

Expression Arrest™ GIPZ lentiviral shRNAmir

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Important Safety Note:

Follow NIH guidelines regarding lentiviral production and transduction; follow Biosafety Level 2 (BL2) or BL2+ laboratory criteria.

NIH Agent Summary Statement: <http://bmbi.od.nih.gov/viral2.htm#retro>

NIH Biosafety Level 2 Description: <http://bmbi.od.nih.gov/sect3bsl2.htm>

NIH/RAC "Guidance on Biosafety Considerations for Research with Lentiviral Vectors":

http://www4.od.nih.gov/oba/RAC/Guidance/LentiVirus_Containment/pdf/Lenti_Containment_Guidance.pdf

Please note that GIPZ vectors are not compatible with third generation packaging systems such as ViraPower from Invitrogen. We recommend the Trans-Lentiviral Packaging system for use with our vectors.

PRODUCT DESCRIPTION

The GIPZ lentiviral shRNAmir library was developed by Open Biosystems in collaboration with Dr. Greg Hannon (CSHL) and Dr. Steve Elledge (Harvard). This library combines the design advantages of microRNA-adapted shRNA (shRNAmir) with the pGIPZ lentiviral vector to create a powerful RNAi trigger capable of producing RNAi in most cell types including primary and non-dividing cells.

DESIGN INFORMATION

Unique microRNA-30 based hairpin design

Expression Arrest™ short hairpin RNA constructs are expressed as human microRNA-30 (miR30) primary transcripts (Figure 1). This design adds a Drosha processing site to the hairpin construct and has been shown to greatly increase knockdown efficiency (Boden, Pusch et al. 2004). The hairpin stem consists of 22nt of dsRNA and a 19nt loop from human miR30. Adding the miR30 loop and 125nt of miR30 flanking sequence on either side of the hairpin results in

greater than 10-fold increase in Drosha and Dicer processing of the expressed hairpins when compared with conventional shRNA designs without microRNA (Silva, Li et al. 2005). Increased Drosha and Dicer processing translates into greater siRNA/miRNA production and greater potency for expressed hairpins.

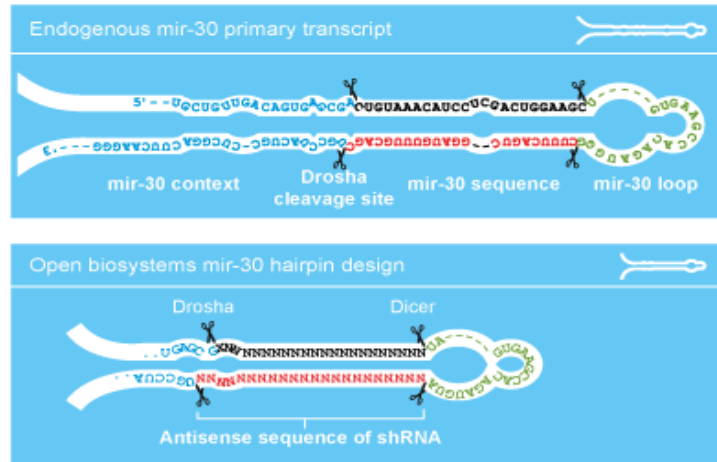


Figure 1. Expression Arrest shRNA are expressed as miR30 primary transcripts

Use of the miR30 design also allowed the use of **'rules-based' designs** for target sequence selection. One such rule is the destabilizing of the 5' end of the antisense strand which results in strand specific incorporation of miRNAs into RISC.

The proprietary design algorithm targets sequences in coding regions and the 3'UTR with the additional requirement that they contain greater than 3 mismatches to any other sequence in the human or mouse genomes.

Each shRNA construct has been sequence verified to ensure a match to the target gene. To assure you the highest possibility of modulating the gene expression level, each gene is represented by multiple shRNA constructs, each covering a unique region of the target gene.

VECTOR INFORMATION

Versatile vector design

Features of the pGIPZ™ lentiviral vector (Figure 2-3, Table 1) that make it a versatile tool for RNAi studies include:

- Ability to perform transfections or transductions using the replication incompetent lentivirus (Shimada, et al. 1995)
- TurboGFP and shRNAmir are part of a bicistronic transcript allowing the visual marking of shRNAmir expressing cells
- Amenable to *in vitro* and *in vivo* applications
- Puromycin drug resistance marker for selecting stable cell lines
- Molecular barcodes enable multiplexed screening in pools

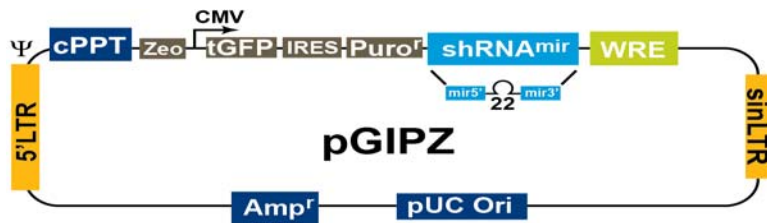


Figure 2: pGIPZ lentiviral vector

Table 1. Features of the pGIPZ vector

Vector Element	Utility
CMV Promoter	RNA Polymerase II promoter
cPPT	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
WRE	Enhances the stability and translation of transcripts
TurboGFP	Marker to track shRNAmir expression
IRES-puro resistance	Mammalian selectable marker
Amp resistance	Ampicillin (carbenicillin) bacterial selectable marker
5'LTR	5' long terminal repeat
pUC ori	High copy replication and maintenance of plasmid in <i>E. coli</i>
SIN-LTR	3' Self inactivating long terminal repeat (Shimada, et al. 1995)
RRE	Rev response element
Zeo resistance	Bacterial selectable marker

