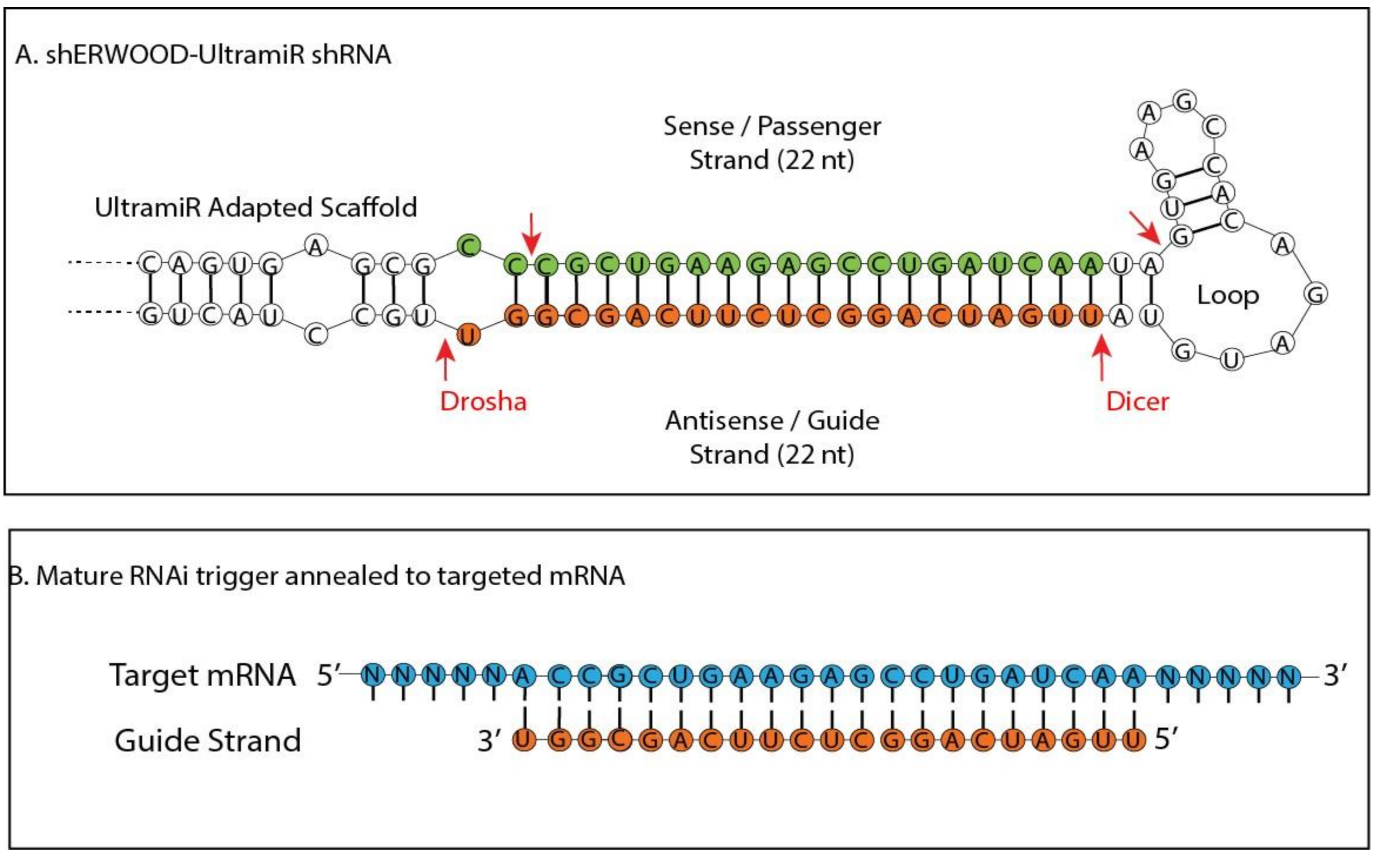


Figure 1. Schematic of shERWOOD-UltramiR shRNA. (A) Passenger (green) and Guide (orange) strand are shown with Dicer and Drosha nuclease cleavage sites are in red. (B) The final step of shRNA processing loads the Guide Strand (orange) into the RISC complex which associates with the target mRNA (blue)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

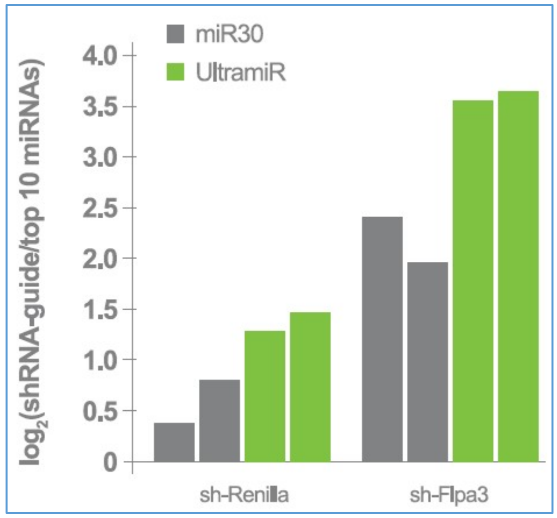


Figure 2. Relative abundances of processed guide sequences for two shRNA as determined by small RNA cloning and NGS analysis when cloned into traditional miR30 and UltramiR scaffolds. Values represent log-fold enrichment of shRNA guides with respect to sequences corresponding to the top 10 most highly expressed endogenous microRNA.

