shERWOOD UltramiR Retroviral shRNA (pLMN)

Bacterial glycerol stock format

TRHSU2000, TRMSU2000

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.

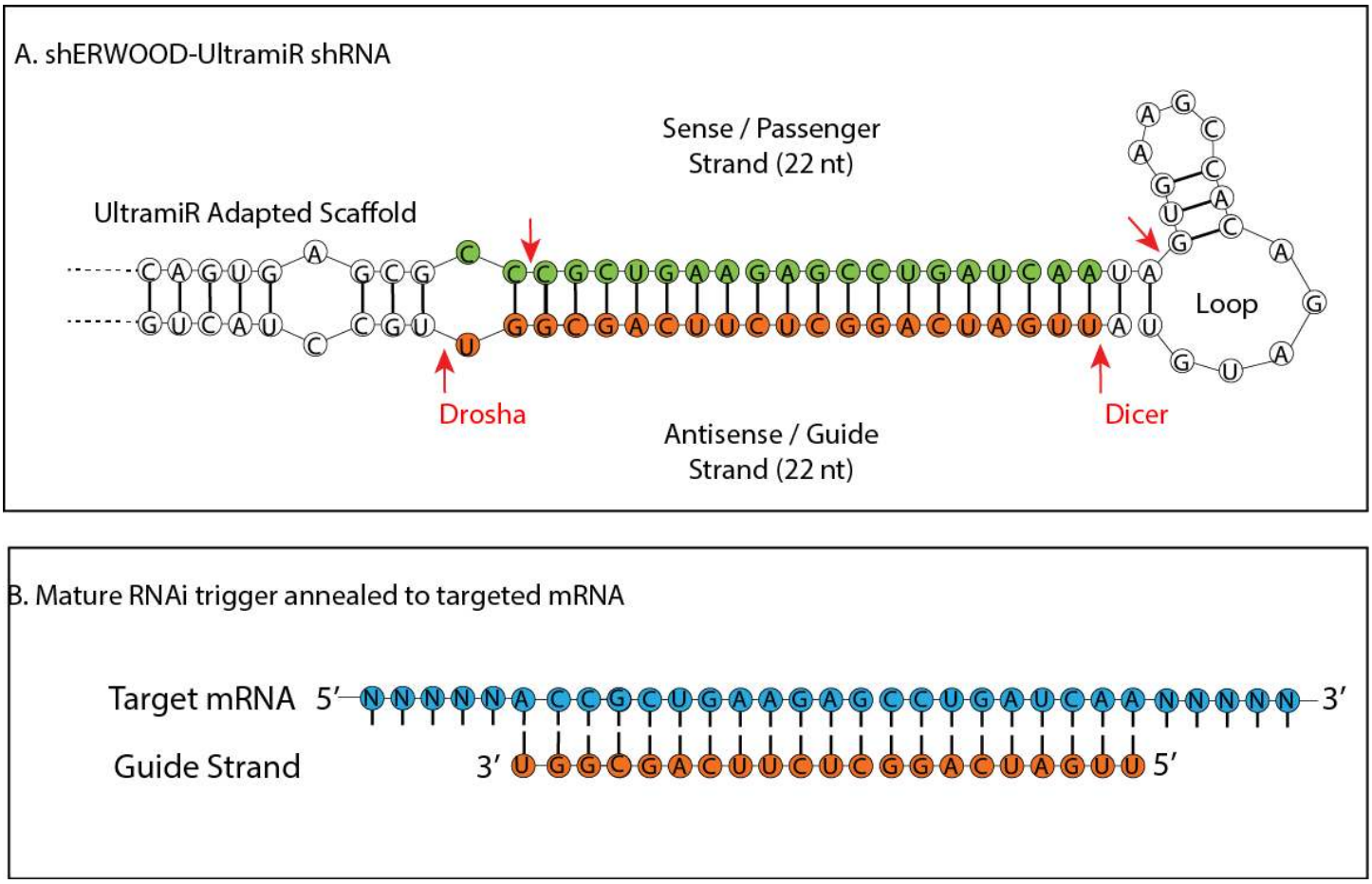

Figure 1. Schematic of shERWOOD-UltramiR shRNA. (A) Passenger (green) and Guide (orange) strand are shown with Dicer and Drosha nuclease cleavage sites are in red. (B) The final step of shRNA processing loads the Guide Strand (orange) into the RISC complex which associates with the target mRNA (blue)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

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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).