VECTOR MAP

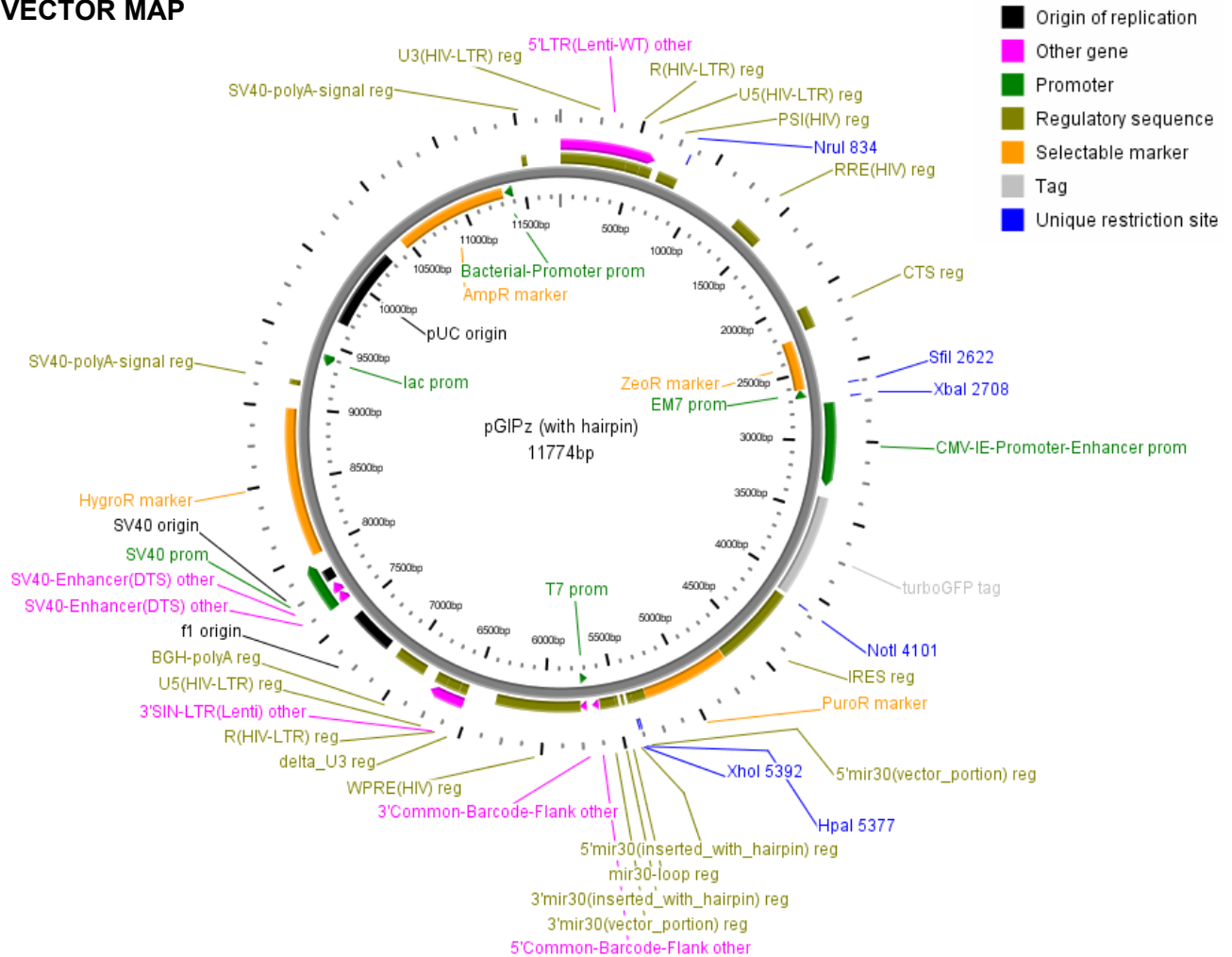


Figure 3. Detailed vector map of pGIPZ™ lentiviral vector.

ANTIBIOTIC RESISTANCE

pGIPZ™ contains 3 antibiotic resistance markers (Table 2).

Table 2. Antibiotic resistances conveyed by pGIPZ

Antibiotic	Concentration	Utility
Ampicillin (carbenicillin)	100µg/ml	Bacterial selection marker (outside LTRs)
Zeocin	25µg/ml	Bacterial selection marker (inside LTRs)
Puromycin	variable	Mammalian selectable marker

QUALITY CONTROL

The GIPZ lentiviral shRNA library has passed through internal QC processes to ensure high quality and low recombination (Figures 4 and 5).

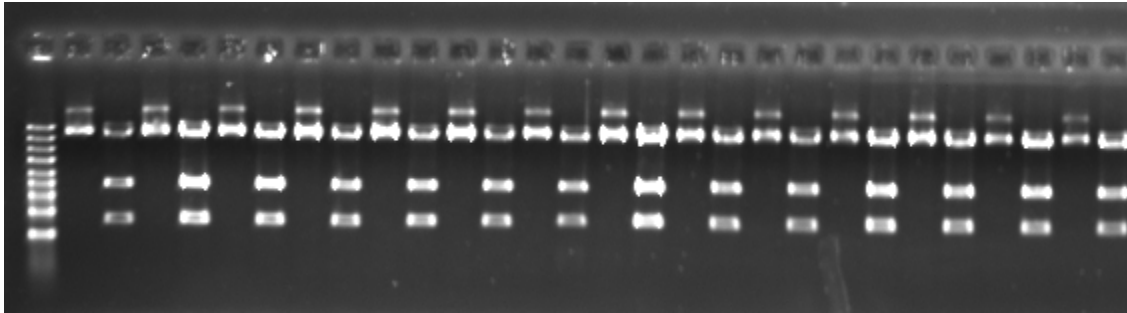


Figure 4. Representative shRNA containing pGIPZ lentiviral clones grown for 16 hours at 30°C and the plasmid isolated and normalized to a standard concentration. Clones were then digested with *Sac*II and run out on a gel. The expected band sizes are **1259bp, 2502bp, 7927bp**. No recombinant products are visible. 10kb molecular weight ladder (10kb, 7kb, 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb)