Section 2: Vector information

shERWOOD UltramiR shRNA allow the stable delivery of the shRNA into host cells via a replication-incompetent lentivirus.

Available Vector Options:

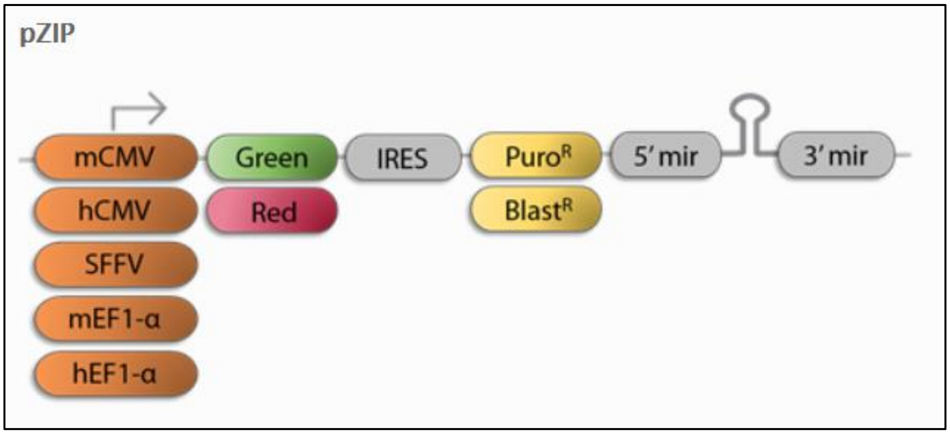
- Constitutive or inducible vectors*
- Antibiotic selection: puromycin or blasticidin
- Fluorescent reporters (ZsGreen or turboRFP)
- mCMV, hCMV, hEF1-alpha, mEF1-alpha, SFFV, and inducible TRE3G*promoter options
  **Refer to Appendix 4 for notes regarding inducible vectors*

Available formats:

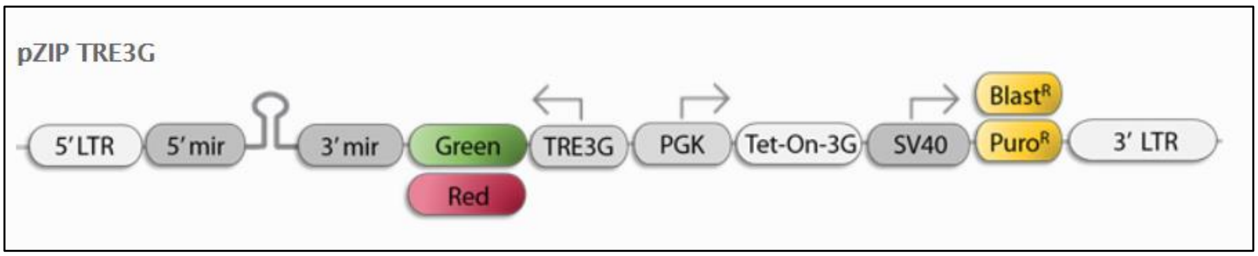
- **Bacterial Glycerol Stock**
- **Viral Particles**

Lentiviral shRNA are provided in the pZIP-mCMV, pZIP-hCMV, pZIP-hEF1-alpha, pZIP-mEF1-alpha, pZIP-SFFV, and inducible pZIP-TRE3G* vectors (**Figure 3**). The protocols refer to pZIP as a general reference to all. Detailed vector graphics are provided in Appendix 1**.**

The pZIP vectors express the fluorescent marker and shRNA on the same transcript allowing the level of fluorescence in the cell to be used as a direct indication of shRNA expression through visual inspection.



pZIP Constitutive Lentiviral Vectors



pZIP Inducible Lentiviral Vectors

Function	Element
Promoter choice	Human CMV (hCMV), human EF1-alpha (hEF1-alpha), mouse CMV (mCMV), mouse EF1-alpha (hEF1-alpha), Spleen Focus Forming Virus (SFFV), and TRE3G inducible*
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 (Puro^R) Blasticidin resistance gene (Blast^R)
shRNA	shERWOOD-UltramiR shRNA showing 5' and 3' flanking UltramiR sequences

Figure 3. Schematic of ZIP lentiviral shRNA vectors. All constitutive vector elements are identical with the exception of the promoter, selection, and fluorescent marker. (LTRs are not shown.) ***Refer to** Appendix 4 for notes regarding inducible vectors

Section 3: Propagation protocols for glycerol stocks

Materials for propagation

LB-Lennox Broth (low salt)	VWR 10128-266
Glycerol	VWR EM-4760
Carbenicillin (or Ampicillin)	VWR 97063-144

Propagate culture for storage

shERWOOD UltramiR 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. A 4 ml starter culture can be inoculated using 5 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. It is a good practice to streak isolate and quality control test the plasmids by sequencing or restriction digest.

Section 4: Plasmid preparation

For transfection and transduction experiments, the **shERWOOD UltramiR shRNA** 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 37°C* overnight or until the culture appears turbid. A 4 ml starter culture can be inoculated using 5 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.

***Note: The temperature for propagation is 30°C while the temperature for plasmid preparation is 37°C**

Section 5: Selection kill curve

The **shERWOOD UltramiR shRNA** have a puromycin or blasticidin resistance marker for selection in mammalian cells. Once transfection/transduction has occurred, the cells can be treated to select for cells expressing antibiotic resistance. Since cell lines differ in their sensitivity to antibiotics, the optimal concentration (pre-transfection/transduction) should be determined. In the following protocol the lowest concentration that provides adequate selection is determined for the experimental cell line. If using an inducible vector, a doxycycline optimization curve also needs to be performed.