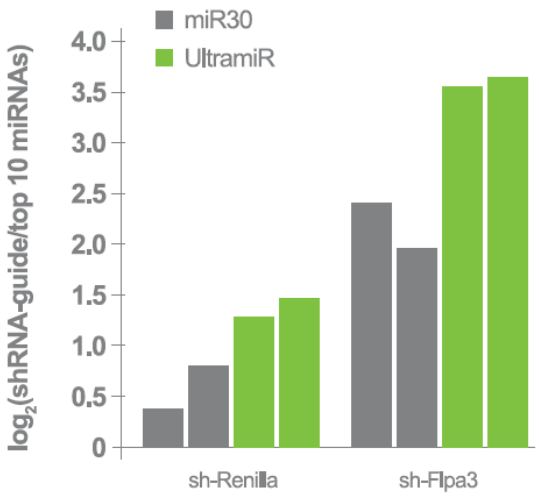

Figure 2. Relative abundances of processed guide sequences for two shRNA as determined by small RNA cloning and NGS analysis when cloned into traditional miR-30 and UltramiR scaffolds. Values represent log-fold enrichment of shRNA guides with respect to sequences corresponding to the top 10 most highly 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 LMN retroviral vector. Review local safety guidelines for complete regulations.

The pLMN shRNA expression vector allows transient and stable transfection; as well as the stable delivery of the shRNA expression into a target cell's genome via a replication-incompetent retrovirus.

Figure 3. Schematic of retroviral shRNA vector. *See appendix 1 for a more detailed vector map.*

The pLMN shRNA expression vector has a number of features allowing both transient and stable transfection; as well as the stable delivery of the shRNA expression cassette into host cells via transduction of replication-incompetent retrovirus. The shRNA expression cassette; sense, loop and antisense elements are all under the control of the viral LTR promoter.

The shRNA sequences have been cloned in to the pLMN vector:

- MSCV-based retroviral vector for delivery and expression in most mammalian cell lines including murine or human hematopoietic and embryonic stem (ES) cells.
- shRNA constructs are expressed from the retroviral LTR promoter.
- The ability to select stable integrants using neomycin selection.
- ZsGreen serves as a marker for retroviral integration.

pLMN is a Murine Stem Cell Virus (MSCV)-based vector derived from the Murine Embryonic Stem Cell Virus (MESV) and the LN retroviral vectors (Hawley *et al.* 1994, Grez *et al.* 1990, Miller and Rosman 1989). Upon transfection of the plasmids into a packaging cell line, replication-incompetent high titer virus can be obtained and used to infect target cells.

II. Propagation and plasmid purification 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

pLMN 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.

Plasmid preparation

For transfection and transduction (infection) experiments the pLMN plasmid DNA will first have to be extracted from the bacterial cells. When transforming directly into an experimental cell line, either a standard plasmid mini-preparation can be used or one that yields endotoxin free DNA. When extracting plasmid DNA to make virus for transduction, more DNA is required and using and endotoxin free kit will generally yield higher viral titers

III. Neomycin selection (Neomycin kill curve)

The pLMN retroviral vector has a neomycin resistance marker for selection in mammalian cells. To establish stable cell lines, once transfection/transduction has occurred, the cells can be placed on neomycin to select for stable integrants. Since each cell line potentially has a different sensitivity to neomycin, the optimal concentration (pre-transfection/transduction) should be determined. Below is the protocol for titrating neomycin (kill curve), using as an example a 24 well tissue culture dish.

Required materials

- Complete media experimental cell line
- Neomycin or G418 (200 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 neomycin.
2. Prepare neomycin dilutions in culture media for antibiotic titration as shown in **Error! Reference source not found.**. Use a neomycin stock solution of 200 µg/µl.

Table 1: Dilutions and volumes required for establishing optimal neomycin concentration

Volume of Neomycin Stock Solution Added (µl)	Total Volume of Media plus Antibiotic per 24 Well (µl)	Final Concentration (µg/ml)
0	500 µl	0
0.25	500 µl	50
0.5	500 µl	100
0.75	500 µl	150
1	500 µl	200
1.5	500 µl	300
2	500 µl	400
2.5	500 µl	500
3	500 µl	600
3.5	500 µl	700
4	500 µl	800
5	500 µl	1000

3. Begin antibiotic selection the following day by replacing antibiotic free media with media containing the appropriate concentrations of neomycin.
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 neomycin every 2 days for 7 days.

Note: The optimal neomycin concentration will kill the cells rapidly (5 - 7 days). This is particularly important for screens involving essential genes that may be selected against prior to the experiment.