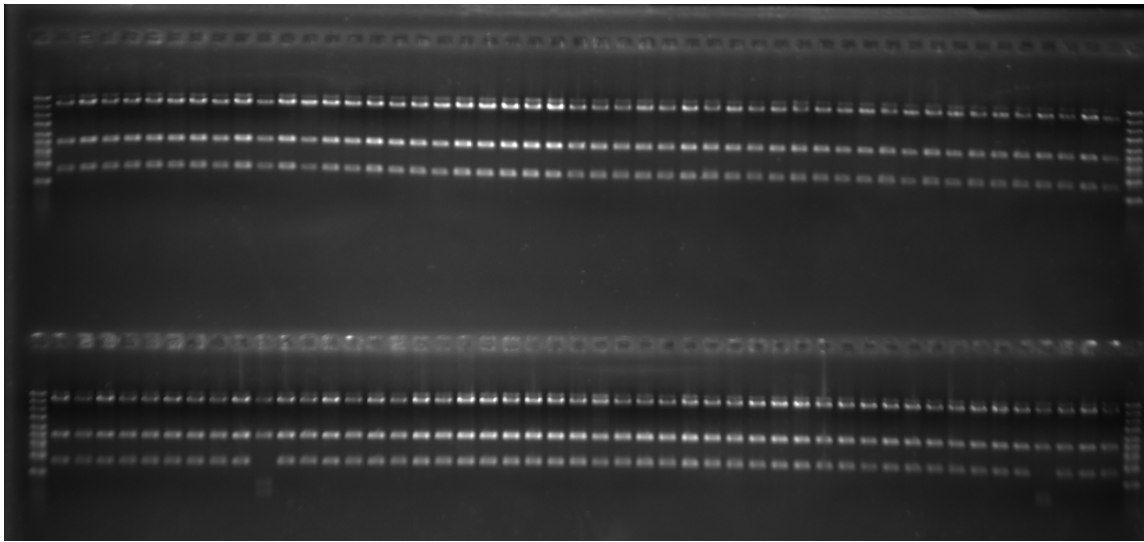


Figure 5. Gel image of a single plate from the GIPZ library cultured for 10 successive generations in an attempt to determine the tendency of the pGIPZ vector to recombine. Each generation was thawed, replicated and incubated overnight for 16 hours at 30°C then frozen, thawed and replicated. This process was repeated for 10 growth cycles. After the 10th growth cycle, plasmid was isolated and normalized to a standard concentration. Clones were then digested with *Sac*II and run on a gel. Expected band sizes **1259bp, 2502bp, 7927bp**. 10kb molecular weight ladder (10kb, 7kb, 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb). The pGIPZ vector appears stable without showing any recombination.

PROTOCOL I - REPLICATION

Table 3. Materials for plate replication

Item	Vendor	Catalog Number
LB-Lennox broth (low salt)	VWR	EM1.00547.0500
Peptone, granulated, 2kg - Difco	VWR	90000-368
Yeast Extract, 500g, granulated	VWR	EM1.03753.0500
NaCl	Sigma	S-3014
Glycerol	VWR	EM-2200 or 80030-956
Carbenicillin or ampicillin	Novagen	69101-3
Zeocin	Invivogen	ant-zn-5p
Puromycin	Cellgro	61-385-RA
96 well microplates	Nunc	260860
Aluminum seals	Nunc	276014
Disposable replicators	Genetix	X5054
Disposable replicators	Scinomix	SCI-5010-OS

For archive replication, grow all pGIPZ™ clones at 30°C in LB-Lennox (low salt) media plus 25µg/ml zeocin and 100µg/ml carbenicillin in order to provide maximum stability of the clones. Prepare media with 8% glycerol* and the appropriate antibiotics.

Replication of plates

Prepare target plates by dispensing ~160µl of LB-Lennox (low salt) media supplemented with 8% glycerol* and appropriate antibiotic (25µg/ml zeocin and 100µg/ml carbenicillin).

Prepare source plates:

1. Remove foil seals while the source plates are still frozen. This minimizes cross-contamination.
2. Thaw the source plates with the lid on. Wipe any condensation underneath the lid with a paper wipe soaked in ethanol.

Replicate:

1. Gently place a disposable replicator in the thawed source plate and lightly move the replicator around inside the well to mix the culture. Make sure to scrape the bottom of the well.
2. Gently remove the replicator from the source plate and gently place in the target plate and mix in the same manner to transfer cells.
3. Dispose of the replicator.
4. Place the lids back on the source plates and target plates.
5. Repeat steps 1-4 until all plates have been replicated.
6. Return the source plates to the -80°C freezer.
7. Place the inoculated target plates in a 30°C incubator without shaking for 18-19 hours.

Freeze at -80°C for long term storage. Avoid long periods of storage at room temperature or higher in order to control background recombination products.

Note: Due to the tendency of all viral vectors to recombine, we recommend keeping the incubation times as short as possible and avoid subculturing.

**Glycerol can be omitted from the media if you are culturing for plasmid preparation. If making copies of the constructs for long term storage at -80°C, 8% glycerol is required.*

PROTOCOL II - PLASMID PREPARATION

Culture conditions for individual plasmid preparations

For plasmid preparation, grow all pGIPZ™ clones at 37°C in 2X-LB broth (low salt) media plus **100µg/ml carbenicillin only**.

2X-LB broth (low salt) media preparation

LB-Broth-Lennox	20g/l
Peptone	10g/l
Yeast Extract	5g/l

Appropriate antibiotic(s) at recommended concentration(s)

Most plasmid mini-prep kits recommend a culture volume of 1-10ml for good yield. For shRNAmir constructs, 5ml of culture can be used for one plasmid mini-prep generally producing 5-10µg of plasmid DNA.

1. Upon receiving your glycerol stock(s) containing the shRNAmir of interest store at -80°C until ready to begin.
2. To prepare plasmid DNA first thaw your glycerol stock culture and pulse vortex to resuspend any *E. coli* that may have settled to the bottom of the tube.
3. Take a 10µl inoculum from the glycerol stock into 3-5ml of 2X-LB (low salt) with 100µg/ml carbenicillin. Return the glycerol stock(s) to -80°C.

Note: If a larger culture volume is desired, incubate the 3-5ml culture for 8 hours at 37C with shaking and use as a starter inoculum. Dilute the starter culture 1:500-1:1000 into the larger volume.

4. Incubate at 37°C for 18-19 hours with vigorous shaking.
5. Pellet the 3-5ml culture and begin preparation of plasmid DNA.
6. Run 3-5µl of the plasmid DNA on a 1% agarose gel. pGIPZ with shRNAmir is 11774bp.

Note: Due to the tendency of all viral vectors to recombine, we recommend keeping the incubation times as short as possible and avoid subculturing. Return to your original glycerol stock for each plasmid preparation.

Culture conditions for 96 well bio-block plasmid preparation

Inoculate a 96 well bio-block containing 1ml per well of 2X-LB (low salt) media with 100µg/ml carbenicillin with 1µl of the culture. Incubate at 37°C with shaking (~170-200rpm). We have observed that incubation times between 18-19 hours produce good plasmid yield. For plasmid preparation, follow the protocols recommended by the plasmid isolation kit manufacturer.

Note: Open Biosystems uses the above 96 well bio-block plasmid preparation protocol in conjunction with a Qiagen Turbo kit (catalog no. 27191). We use 2 bio-blocks combined, do not perform the optional wash and elute the DNA in water.

