shERWOOD UltramiR lentiviral inducible shRNA (pZIP-TRE3GS)

Bacterial glycerol stock
TRHSU2300, TRMSU2300, TRRSU2300

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.

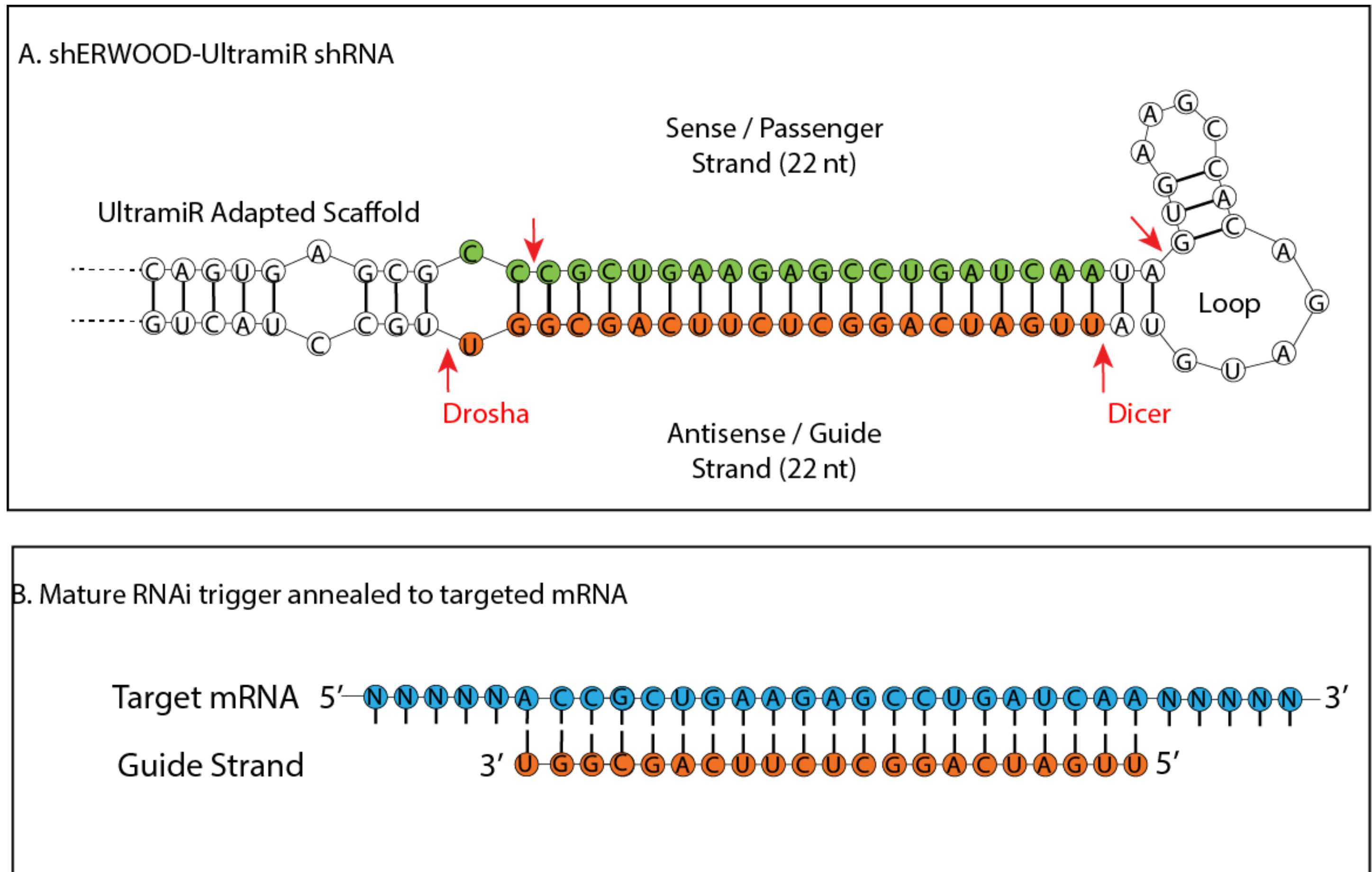


Figure 1. Schematic of shERWOOD-UltramiR shRNA. (A) Passenger (green) and Guide (orange) strand are shown with Dicer and Drosha nuclease cleavage sites are in red. (B) The final step of shRNA processing loads the Guide Strand (orange) into the RISC complex which binds to the target mRNA (blue) in a sequence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