IV. 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 2):

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.*

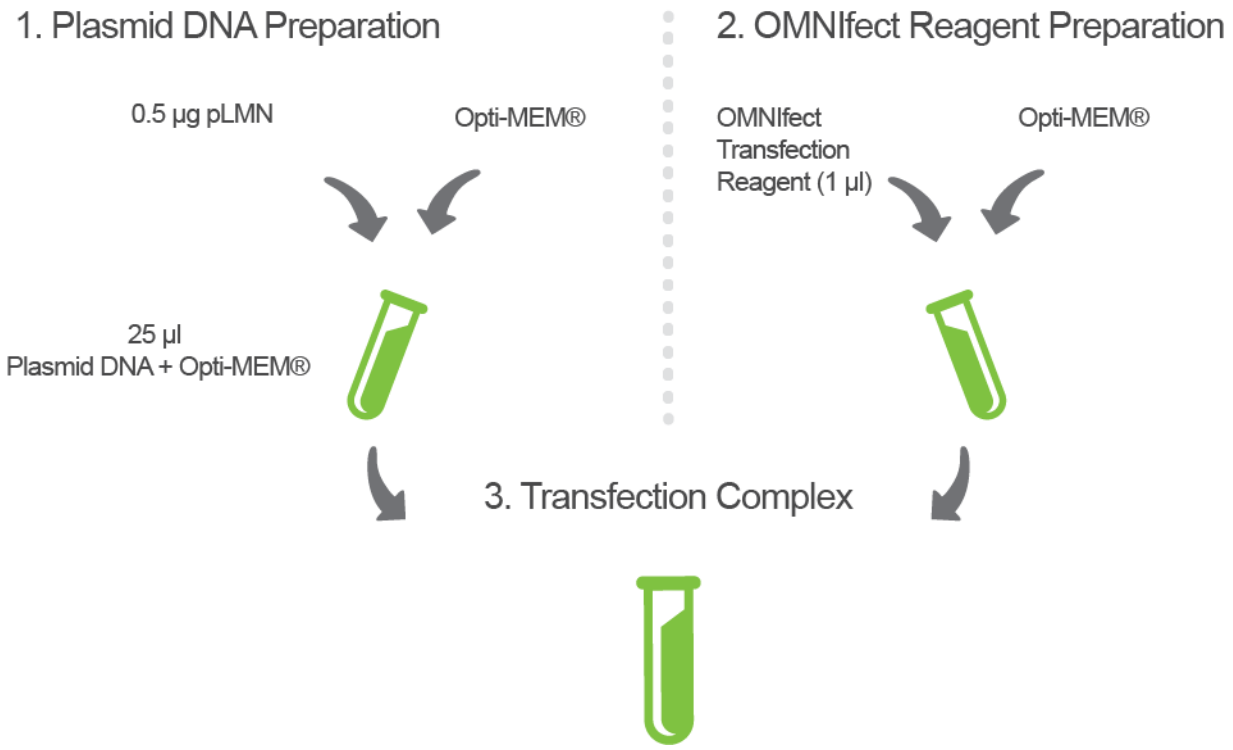

Figure 4. Transfection protocol for 24 well plates (volumes indicated are per well). To transfect the entire plate multiply all volumes and DNA amount by 24.

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.

Neomycin selection of transfected cells

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

1. After incubating for 48-72 hours, examine the cells microscopically for ZsGreen expression.
2. Begin the antibiotic selection by replacing the medium with complete medium supplemented neomycin.
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 neomycin. Observe the cells for approximately 7 days until you see single colonies surviving the selection. Colonies can be isolated and expanded for analysis.