PROTOCOL III - CLONING

Moving shRNAmir constructs from pSM2 to pGIPZ

1. Order the pSM2 vector already expressing the shRNAmir of interest from [Open Biosystems](#).

2. Order the following PCR primers:
 pSM2 forward - 5' aagccctttgtacaccctaagcct 3'
 pSM2 reverse - 5' actggtgaaactcaccaggatt 3'
3. Order a KOD Hotstart Polymerase kit from Novagen (catalog no. 71086-5 for 20U)
4. Resuspend the PCR primers at a stock concentration of 50pmol/μl in sterile DEPC water. Dilute the stock 1:10 for a working concentration of 5pmol/μl in sterile DEPC water.
5. Set up the following PCR reaction at room temperature (Table 4). Add the components in the order listed. The following is for one 50ul reaction. To do more reactions simply multiply the master mix components by the desired number of reactions plus 10%. We recommend doing 4 reactions to ensure enough fragment will be available for cloning.

Table 4. PCR reaction

Component	volume in μl
H2O (DEPC)	25
5M Betaine	5
10X PCR buffer for KOD Hotstart Polymerase	5
dNTP's (2mM each)	5
MgSO4 25mM	2
pSM2 Forward Primer (5pmol/μl)	3
pSM2 Reverse Primer (5pmol/μl)	3
KOD Hotstart Polymerase (1U)	1
Total volume	49
Template (1ul of glycerol stock from your pSM2 clone of interest)	1

6. Input the following program into your thermocycler (Table 5):

Table 5. PCR program

PCR Program HS KOD		
	Temperature	Time
Hot Start Enzyme Activation	94°C	2min
Melt	94°C	15sec
Anneal	58°C	30sec
Extension	72°C	25sec
Cycles	30°C	
Expected Product Size	1735bp	

7. Put the four PCR reactions through a Wizard SV Gel and PCR Clean-up System column according to the kit directions (Promega catalog no. A9281 for 50 preps), with the exception of eluting with 110μl of the provided nuclease free water. All four reactions can be run on a single SV Gel and PCR Clean-up column.
8. Set up the following restriction digest using the Clean-up column eluent (Table 6) and incubate at 37°C for 3 hours.

Table 6. Restriction digest

Component	volume in μ l
PCR eluent	100
10X Restriction Enzyme Buffer	20
MluI	2
XhoI	2
Sterile Water	76
Total volume	200

9. Run the entire digest on a 1.2%-1.5% agarose gel. Three bands should be seen (789bp, 683bp, and 345bp) (Figures 6 and 7). Three bands will appear only if both *MluI* and *XhoI* have cut. Therefore the digest is diagnostic of the enzyme cuts in the following fashion (Table 7).

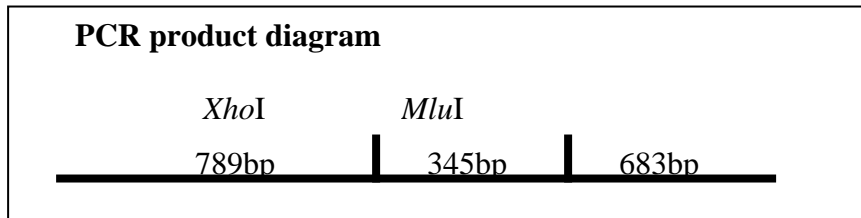


Figure 6. PCR product diagram

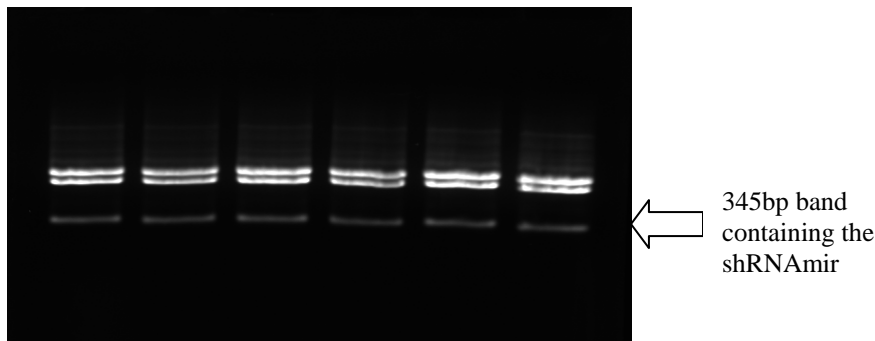


Figure 7. *MluI* and *XhoI* digest of PCR eluent with the expected digestion pattern (789bp, 683bp, 345bp)

Table 7. Possible digestion patterns

Band sizes seen	Conclusion
1735bp only	Neither <i>MluI</i> or <i>XhoI</i> cut
789bp, 683bp, and 345bp	Both <i>MluI</i> and <i>XhoI</i> cut
1028bp and 789bp	Only <i>XhoI</i> cut. <i>MluI</i> did not cut.
1134bp and 683bp	Only <i>MluI</i> cut. <i>XhoI</i> did not cut.

10. Excise the 345bp band containing the shRNA insert of interest and purify on a Wizard SV Gel and PCR Clean-up System column according to the kit directions (Promega catalog no. A9281 for 50 preps). Elute in 50 μ l nuclease free water.
11. Quantitate the insert fragment.
12. Prepare the pGIPZ empty vector for ligation to the shRNA insert. Set up the following restriction digest (Table 8).

Table 8. Restriction digest

Component	volume in μ l
pGIPZ empty vector (250ng/ μ l)	12
10X Restriction Enzyme Buffer	10
<i>Mlu</i> I	2
<i>Xho</i> I	2
Sterile Water	74
Total volume	100

13. Mix the solution by pipetting and then gently spin the reaction for approximately 10 seconds to collect all the solution in the bottom of the tube. This will aid in decreasing contamination of uncut vector in your vector prep to follow. Incubate at 37°C for 3 hours.
14. Run the entire digest on a 0.8% agarose gel. Make sure to run the gel through no less than 3cm length of agarose. This will also aid in decreasing contamination of uncut vector in your vector prep.
15. Gel isolate the 11429bp band using a Wizard SV Gel and PCR Clean-up System column according to the kit directions (Promega catalog no. A9281 for 50 preps). Elute in 50 μ l nuclease free water. You will likely not see a band representing the excised portion of the vector as it is too small (~259bp).
16. Quantitate the amount of cut vector per μ l you have isolated.
17. Set up the following ligation reactions (Table 9):

Table 9. Ligation reactions

Component	No Insert control	shRNAmir ligation
shRNAmir insert cut with <i>Mlu</i> I and <i>Xho</i> I (total 7.4ng)	XXXXXXXXXX	_____ μ l
pGIPZ vector cut with <i>Mlu</i> I and <i>Xho</i> I (total 250ng)	_____ μ l	_____ μ l
DEPC water	_____ μ l	_____ μ l
10X ligase buffer	2 μ l	2 μ l
Ligase	0.5 μ l	0.5 μ l
Total volume	20 μ l	20 μ l

Note: This setup yields a molar ratio of 1 vector to 1 insert.