Materials

- Complete media for experimental cell line
    - Blasticidin S HCl antibiotic (Life Technologies, Catalog# A11139-03) (1.25 µg/µl stock solution)
    - Puromycin Dihydrochloride (Life Technologies, Catalog# A11138-03) (1.25 µg/µl stock solution)
- 24-well tissue culture plate

Puromycin and blasticidin have a similar range of concentration that is toxic to most cell lines. The same kill curve can be used for both as shown in the example in **Table 1**.

Equipment

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

Protocol

1. Make a 1.25 µg/µl stock solution of puromycin or blasticidin

2. Plate 5 x 10^4 cells per well in 11 wells of a 24-well tissue culture plate using media without antibiotics.
3. Prepare antibiotic dilutions in culture media for titration as shown in **Table 1** below:

Volume of Puromycin or Blasticidin 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

Table 1. Example dilutions and volumes required for establishing optimal antibiotic concentration for puromycin and blasticidin

4. Begin antibiotic selection the following day by replacing antibiotic free media with media containing the appropriate concentrations of antibiotic.
5. Incubate cells with 5% CO_2 at 37°C, or use conditions normal for your target cells.
6. Check cells daily to estimate rate of cell death.
7. Replenish the media containing the appropriate concentrations of antibiotic every 2 days for 6 days.

Note: The optimal antibiotic 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.

Section 6: 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 transfection 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 doubling time.

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

Materials

- 24-well tissue culture plates
- Transfection Reagent (Examples: Lipofectamine®, Fugene®)
- Cell culture complete medium for maintenance and passaging of experimental cell line (including serum and supplements)
- Antibiotic-free complete medium for maintenance and passaging of experimental cell line (including serum or supplements) without antibiotics such as pen-strep.-(i.e. Opti-MEM® I-Gibco Catalog # 51985034)
- Puromycin Dihydrochloride (Life Technologies, Catalog# A11138-03)
- Blasticidin S HCl antibiotic (Life Technologies, Catalog# A11139-03)
- Sterile 1.5 ml microfuge tubes
- Assays for assessing knockdown (e.g. Surveyor Assay, sequencing, etc.)

Equipment

- Automatic pipette/Pipette-aid (for tissue culture)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Fluorescent microscope

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. **Transfection reagent preparation**: In a separate microfuge tube, add 1 µL of transfection reagent 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 transfection 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.*