Optimized microRNA scaffold sequence increases small RNA processing

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

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

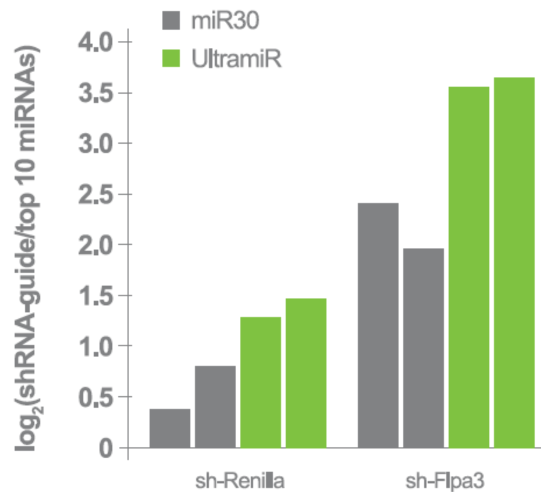


Figure 2. Relative abundances of processed guide sequences for two shRNA as determined by small RNA cloning and NGS analysis when cloned into traditional miR-30 and UltramiR scaffolds. Values represent log-fold enrichment of shRNA guides with respect to sequences corresponding to the top 10 most highly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

Guaranteed knockdown

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

I. Introduction

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

The inducible ZIP (all-in-one) vector contains the components necessary for regulated expression of the shRNA-mir (Figure 1), including the TRE3GS inducible promoter positioned upstream of the shRNA, 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 ZsGreen and the shRNA-mir. This allows for direct visual confirmation of induced shRNA expression. A puromycin resistance gene ($Puro^R$) is also encoded for rapid selection of transduced cells.

Function	Element
Promoter	TRE3GS • 3rd generation Tet-inducible promoter with reduced background expression • TRE3GS promoter that was modified for higher performance in a single vector context • Expresses shRNA and ZsGreen PGK • Expresses Tetracycline induced transactivator (Tet-On 3G TA) SV40 • Expresses Puromycin resistance
Fluorescent marker	ZsGreen expressed when induced with shRNA excitation maximum = 496 nm; emission maximum = 506 nm
Mammalian selection marker	Puromycin resistance gene (PuroR)
shRNA	shERWOOD-UltramiR shRNA showing 5' and 3' flanking UltramiR sequences

Figure 3. Schematic of Lentiviral Inducible shRNA vector with vector element information.

II. Safety

See Appendix 2 for safety considerations.

III. Overview of Transduction and Titer

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

Various factors affect the transduction efficiency in any given cell line. The titer, or number of viral particles in a particular volume, has the most influence on the number of cells transduced. Viral titers can be measured in a number of ways. One approach is simply to measure the number of viral particles, usually by ELISA, which detects and quantifies a component of the viral particle. In the case of HIV-based lentiviral vectors a p24 ELISA is used to measure the amount of HIV capsid protein in the sample. This is referred to as p24 titer or particle titer. Because p24 ELISAs detect capsid antigen present on both functional and non-functional viral particles, p24 titers overestimates the true viral titer, as most lentiviral vector viral particles are non-functional (missing one or more required vector components). Another approach is to use RT-PCR to quantify the number of viral genomic RNAs packaged into viral particles. Titers determined by this method are usually provided as copies per ml, and like p24 titer it too is a measure of the number of viral particles, not a transduction unit, and overestimates true titer (~100-fold). A more meaningful measurement is referred to as functional titer, which measures the number of cells that are transduced and expresses 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-mir 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.

4. 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 doubling 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.*

1. Plasmid DNA Preparation

0.5 µg pZIP

Opti-MEM®

25 µl
Plasmid DNA + Opti-MEM®

2. OMNIfect Reagent Preparation

OMNIfect Transfection Reagent (1 µl)

Opti-MEM® (24 µl)

25 µl
OMNIfect + Opti-MEM®

3. Transfection Complex
*50 µl

10 minute incubation
Room temperature

Add complexes to cells

*50 µl is sufficient for 1 well of a 24-well plate

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.