18. Ligate for 3 hours at room temperature. Dilute the ligation mix by adding 160 μ l DEPC water.
19. Transform 5 μ l of the diluted ligation mix into PrimePlus™ competent *E.coli* (Open Biosystems catalog no. MBC4246). Follow the transformation protocol for the competent cells. Plate the transformed cells onto agar plates containing 100 μ g/ml carbenicillin and 25 μ g/ml zeocin. Be sure to transform the same volume of ligation mix and plate the same volume of cells for both the control and the experimental sample. Plating 100 μ l, 50 μ l, and 10 μ l aliquots is recommended.
20. Incubate plates at 30°C overnight. Count colonies and determine the ratio of colonies on the control plate versus the experimental plates. Determine the number of colonies to screen.

21. Order the following PCR primers to screen your clones for insertion of the shRNAir sequence of interest.

X76 Forward - 5' acgtcgaggtgccccgaagga -3'

M100 Reverse - 5' aagcagcgtatccacatagcgt -3'

22. Set up the following PCR reaction (Table 10). For template simply pick a colony with a toothpick, swirl in a small broth culture containing ampicillin and zeocin to maintain a stock and then swirl the same toothpick into your PCR well containing the appropriate amount of master mix. For a no insert control simply use 1ng of empty pGIPZ™ vector. The amounts in the table below are for a single 50µl reaction.

Table 10. PCR reaction

Component	volume in µl
H2O (DEPC)	26
5M Betaine	5
10X PCR buffer for KOD Hotstart Polymerase	5
dNTP's (2mM each)	5
MgSO ₄ 25mM	2
X76 Forward Primer (5pmol/µl)	3
M100 Reverse Primer (5pmol/µl)	3
KOD Hotstart Polymerase (1U)	1
Total volume	49
Template colony picked with toothpick	---

23. Run the following PCR program (Table 11). Note the annealing temperature has changed.

Table 11. PCR program

PCR Program HS KOD#2		
	Temperature	Time
Hot Start Enzyme Activation	94°C	2min
Melt	94°C	15sec
Anneal	56°C	30sec
Extension	72°C	25sec
Cycles	30	

24. Expected band sizes are as follows:

shRNA inserted = 603bp (clone with barcode); 543bp (clone without barcode)

No shRNA inserted = 516bp

25. Sequence verify clones. The pGIPZ sequencing primer is as follows:

5'- GCATTAAAGCAGCGTATC -3'

Note: The binding site lies from base 5820-5842 and runs in the reverse complement direction. The melting temperature of this 18mer=52.7°C.

PROTOCOL IV - RESTRICTION DIGEST

The following is a sample protocol for restriction enzyme digestion using *KpnI*, *SacII*, *SalI*, *XhoI* and/or *NotI* for diagnostic quality control of GIPZ lentiviral vectors.

1. Using filtered pipette tips and sterile conditions add the following components (Table 12), in the order stated, to a sterile PCR thin-wall tube.

Table 12. PCR Components

Component	Amount
Sterile, nuclease-free water	X μ l
Restriction enzyme 10X buffer	1 μ l
BSA (10X, 10mg/ml) if required	1 μ l
DNA sample 80-240ng, in water or TE buffer	X μ l
Restriction enzyme 20U	0.25 μ l
Final volume	10 μ l

2. Mix gently by pipetting.
3. Incubate in a thermalcycler at 37°C for 2 hours to digest
4. Load the gel with 10 μ l of each of the digested samples (*KpnI*, *SacII*, *SalI*, *XhoI* and/or *NotI*) on a 1% agarose gel. Run uncut sample alongside the digested samples. (Figure 8)

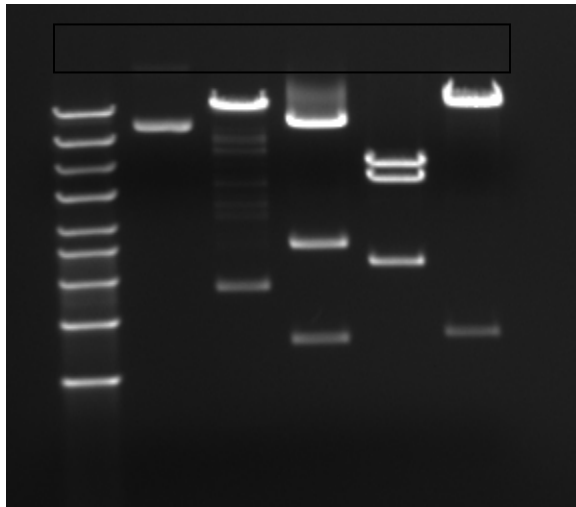


Figure 8. Restriction digests with pGIPZ. Lane 1- 10kb molecular weight ladder (10kb, 7kb, 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb). Lane 2 - Uncut pGIPZ vector. Lane 3 - *KpnI* digested pGIPZ produces 2 bands at 1750bp and 9860bp. Lane 4- *SacII* digest produces 3 bands at 1178bp, 2502bp and 7930bp. Lane 5 -*SalI* produces 3 bands at 2188bp, 4298bp and 5124bp. Lane 6 - *XhoI*, *NotI* double digest produces 2 bands at 1210bp and 10400bp.

PROTOCOL V - PUROMYCIN SELECTION

Puromycin Kill Curve and Puromycin Selection

In order to generate stable cell lines, it is important to determine the minimum amount of puromycin required to kill non-transfected/transduced cells. This can be done by generating a puromycin kill curve.

Puromycin Kill Curve

1. On day 0 plate 5×10^4 cells per well in a 24 well plate in enough wells to carry out your puromycin dilutions. Incubate overnight.
2. Prepare media specifically for your cells containing a range of antibiotic, for example: 0 - 15 μ g/ml puromycin.
3. The next day (day 1) replace the growth media with the media containing the dilutions of the antibiotic into the appropriate wells.
4. Incubate at 37°C.
5. Approximately every 2-3 days replace with freshly prepared selective media.
6. Monitor the cells daily and observe the percentage of surviving cells. Optimum effectiveness should be reached in 3-6 days under puromycin selection.
7. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 3-6 days from the start of antibiotic selection.