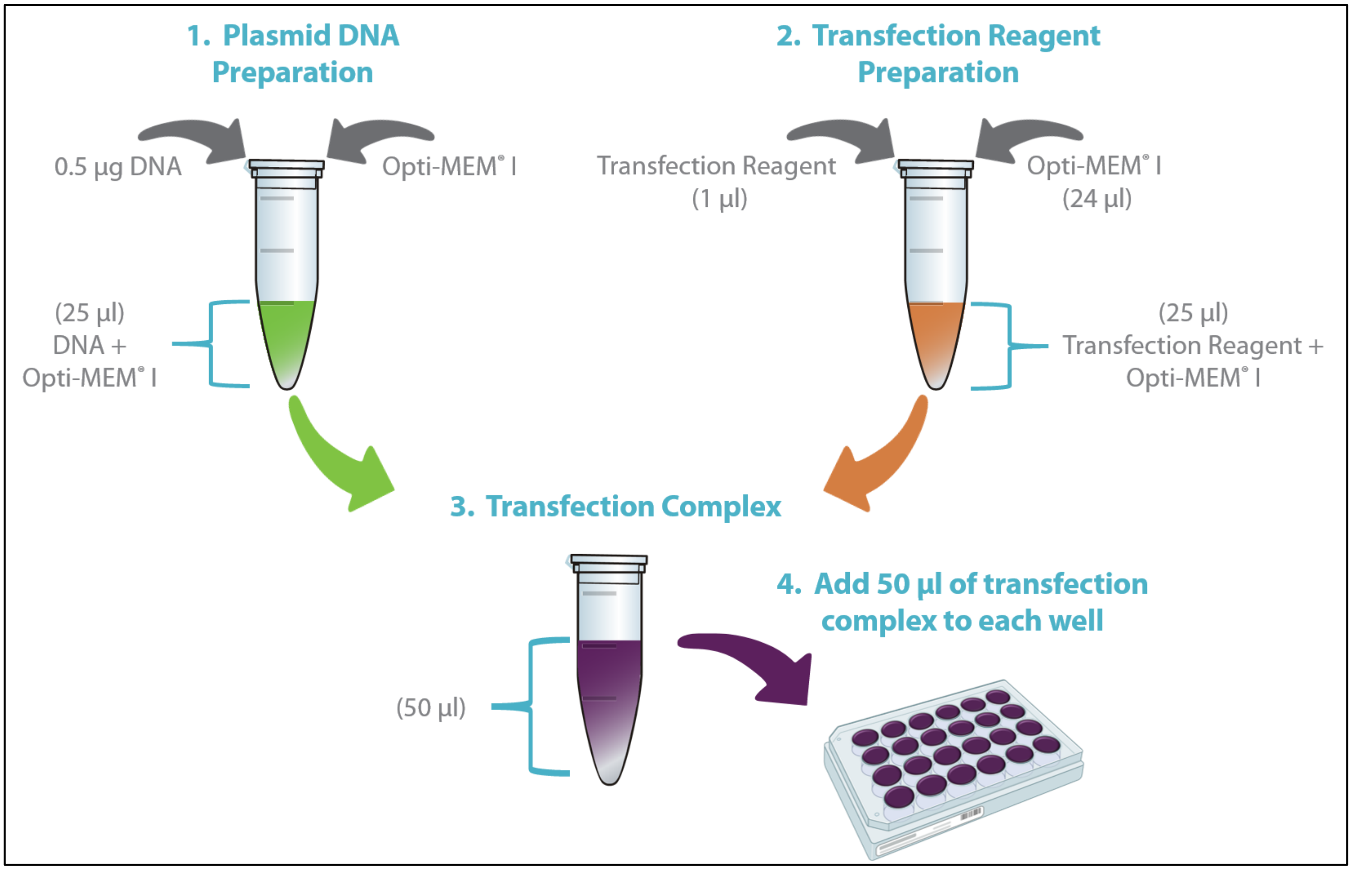


Figure 4: Transfection protocol for 24 well plates (volumes indicated are per well). To transfect the entire plate multiply all volumes and DNA amount by 24.

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

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

† Total volume of the transfection complex is made up of equal parts of DNA solution and transfection reagent.

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 transfection reagent 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 to transfection reagent (at 1 µg DNA:2 µl transfection reagent) 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 transfection 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.

Antibiotic selection of transfected cells

Use antibiotic selection to reduce background from untransfected cells. Refer to the protocol for the antibiotic kill curve in section 5 to determine the optimal concentration for each cell line.

1. Begin the antibiotic selection by replacing the medium with complete medium supplemented with the appropriate antibiotic.
2. 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 gone within 3-5 days.
3. Collect samples for assay.

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

Fluorescence selection of transfected cells

To assay for a fluorescent protein, incubate for **24-72 hours** following transfection and then examine the cells microscopically for fluorescence expression. Sort the cells based on level of fluorescence and select the highest expressing population.

Section 7. Packaging lentiviral particles

Some cell lines are resistant to transfection. Lentiviral particles offer an alternative delivery method. The **shERWOOD UltramiR shRNA** can be packaged into lentiviral particles for efficient delivery into target cell lines. Constitutive vectors may be packaged with 2nd, 3rd, or 4th generation packaging plasmids. Inducible vectors must be packaged with 2nd or 4th generation packaging plasmids as they are Tat-dependent.