5. Packaging lentiviral particles

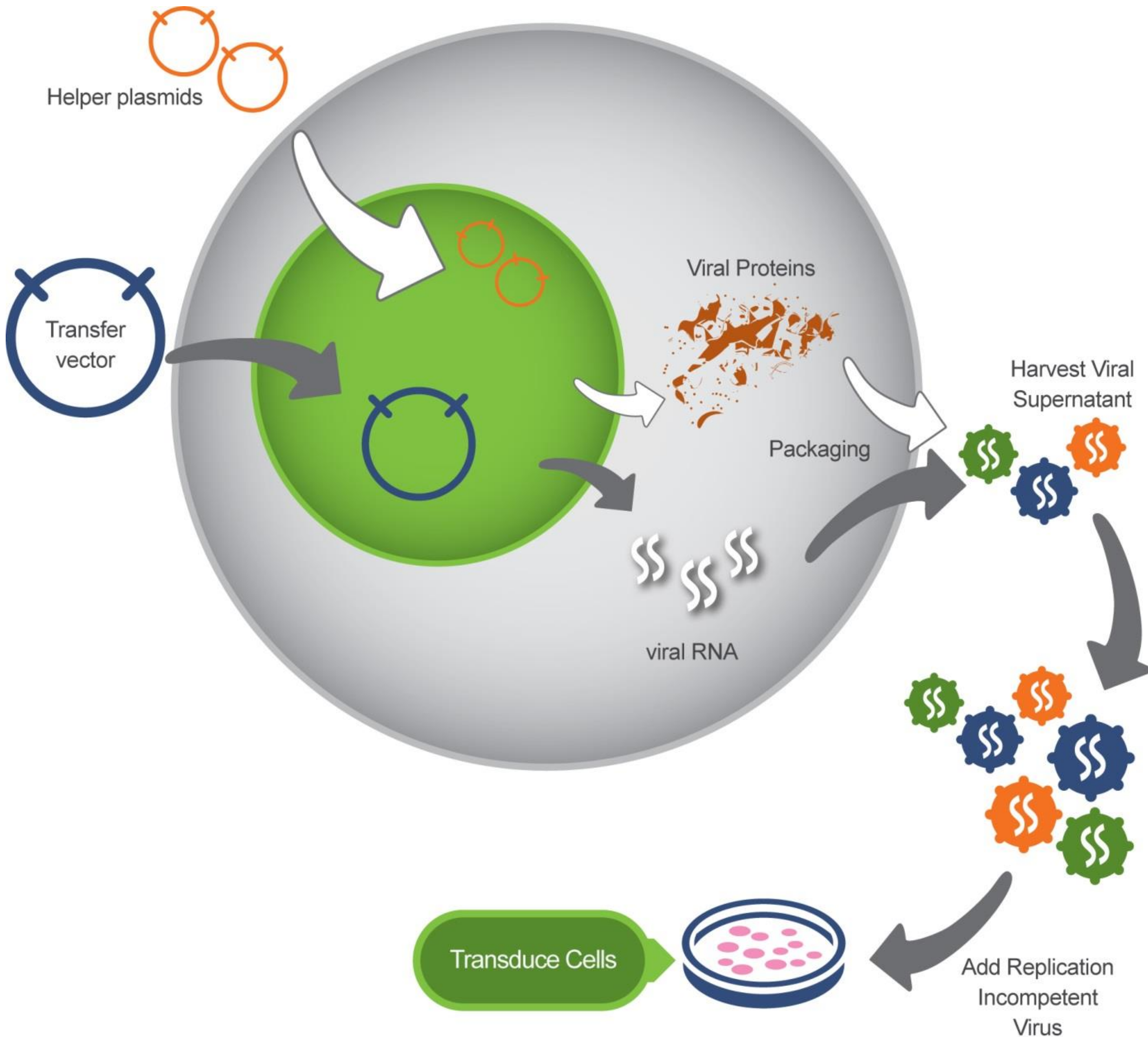


Figure 5. Schematic depicting lentiviral packaging of pZIP lentiviral vectors

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-mir 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-mir 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 150 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℃ overnight prior to titering to reflect any loss of function due to freeze/thaw cycle that will occur for the transduction aliquots.
The inducible lentiviral shRNA vector is complex may not package as efficiently as other lentiviral vectors. Freshly harvested viral particles from well-transfected cells should have a titer of approximately 1-5 x 10^4 TU/ml when measured on HEK293T cells. Concentrating the viral particles and/or applying selection to the transduced target cell culture will likely be beneficial.