V. Packaging retroviral particles

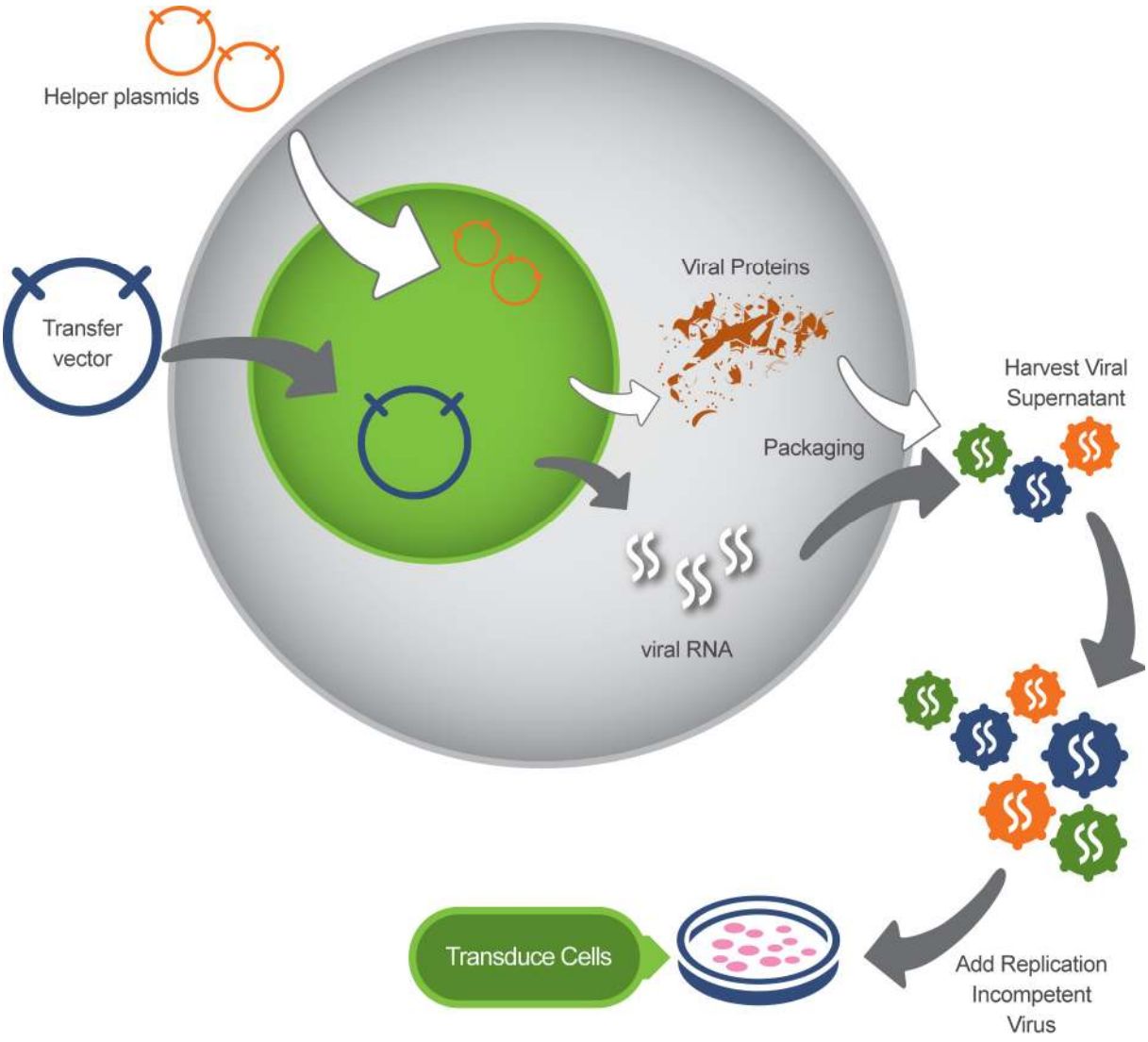

Figure 5. Schematic depicting retroviral packaging of pLMN retroviral vectors

A retroviral transfer vector (pLMN) is co-transfected with the desired helper vector encoding the *env* protein into a packaging cell line. In most packaging systems the gag and pol genes, essential for virus production, are stably integrated into the cell line's genome and constitutively expressed. *gag*, *pol* and *env* provide the proteins necessary for viral assembly and integration. The transfer vector contains 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. This virus can be concentrated or used as is.

Considerations for packaging the pLMN shRNA retroviral vector:

- Due to the increased processing efficiency of shERWOOD UltramiR shRNA an siRNA targeting Pasha/DGCR8 should be transfected with the transfer vector during packaging. (**See note below**)
- pLMN may be packaged with most common packaging cell lines and commercially available packaging systems.
- The Non-Targeting Control should be used to optimize packaging and determine transduction efficiency of the target cell.
- Make 2 x 50 µl aliquots for titering and divide the rest into transduction aliquots.
  - Store in cryovials with other aliquots at -80°C prior titer to properly reflect the change in titer from freezing that will occur in the other aliquots.
  - Store in cryovials 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 HEK293T cells.

NOTE: Using an siRNA targeting DGCR8 (Pasha) can increase titers by several fold.

- Transfection reagent used for packaging must efficiently deliver both plasmid and siRNA.
- Sequence for the Pasha/DGCR8 siRNA - CGGGTGGATCATGACATTCCA
- Use 1.8 µg of siRNA for packaging in a 100 mm cell culture dish. Adjust the amount of siRNA added based on cell count if packaging in alternate size cultures.

Appendix 3 has instructions for ordering.