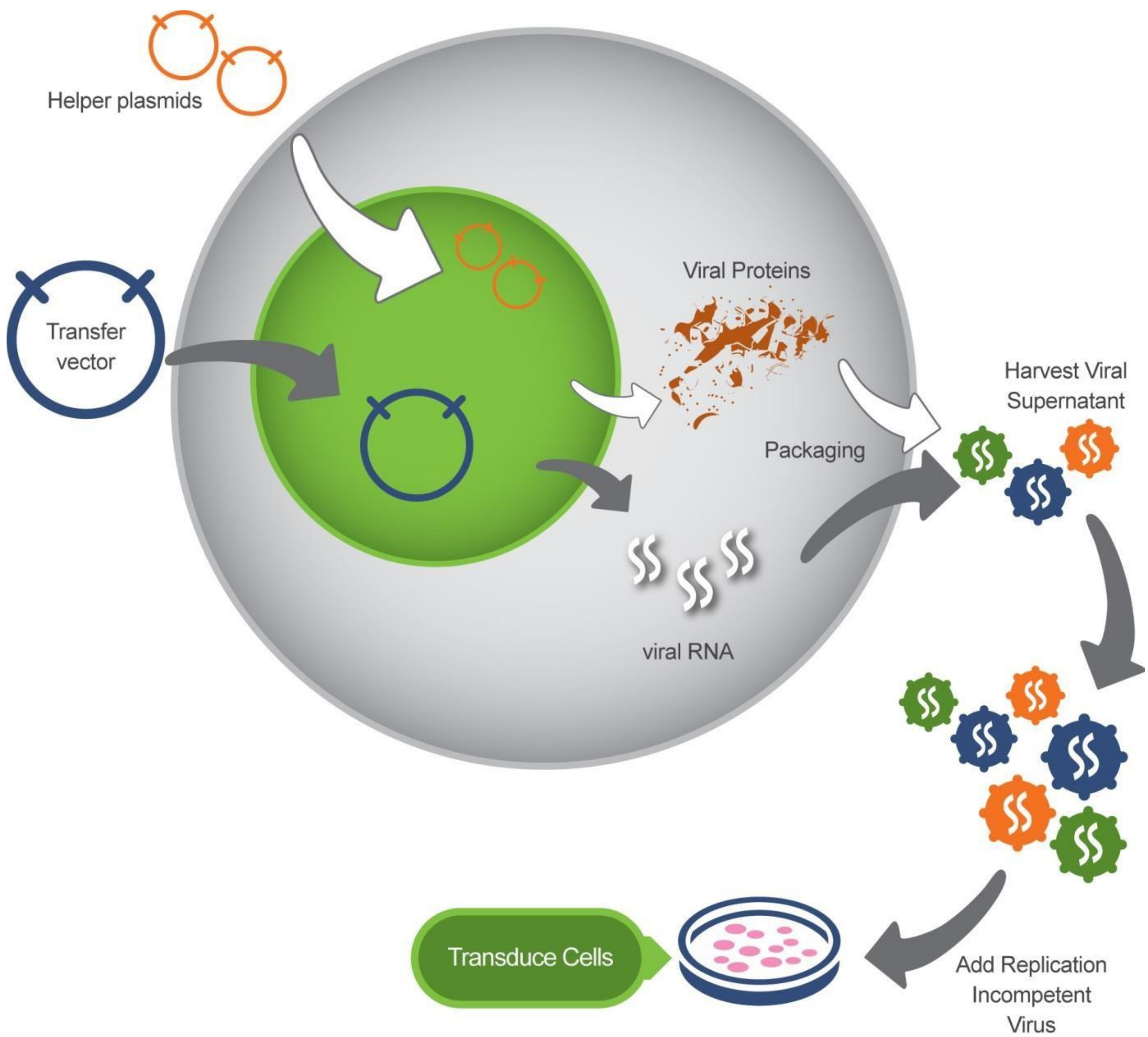


Figure 5. Schematic depicting lentiviral packaging of pZIP lentiviral vectors

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 is co-transfected with the desired packaging vectors (helper plasmids) 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 targeted 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 pZIP shRNA lentiviral vectors:

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. Refer to Section 8 to determine the functional viral titer.

Note: The Non-Targeting Control should be used to determine the packaging and transduction efficiency of the target cell used.

Materials

- pCMV-dR8.2 Packaging Plasmid (Addgene, Plasmid 8455) (2^{nd} generation)
- pCMV-VSVG Envelope Plasmid (Addgene, Plasmid 8454)
- 6-well tissue culture plate
- HEK293T cells
- Complete cell culture medium – (DMEM supplemented with 10% fetal calf serum, 1X L-Glutamine, and 1X Pen-Strep)
- Antibiotic-free complete medium – (DMEM supplemented with 10% fetal calf serum, 1X L-Glutamine)
- Transfection Reagent
- OPTI-MEM® I + GlutaMAX Reduced Serum Media (Gibco, Catalog # 51985-034)
- Sterile 1.5 ml microfuge tubes

Equipment

- Automatic pipette/Pipette-aid (for tissue culture)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

Protocol

1. Plate your target cells and HEK293T cells 18-24 hours prior to transduction in a 6-well plate. Plate at a density of 800,000 to 1,000,000 cells per well in 2 ml complete cell culture medium. It is important to seed enough cells so that the cell confluency ranges between 70 and 80% at the time of transfection.
2. Incubate overnight with 5% CO_2 at 37°C.
3. Two hours prior to transfection, remove the culture media and replace with 2 ml fresh, antibiotic-free culture medium.
4. Preparation of **shERWOOD UltramiR shRNA** plasmids and lentiviral vector packaging mix for transfection (note, all plasmids are re-suspended in dH_2O):
   - Transfer vector (**shERWOOD UltramiR shRNA**) – dilute plasmid to 0.2 µg/µl
   - Lentiviral packaging mix (0.5 µg/µl):
     - 100 µl pCMV-dR8.2 (0.5 µg/µl)
     - 50 µl pCMV-VSVG (0.5 µg/µl)
5. Just prior to transfection, allow transfection reagent and OPTI-MEM® I to come to room temperature.
6. Plasmid DNA preparation:
   - Add 5 µl of **shERWOOD UltramiR shRNA** (1.0 µg) and 3 µl lentiviral packaging mix (1.5 µg) in a sterile microfuge tube containing OPTI-MEM© I Reduced Serum Media to a total volume of 100 µl.

7. Transfection reagent preparation: In a separate microfuge tube, add 5 μl of transfection reagent into 95.0 μl OPTI-MEM© I Reduced Serum Media for a total volume of 100 μl.
8. Final transfection complex: Transfer the diluted DNA solution to the diluted transfection reagent (total volume = 200 μl. Mix gently and incubate at room temperature for 10 minutes.
9. Add the 200 μl of transfection complex to each well containing HEK293T cells and medium.
10. Incubate cells at 37°C in a CO_2 incubator.
11. Collect viral particles (supernatant) 48-60 hours post-transfection.
12. Clarify supernatant by low-speed centrifugation (800xg) for 10 minutes using a tabletop centrifuge.

Aliquot supernatant into sterile cryovials and store at -80°C. *Note: 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.*

Section 8. 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. Antibiotic selection may be used to remove untransduced cells. A kill curve should be performed as described in Section 5 in this product manual.

Increasing transduction efficiency:

Optimizing transduction conditions can extend the utility of viral particles and limit cell toxicity. Several 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 positive colonies.
- If the packaging protocol was followed in Section 7, use the titering aliquots made to determine the titer.
- Transduction optimization should be done with empty vector control viral particles.
- HEK293T cells are readily transduced under standard conditions and are included in the protocol as a positive control for transduction.