6. 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

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

Equipment

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

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 14 wells with complete media. Incubate overnight with 5% CO_2 at 37°C. It is important to seed enough cells so that the cell confluency ranges between 40 and 50% at the time of transduction.
2. Prepare a serial dilution series with serum free media and viral supernatant as shown in Table 3 and Figure 4.
    a. Set up 5 sterile microcentrifuge tubes.
    b. Add 150 µl of undiluted viral supernatant to the first tube from the titration aliquots generated while following the packaging protocol.
        i. Add Polybrene to the first tube for a final concentration of 5-8 µg/ml.
    c. Add 160 µl of serum free medium containing 5-8 µg/ml Polybrene to each remaining tube.
    d. Add 40 µl of viral stock to the first microfuge tube.
    e. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
    f. Transfer 40 µl from the first microcentrifuge tube to the second tube. Mix well and discard the tip.
    g. Repeat the procedure for the 4 remaining tubes.
    h. 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 containing 1.0 – 2.0 µg/ml doxycycline and allow cells to grow for 72-96 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 $\div$ 0.025 ml = 1.75 X 10^6 TU/ml

Dilution table and schematic for titration protocol

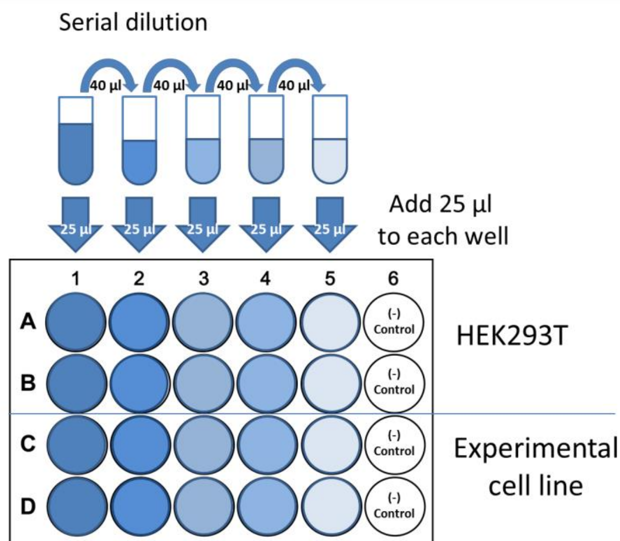


Figure 4 Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

Table 3 Dilution factors for calculating viral titer

Tube	Viral particles	Dilution medium	Dilution factor
1	150 µl (from titer aliquot)	0 µl	1
2	40 µl (from Tube 1)	160 µl	5
3	40 µl (from Tube 2)	160 µl	25
4	40 µl (from Tube 3)	160 µl	125
5	40 µl (from Tube 4)	160 µl	625
6	0 µl		n/a

Example:
Typical virus production will yield 1-5 x 10^4 TU/ml. The expected number of fluorescent colonies for a viral titer of 5 x 10^4 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	50,000	10,000	2,000	400	80	0
ml transduced cells	0.025	0.025	0.025	0.025	0.025	0
Fluorescent colonies expected	1,250	250	50	10	2	0

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

5. Transduction and Induction Guidelines and Protocol

Considerations for transduction and inducible expression

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 (see calculation below). The MOI is the number of transducing units per cell in a culture. To get maximum knockdown of a single gene target, generally an MOI $\geq$ 5 is recommended. Some cell types, such as primary cell cultures, may require an MOI $\geq$ 25.

For inducible shRNA expression vectors, a high MOI may lead to an unacceptable level of leakiness. Therefore, it may be necessary to transduce cells at a lower MOI, followed by selection of the culture in puromycin to remove non-transduced cells or those cells where the vector integrations are in regions that are transcriptionally silent.

Determining optimal MOI

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. ZsGreen expression is 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:

$$TU_{total} = (\text{MOI x Cell Number})/\text{Viral titer}$$

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; Viral Titer = TU/µl.