PROTOCOL VI - TRANSFECTION

The protocol below is optimized for transfection of the shRNA plasmid DNA into HEK293T cells in a 24 well plate using serum free media. If a different culture dish is used, adjust the number of cells, volumes and reagent quantities in proportion to the change in surface area (Table 13). It is preferable that transfections be carried out in medium that is serum free and antibiotic free. A reduction in transfection efficiency occurs in the presence of serum, however it is possible to carry out successful transfections with serum present (see Transfection Optimization).

Warm Arrest-In™ to ambient temperature (approximately 20 minutes at room temperature) prior to use. Always mix well by vortex or inversion prior to use.

Maintain sterile working conditions with the DNA and Arrest-In mixtures as they will be added to the cells.

Table 13. Suggested amounts of DNA, medium and Arrest-In reagent for transfection of shRNA plasmid DNA into adherent cells.

Tissue Culture Dish	Surface area per plate or well (cm ²)	Total serum free media volume per well (ml)	Plasmid DNA (μ g)*	Arrest-In (μ g)**
60 mm	20	2	4	21
35 mm	8	1	2	10
6 well	9.4	1	2	10
12 well	3.8	0.5	1	5
24 well	1.9	0.25	0.5	2.5
96 well	0.3	0.1	0.1 - 0.2	0.5 - 1

*Recommended starting amount of DNA. May need to be optimized for the highest efficiency

**Recommended starting amount of Arrest-In reagent. See Transfection Optimization.

1. The day before transfection (day 0), plate the cells at a density of 5×10^4 cells per well of a 24 well plate.

Full medium (i.e. with serum and antibiotics) will be used at this stage.

2. On the day of transfection form the DNA/Arrest-In transfection complexes.

- a. For each well to be transfected, dilute 500ng shRNA plasmid DNA into 50µl (total volume) of serum free medium in a microfuge tube.
- b. For each well to be transfected, dilute 2.5µg (2.5µl) of Arrest-In into 50µl (total volume) serum free medium into a separate microfuge tube.
- c. Add the diluted DNA (step a) to the diluted Arrest-In™ reagent (step b), mix rapidly then incubate for 20 minutes at room temperature.

This will give a 1:5 DNA:Arrest-In ratio by mass which is recommended for optimal transfection into HEK293T cells. Your total volume will be 100µl at this stage.

3. Aspirate the growth medium from the cells. Add an additional 150µl of serum free medium to each of the tubes containing transfection complexes (100µl). Add the total volume of the tube (250µl) to the cells and incubate for 5-6 hours in a CO₂ incubator at 37°C.

Your total volume will be 250µl at this stage (150µl serum-free medium + 100µl DNA:Arrest-In mixture).

4. Following the 5-6 hour incubation, add an equal volume of growth medium (250µl) containing twice the amount of normal serum to the cells (i.e. to bring the overall concentration of serum to what is typical for your cell line). Alternatively, the transfection medium can be aspirated and replaced with the standard culture medium (see note). Return the cells to the CO₂ incubator at 37°C.

*Note - Arrest-In has displayed low toxicity in the cell lines tested, therefore removal of transfection reagent is not required for many cell lines. In our hands higher transfection efficiencies have been achieved if the transfection medium is **not** removed. However, if toxicity is a problem, aspirate the transfection mixture after 5-6 hours and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.*

5. After 24-72 hours of incubation, examine the cells microscopically for the TurboGFP expression.

Note: When visualizing TurboGFP expression, if less than 90% of all cells are green, it is recommended in these cases to utilize puromycin selection in order to reduce background expression of your gene of interest from untransfected cells (see Figure 11).

The working concentration of puromycin varies between cell lines. We recommend you determine the optimal concentration of puromycin required to kill your host cell line prior to selection for shRNAmir transfectants (see puromycin kill curve protocol, page 20). Typically, the working concentration ranges from 1-10µg/ml. You should use the lowest concentration that kills 100% of the cells in 1-4 days from the start of puromycin selection.

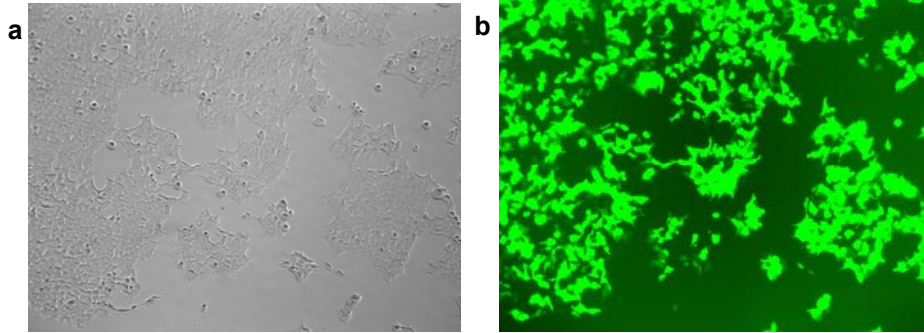


Figure 9. pGIPZ shRNAmir to GAPDH transfected into HEK293T cells 24 hours post transfection. a) Phase b) TurboGFP fluorescence. Note the high percentage of cells successfully transfected.