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 (Harvested or purchased)
- Sterile Microcentrifuge tubes
- Polybrene
- Puromycin Dihydrochloride (Life Technologies, Catalog# A11138-03) for selection
- Blasticidin S HCl antibiotic (Life Technologies, Catalog# A11139-03)
- **For Inducible vectors only**: Doxycycline (1.0-2.0 µg/ml as determined in kill curve)

Equipment

- Automatic pipette /Pipette-aid (for tissue culture)
- Pipette (for dilutions and handling of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

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 and 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 7×10^4 cells per well in 12 wells for each cell line with complete media (see **Figure 6**). Incubate overnight with 5% CO_2 at 37°C. It is important to seed enough cells so that the cell confluency ranges between 30 and 40% at the time of transduction.
2. Prepare a serial dilution series with serum free media and viral supernatant as shown in **Table 3** and **Figure 6**.
    a. Serial dilutions can be set up in a sterile 96-well plate or in sterile micro centrifuge tubes. The number of wells or tubes needed depends on the expected titer of the viral particles (generally 5-8 wells/tubes). The higher the expected titer, the more wells/tubes needed for the dilutions.
    b. Make Dilution Media by taking serum-free cell culture media and adding Polybrene to a final concentration of 5-8 µg/ml.
    c. Add 80 µl of Dilution Media to Tube/Well 1 and then 160 µl of Dilution Media to each remaining tube (Tubes/Wells 2-5).
    d. Add 20 µl of viral particles to Tube/Well 1 and mix well by gently pipetting up and down (10 - 15 times) without creating bubbles, and discard the tip.
    e. Transfer 40 µl from Tube/Well 1 to Tube/Well 2. Mix well and discard the tip.
    f. Transfer 40 µl from tube 2 to tube 3. Mix well and discard the tip.
    g. Repeat the procedure for the remaining tubes.
    h. Incubate at room temperature for 10-15 minutes.
3. Remove media from each well.
4. Add 200 µl of culture media containing 1% serum to each well containing cells.
5. Add 25 µl from each viral dilution to two wells for each cell line (225 µl final volume) for a total of 10 wells per cell line. The remaining 4 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 containing the appropriate antibiotic and allow cells to grow for 72-96 hours.
    a. **Note**: Add 1.0-2.0µg/ml doxycycline for inducible vectors ONLY

9. Colony counting: (**Note**: Counting 50-200 colonies in a well is sufficient to provide accurate titers.)
   a. Antibiotic titering by selection and colony counting:
      i. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal concentration determined in "kill curve"
      ii. 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.
      iii. Use a microscope to count the number of surviving colonies.
   b. Fluorescent colony counting
      i. Replace the viral supernatant with complete media (including doxycycline for inducible vectors) and allow growth for 48 hours.
      ii. Count the number of colonies expressing the fluorophore. A colony consisting of multiple cells should be counted as a single transduction event.

10. Use the calculation below and **Table 3** to determine functional titer. (Alternate methods for calculating are described in Appendix 3.)

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

Example:
If the average number of colonies counted in well A5 and B5 is 70 the titer is calculated as follows:
70 colonies X 3125 ÷ 0.025 ml = 8.75 X 10^6 TU/ml

Dilution table and schematic for titration protocol

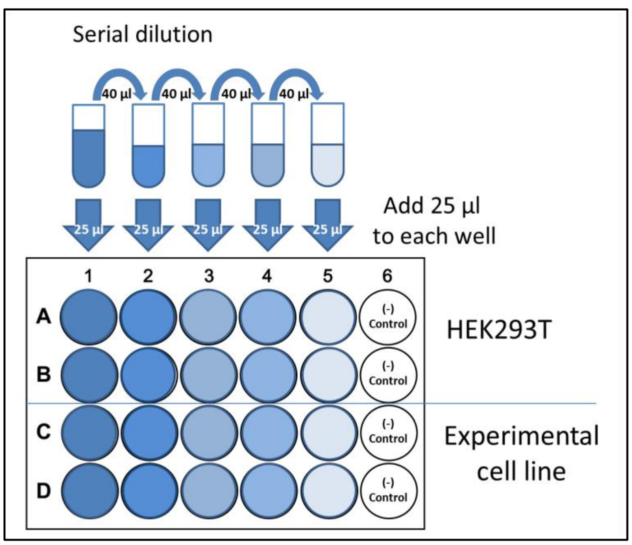


Figure 6. Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

Table 3. Dilution factors for calculating viral titer

Tube/Well	Viral particles	Dilution medium	Dilution factor
1	20 µl (from virus aliquot)	80 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 unconcentrated virus production will yield 1-5 x 10^5 TU/ml. The expected number of fluorescent colonies for a viral titer of 5 x 10^5 TU/ml 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	100,000	20,000	4,000	800	160	0
ml transduced cells	0.025	0.025	0.025	0.025	0.025	0
Fluorescent colonies expected	2500	500	100	20	4	0

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

Section 9: Determining Multiplicity of Infection (MOI)

After the functional titer has been determined in the experimental cell line, the volume of virus required for a particular multiplicity of infection (MOI) can be calculated. The MOI is the number of transducing units per cell in a culture. The necessary MOI needed is dependent on the cell line being used and can vary widely.

Calculating volume of viral particles for a given MOI

Calculate the total number of transducing units (TU_{total}) that would be added to a well for a given MOI with the following equation:

TU_{total} = (MOI x Cell Number)/Viral titer (TU/µl)

Where:

- MOI = the desired MOI in the well (units are TU/cell)
- Cell number = number of cells in the well at the time of transduction

For example, if the experiment requires:

- MOI of 10 (highest MOI)
- Cell density of 10,000 cells per well at time of transduction
- Viral Titer is 1 x 10^7 TU/ml

Then, TU_{total} per well is calculated:

TU_{total} = (10 TU/cell) x (10,000 cells/well)] / 1 x 10^4 TU/µl = 10 µl of viral stock/well.

Therefore, the volume of viral particles with a titer of 1x10^7 TU/ml required for an MOI of 10 is 10 µl per well.