VI. Functional titer and transduction optimization

The number of viral particles used and the transduction efficiency will determine the average number of retroviral integrations into the target cell genome. The following protocol is designed to evaluate functional titer of the virus produced in the previous section. Neomycin selection may be used to remove untransduced cells. A kill curve should be performed as described in section 3.

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 2.
- Transduction optimization should be done with the with Non-Targeting Control viral particles.
- Virus used to calculate the titer should be frozen with the aliquots that will be used in the experiment to reflect any changes in titer to freeze-thaw cycles.
- 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
- Retroviral 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 a GFP filter (Refer to Appendix 2 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 2.)

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

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

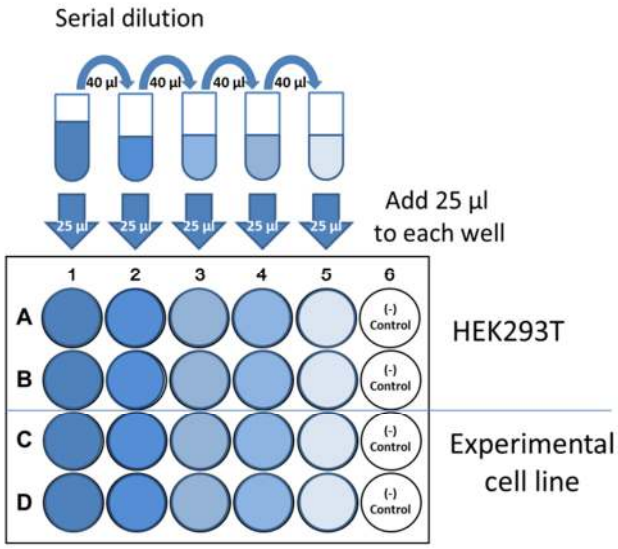

Figure 6 Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

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 neomycin

Required materials

- Complete media for experimental cell line
- Selection media: complete media for experimental cell line supplemented with neomycin
- 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 neomycin).

Neomycin 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 1 –pLMN vector information

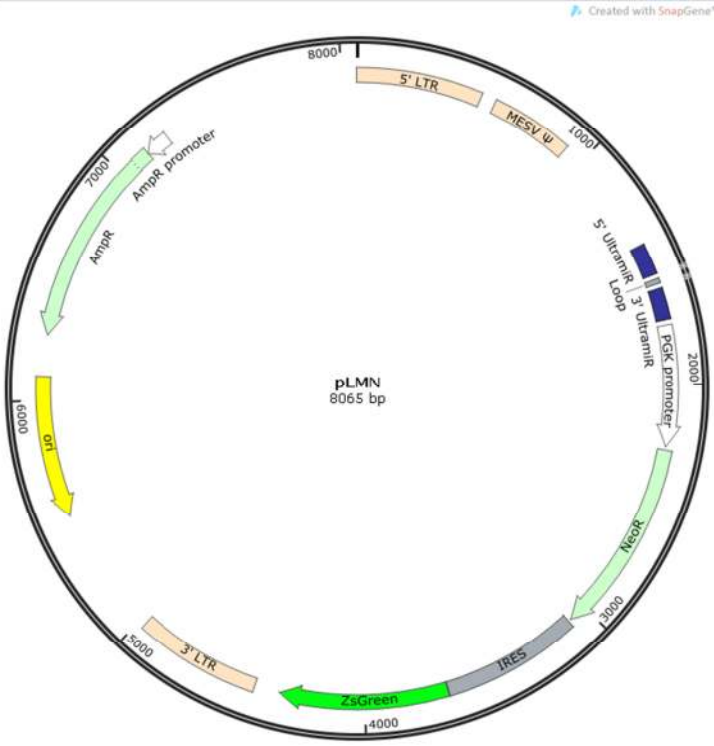

Figure 1: Detailed map of the pLMN vector.

Appendix 2 – Alternate methods for titering

1. Neomycin titering by selection and colony counting:
   a. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal neomycin concentration determined in neomycin 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 3 – 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 4 - References

Safety guidelines for working with retrovirus

http://oba.od.nih.gov/rdna/nih_guidelines_oba.html

www.fda.gov/downloads/AdvisoryCommittees/.../UCM232592.pdf

Biosafety in Microbiological and Biomedical Laboratories, Fourth Edition (May 1999) HHS Pub. No. (CDC) 93-8395. U.S. Department of Health and Human Services, PHS, CDC, NIH.

shRNA–mir and design

Knott et al., A computational algorithm to predict shRNA potency. Molecular Cell (2014), http://www.cell.com/molecular-cell/pdfExtended/S1097-2765%2814%2900835-1

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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