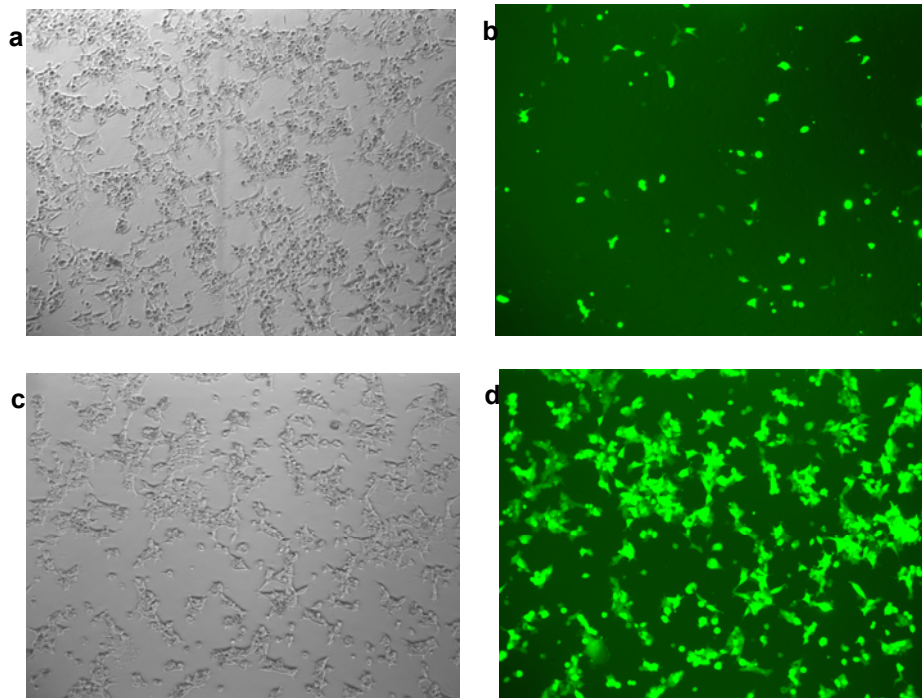


Figure 10. pGIPZ shRNAmir to GAPDH transfected into HEK293T cells 14 days post transfection. a) Phase. No puromycin selection. b) TurboGFP fluorescence. No puromycin selection. c) Phase. Puromycin selected. d) TurboGFP fluorescence. Puromycin selected. Note the decrease in the number of cells expressing TurboGFP when puromycin selection is not applied. When puromycin selection is maintained TurboGFP expression remains in all cells at high levels.

- a. If adding puromycin, use the appropriate concentration as determined based on the above kill curve. Incubate.
- b. Approximately every 2-3 days replace with freshly prepared selective media.
- c. Monitor the cells daily and observe the percentage of surviving cells. At some time point almost all of the cells surviving selection will be harboring the shRNAmir. Optimum effectiveness should be reached in 3-6 days with puromycin.

6. If selecting for stably transfected cells (optional), change the medium on the cells to that containing puromycin for selection. It is important to wait at least 24 hours before beginning selection.
7. Once cells not expressing TurboGFP are virtually eliminated and/or you have selected for stably transfected cells (optional), you can proceed to assay cells for reduction in gene or reporter activity by quantitative/real-time RT-PCR, western blot or other appropriate functional assay; compare to untreated, reporter alone, non-silencing shRNAmir or other negative controls.

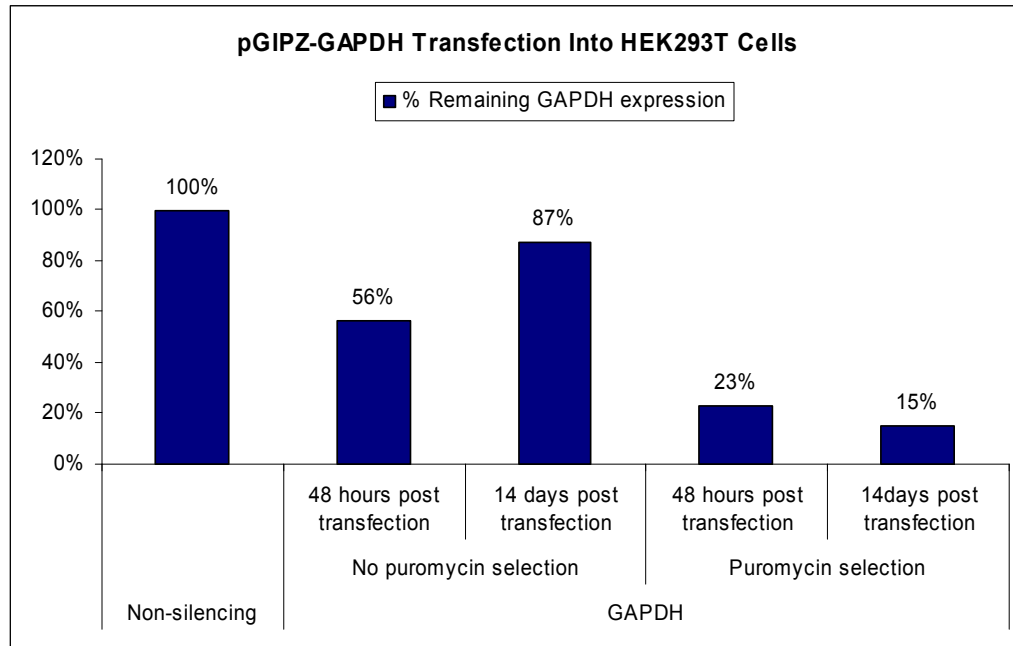


Figure 11. mRNA to GAPDH as measured by quantitative/real-time RT-PCR of GAPDH post transfection into HEK293T cells (48 hours and 14 days post transfection/puromycin selection). While transfection efficiency is high (Figure 9) the number of cells without the shRNA present is still high enough to mask the knockdown readout. These cells are eliminated via puromycin selection and the knockdown readout is increased.

Optimal length of incubation from the start of transfection to analysis is dependent on cell type, gene of interest, and the stability of the mRNA and/or protein being analyzed. Quantitative/real-time RT-PCR generally gives the best indication of expression knockdown. The use of western blots to determine knock-down is very dependent on quantity and quality of the protein, its half-life, and the sensitivity of the antibody and detection systems used.

Factors affecting transfection efficiency are not limited to but include purity of plasmid DNA, health of transduced cells, and inconsistencies in number of cells plated.

Transfection Optimization using Arrest-In™

It is essential to optimize transfection conditions to achieve the highest transfection efficiencies and lowest toxicity with your cells. The most important parameters for optimization are DNA to transfection reagent ratio, DNA concentrations and cell confluency. We recommend that you initially begin with the Arrest-In and DNA amount indicated in Table 13 and extrapolate the number of cells needed for your vessel size from the number of cells used in a well of a 24 well plate as listed in step 1 of the protocol for delivery of plasmid DNA.

Cells Grown In Suspension

Transfection of cells in suspension would follow all the above principles and the protocol would largely remain the same, except that the DNA/Arrest-In mixture should be added to cells (post 20 minute incubation for complex formation) to a total volume of 250µl serum free medium or to a total volume of 250µl of medium with serum (no antibiotics).

PROTOCOL VII - PACKAGING LENTIVIRUS

The pGIPZ vector is *tat* dependant, so you must use a packaging system that expresses the *tat* gene. For packaging our lentiviral shRNAmir constructs, we recommend the Trans-Lentiviral™ shRNA Packaging System (TLP4614, TLP4615). The Trans-Lentiviral shRNA Packaging System allows creation of a replication-incompetent (Shimada, et al. 1995), HIV-1-based lentivirus which can be used to deliver and express your gene or shRNAmir of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral shRNA Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes (Kappes and Wu 2001). For protocols and information on packaging pGIPZ™ with our Trans-Lentiviral shRNA Packaging System, please see the product manual available at the following link:

http://www.openbiosystems.com/collateral/rnai/pi/Trans-Lentiviral_GIPZ_Packaging_System.pdf

PROTOCOL VIII - TITERING

Viral Titering

Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of choice. This protocol uses the TLA-HEK293T™ cell line that is available as part of our Trans-Lentiviral shRNA Packaging System. You can use a standard HEK293T cell line as an alternative.