Protocol for determining optimal MOI

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 appropriate antibiotic

Materials

- Experimental cells
- Complete media for experimental cell line
- Serum free media for each cell line
- 24-well tissue culture plate
- Lentiviral particles
- Sterile Microcentrifuge tubes
- Polybrene
- Doxycycline (for inducible vectors ONLY)

Equipment

- Automatic pipette /Pipette-aid (for tissue culture)
- Pipette (for dilutions and handling of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Fluorescent microscope with appropriate filter

Protocol

The following protocol represents the standard procedure followed for determining optimal MOI in HEK293T cells. Cell numbers, serum and polybrene concentrations, times, and culture conditions are likely to be different for the experimental cell line. The optimal conditions for a target cell line can be determined using the protocol for functional titer and transduction optimization (Section 8).

1. Plate cells 18-24 hours prior to transduction in a 24 well plate with complete media. Plate at a density of cells so that the cell confluency ranges between 30 and 40% at the time of transduction.
2. Incubate overnight with 5% CO_2 at 37°C.
3. Prepare viral particles:
   a. Set up 8 sterile microcentrifuge tubes and label two tubes each with MOI. For example: 1, 2, 5, and 10.
   b. Add 50 µl of medium containing 1% serum and appropriate level of Polybrene.
   c. Add the volume of viral stock that corresponds to the MOI (use the calculation above for determining volume for the desired MOI).
   d. Bring volume in each tube up to 100 µl with medium containing 1% serum and appropriate level of Polybrene.
   e. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
   f. Incubate for 10 minutes at room temperature.
4. While viral particles are incubating, remove media from cells in each well.
5. Add to each well 125 µl of 1% serum media containing NO Polybrene.

6. After the 10-minute incubation, transfer all (100 µl) of virus from tubes to the corresponding wells (225 µl final volume) for a total of 8 wells (two well for each MOI). The remaining wells (without viral particles) should be evaluated as negative controls.
7. Rock plate gently a few times to distribute the viral particles across the well.
8. Incubate overnight with 5% CO_2 at 37°C (12-24 hours).
9. Replace the viral supernatant with complete media
    a. **For inducible vectors ONLY**, include 1.0 - 2.0 µg/ml doxycycline for one set of MOIs (induced) and no doxycycline for the other set (non-induced)).
10. Incubate cells in culture for 72-96 hours.
11. Using a fluorescent microscope, assess fluorescent expression in the wells.
    a. **For inducible vectors**: Choose an MOI that results in a high level of induction (bright fluorescent expression in the induced set) and low level of leakiness (no or faint fluorescent expression in the non-induced set).

Note: levels of fluorescent protein expression will vary greatly across a culture due to random integration of lentiviral vectors into regions of the chromosomes with varying levels of transcriptionally active and non-active states.

Section 10: 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 antibiotic

Required materials

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

Equipment

- Automatic pipette/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 30 - 40% 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 8 for information on transduction optimization.

5. Incubate cells 12 - 24 hours in transduction media.
6. Replace transduction media with complete media (no selection reagent).

Note: to improve transduction for non-adherent cells, cells can be moved to a round bottom tube and incubated with rotation. Rotation allows the cells and viral particles to come into contact.

Antibiotic 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.

Induction (Applies only to inducible vectors)

10. Induce expression using 1.0 – 2.0 µg/ml of doxycycline. Allow cells to culture for 48-96 hours post induction. If cells need passaging during this incubation period, maintain the same concentrations of the appropriate selection antibiotic and doxycycline.

Analysis

11. Analyze knockout efficiency in population. Determine cellular phenotype or harvest cell for gene expression analysis according to your experimental design.

Section 11: Selection after transduction - Enrichment for increased knockdown

After 24-48 hours, transduced cells can be selected using antibiotic resistance or fluorescent protein expression. **shERWOOD UltramiR Lentiviral Pooled shRNA pZIP vectors** express a fluorophore (ZsGreen or RFP) and mammalian selection marker (puromycin or blasticidin), depending on the vector chosen. Antibiotic selection ensures the removal of untransduced cells. Using FACS analysis to select for cells with highest fluorescent protein expression can further enrich for the population of cells with the highest frequency of gene knockdown.

Antibiotic selection: Refer to the protocol for the puromycin kill curve in section 5 to determine the optimal concentration for each cell line.

1. Incubate for 24-72 hours following transduction and then examine the cells microscopically for growth.
2. Begin the antibiotic selection by replacing the medium with complete medium supplemented with the appropriate antibiotic.
3. Replace the selective media every 2-3 days. Monitor the cells daily and observe the percentage of surviving cells.
    a. All untransduced cells should be removed within 3-5 days.
4. Collect samples for assay.

Fluorescence analysis: To assay for a fluorescent protein, incubate for 24-72 hours following transduction and then examine the cells microscopically for fluorescence expression. Sort the cells based on level of fluorescence and select the highest expressing population.

Appendix 1-Vector information

Full vector sequences and maps are available at https://www.transomic.com/cms/Product-Support/Vector-Maps-and-Sequences/CRISPR-Vector-Maps.aspx

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 (https://osp.od.nih.gov/wp-content/uploads/2014/01/Lenti_Containment_Guidance_0.pdf)

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 - Methods for titering

1. Antibiotic titering by selection and colony counting:
    a. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal concentration determined in the previously performed "kill curve"(Section 5)

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. Fluorescent 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.