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 puromycin

Required 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 pipettor /Pipette-aid (for tissue culture)
- Pipettor (for dilutions and handling of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Fluorescent microscope with GFP 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 (previous section) 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 above calculation 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 ZsGreen expression in the induced and non-induced wells. Choose MOI that results in high level of induction (bright ZsGreen expression) and low level of leakiness (faint ZsGreen expression). Note, levels of ZsGreen 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 shRNA expression off

Note: Doxycycline adheres to many cells and culture plates. Turning ZsGreen 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: ZsGreen protein will dissipate within 72 hours. Expression of the target gene may return to expected levels prior to this.

Transduction with lentiviral inducible shRNA viral particles

The following should be optimized prior to transduction:

- Transduction media: % Serum, Polybrene µg/ml
- Time exposed to transduction media: hours or overnight
- Selection media: µg/ml puromycin
- Optimal MOI: low-level leakiness, high-level induction
- Doxycycline concentration µg/ml

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)
- Induction media (with Dox)

Equipment

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

Protocol:

Prepare cells

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

Transduce cells

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

Puromycin selection

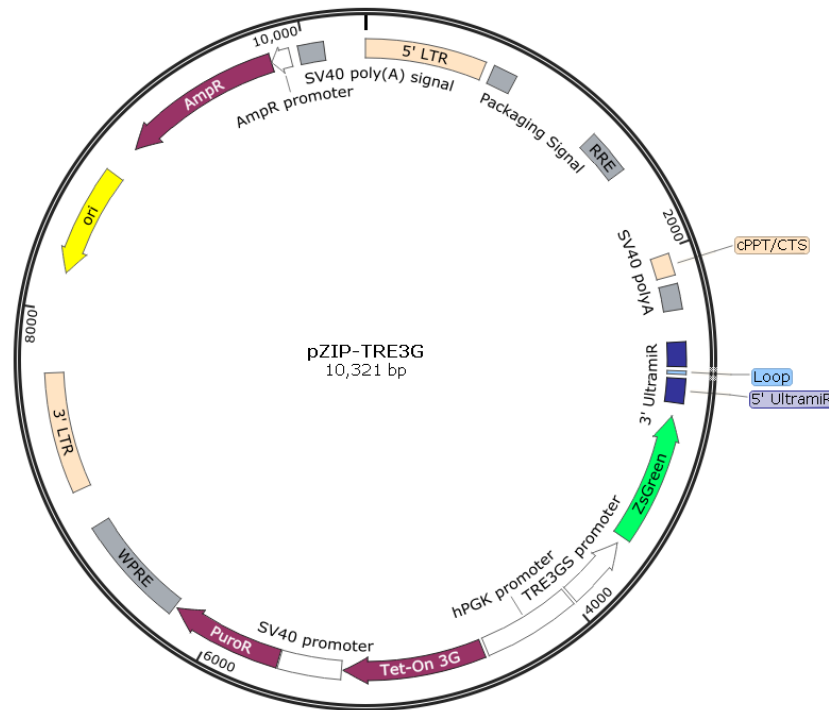
7. Allow cells to culture for 48 hours.
8. Replace media with selection media. (Use information from the puromycin kill curve concentration of puromycin that is determined)
9. Continue feeding cells selection media until untransduced cells have been removed.

Induction

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

I. Appendix

Appendix 1. Vector map



Element	Start	Stop
5' LTR	1	635
cPPT/CTS	2028	2143
SV40 polyA	2187	2321
3' UltramiR	2489	2617
Loop	2640	2657
5' UltramiR	2679	2807
ZsGreen	2879	3574
TRE3GS promoter	3614	3981
hPGK promoter	4002	4512
Tet-On 3G	4531	5277
SV40 promoter	5288	5617
PuroR	5626	6225
WPRE	6243	6831
3' LTR	7039	7672
SV40 poly(A) signal	9972	10106

Figure 5: Detailed map of the pZIP-TRE3GS vector.

Appendix 2. Safety

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

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

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

Summary of Biosafety Level 2 Practices

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

Standard microbiological practices

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

Safety equipment

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

Facilities

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

Appendix 3. Alternate methods for titering

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

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

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

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

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)	**None (default)**

Appendix 5 – References

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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