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

Appendix 4 - Notes regarding inducible vectors

Our inducible vectors use an all-in-one TRE3G system that allows the Tet promoter and transactivator to be present on the same vector, thus allowing for less manipulation of the cells. The inducible vectors include the TRE3GS inducible promoter positioned upstream of the gene coding sequence, and the Tet-On 3G transcriptional activator (Tet-On 3G TA), which is expressed constitutively from an internal promoter. The Tet-On 3G TA binds to the TRE3GS promoter in the presence of doxycycline and induces expression of the gene plus any associated fluorophores and/or tags. The inducible vectors must be packaged using a 2nd or 4th generation packaging line since the vectors are Tat dependent. We use a 2nd generation packaging line for viral production.

Considerations for transduction and inducible expression

Prior to inducible gene expression experiments, perform a doxycycline kill curve in your cell line to optimize the concentration of doxycycline to ensure there is enough for induction but not so much that is toxic to the cell. Also ensure that you perform a kill curve for any selectable markers present in the vector being used.

It is important to determine the optimal MOI for inducible expression vectors to ensure that the level induction is high while the level of leakiness is low. Fluorescent expression can be used to assess both induction and leakiness.

To determine the optimal MOI, cells will be transduced at different MOIs: MOI = 1, MOI = 2, MOI = 5, and MOI = 10. Two wells will be transduced per MOI: one well that will be induced with doxycycline (to assess level of induction); and one well that is not treated with doxycycline (to assess level of leakiness).

Calculating volume of viral particles for a given MOI

Calculate the total number of transducing units (TU_{total}) that would be added to a well for a given MOI with the following equation:

TU_{total} = (MOI x Cell Number)/Viral titer (TU/µl)

Where:

- MOI = the desired MOI in the well (units are TU/cell);
- Cell number = number of cells in the well at the time of transduction

For example, if the experiment requires:
- MOI of 10 (highest MOI)
- Cell density of 10,000 cells per well at time of transduction
- Viral Titer is 1×10^7 TU/ml

Then, TU_{total} per well is calculated:

TU_{total} = (10 TU/cell) x (10,000 cells/well)] / 1×10^4 TU/µl = 10 µl of viral stock/well.

Therefore, the volume of viral particles with a titer of 1×10^7 TU/ml required for an MOI of 10 is 10 µl per well.

Protocol for determining optimal MOI for inducible vectors

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 appropriate antibiotic

Materials

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

Equipment

- Automatic pipette/Pipette-aid (for tissue culture)
- Pipette (for dilutions and handling of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Fluorescent microscope with appropriate filter

Protocol

The following protocol represents the standard procedure followed for determining optimal MOI in HEK293T cells. Cell numbers, serum and polybrene concentrations, times, and culture conditions are likely to be different for the experimental cell line. The optimal conditions for a target cell line can be optimized following the protocol for determining the relative transduction efficiency (Section 8) while varying transduction conditions.

1. Plate cells 18-24 hours prior to transduction in a 24 well plate with complete media. Plate at a density of cells so that the cell confluency ranges between 40 and 50% at the time of transduction.
2. Incubate overnight with 5% CO_2 at 37°C.
3. Prepare viral particles:
   a. Set up 8 sterile microcentrifuge tubes and label two tubes each with MOI. For example: 1, 2, 5, and 10.
   b. Add 50 µl of medium containing 1% serum and appropriate level of Polybrene.
   c. Add the volume of viral stock that corresponds to the MOI (use the calculation above for determining volume for the desired MOI).
   d. Bring volume in each tube up to 100 µl with medium containing 1% serum and appropriate level of Polybrene.
   e. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
   f. Incubate for 10 minutes at room temperature.
4. While viral particles are incubating, remove media from cells in each well.
5. Add to each well 125 µl of 1% serum media containing NO Polybrene.
6. After the 10-minute incubation, transfer all (100 µl) of virus from tubes to the corresponding wells (225 µl final volume) for a total of 8 wells (two well for each MOI). The remaining wells (without viral particles) should be evaluated as negative controls.
7. Rock plate gently a few times to distribute the viral particles across the well.
8. Incubate overnight with 5% CO_2 at 37°C (12-24 hours).
9. Replace the viral supernatant with complete media containing 1.0 - 2.0 µg/ml doxycycline for one set of MOIs (induced) and no doxycycline for the other set (non-induced). Incubate cells in culture for 72-96 hours.
10. Using fluorescent microscope, assess fluorescent expression in the induced and non-induced wells. Choose an MOI that results in a high level of induction (bright fluorescent expression in the induced set) and low level of leakiness (no or faint fluorescent expression in the non-induced set).

Note: Levels of fluorescent expression will vary greatly across a culture due to random integration of lentiviral vectors into regions of the chromosomes with varying levels of transcriptionally active and non-active states.

Turning expression off

Note: Doxycycline adheres to many cells and culture plates. Turning gene expression and/or fluorescence off after doxycycline induction requires cell to be split, rinsed and transferred to a new plate or well that has not been exposed to tetracycline.

Protocol

1. Wash the cells in PBS.
2. Split cells and transfer to new plate using doxycycline-free media.
3. After splitting the cells into fresh media without doxycycline, incubate for 3 hours.
4. When cell are adherent, rinse cell with PBS three times.
5. Continue to feed cells with doxycycline-free media.

Note: It may take time for fluorescent proteins to dissipate, typically within 72 hours. Expression of the target gene may return to expected levels prior to this.

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