Note: If you have generated a lentiviral stock of the expression control (e.g. pGIPZ Non-Silencing), we recommend titering this stock as well.

1. The day before transduction, seed a 24 well tissue culture plate with TLA-HEK293T cells at 5×10^4 cells per well in DMEM (10% FBS, 1% pen-strep).

The following day, the well should be no more than 40-50% confluent. TLA-HEK293T (Open Biosystems catalog no. HCL4517).

2. Make dilutions of the viral stock in a round bottom 96 well plate using serum-free media. Utilize the plate as shown in Figure 12 using one row for each virus stock to be tested. Use the procedure below (starting at step 4) for dilution of the viral stocks. The goal is to produce a series of 5-fold dilutions to reach a final dilution of 390625-fold.

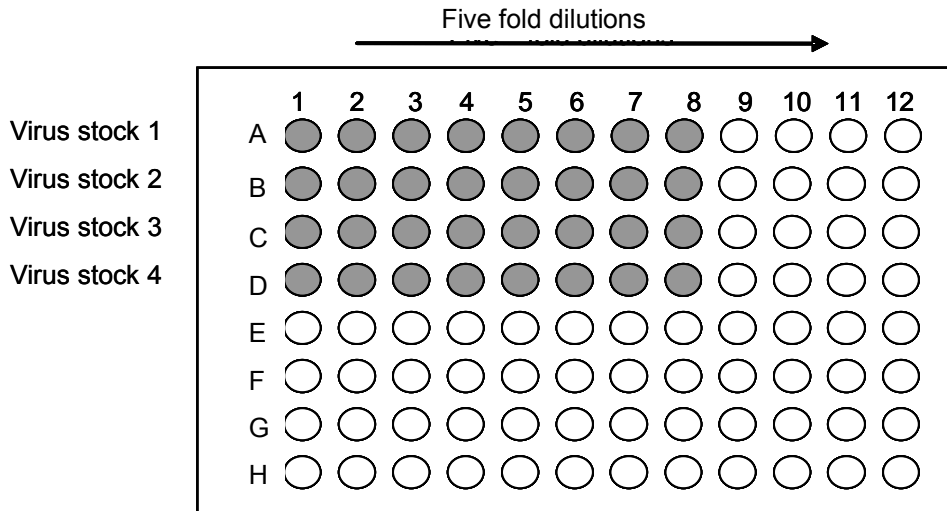


Figure 12. Five-fold serial dilutions of virus stock.

- To each well add 80µl of serum-free media.
- Add 20µl of thawed virus stock to each corresponding well in column 1 (5 fold dilution).

Pipette contents of well up and down 10-15 times. Discard pipette tip.

- With new pipette tips, transfer 20µl from each well of column 1 to the corresponding well in column 2.

Pipette 10-15 times and discard pipette tips.

- With new pipette tips, transfer 20µl from each well of column 2 to the corresponding well in column 3.

Pipette 10-15 times and discard pipette tip.

- Repeat transfers of 20µl from columns 3 through 8, pipetting up and down 10-15 times and changing pipette tips between each dilution.

It is strongly recommended that you use a high quality multichannel pipettor when performing multiple dilutions. Pre-incubate the dilutions of the virus stock for 5 minutes at room temperature.

- Label 24 well plate as shown in Figure 13 using one row for each virus stock to be tested.

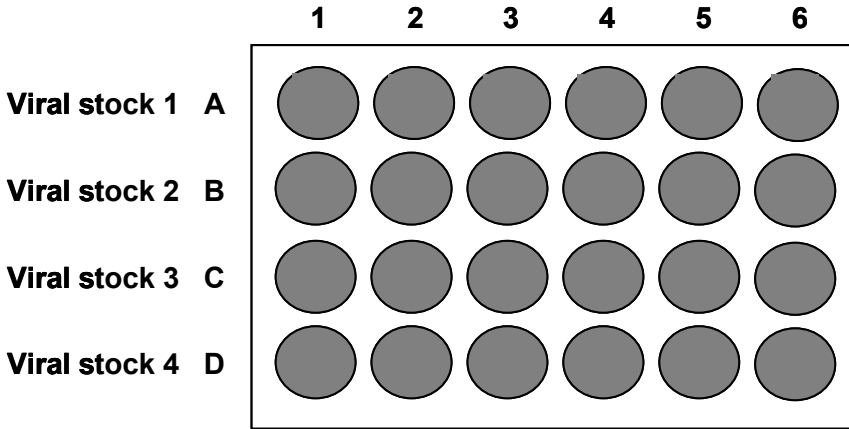


Figure 13. Twenty four well tissue culture plate, seeded with TLA-HEK293T™ cells, used to titer the virus.

9. Remove culture media from the cells in the 24 well plate.
10. Add 225µl of serum-free media to each well.
11. Transduce cells by adding 25µl of diluted virus from the original 96 well plate (Figure 12) to a well on the 24 well destination plate (Figure 13) containing the cells.

For example, transfer 25µl from well A2 of the 96 well plate into well A1 in the 24 well plate (Table 14).

Table 14. Example of set up for dilutions

Well (Row A, B, C, or D)		Volume diluted virus used	Dilution Factor
Originating (96 well plate)	Destination (24 well plate)		
A1		25µl	5 *
A2	A1	25µl	25
A3	A2	25µl	125
A4	A3	25µl	625
A5	A4	25µl	3125
A6	A5	25µl	15625
A7	A6	25µl	78125
A8		25µl	390625 *

**Please note that when expecting very high or very low titers, it would be advisable to include either well 8 or well 1 respectively.*

12. Incubate transduced cultures at 37°C for 4 hours.
13. Remove transduction mix from cultures and add 1ml of DMEM (10% FBS, 1% pen-strep).
14. Culture cells for 48 hours.

15. Count the TurboGFP expressing cells or colonies of cells (Figure 14).

Count each multi-cell colony as 1 transduced cell, as the cells will be dividing over the 48 hour culture period. Figure 14 illustrates this principle of counting.

16. Transducing units per ml (TU/ml) can be determined using the following formula:

of TurboGFP positive colonies counted x dilution factor x 40 = #TU/ml

Example: 55 TurboGFP positive colonies counted in well A3.

55 (TurboGFP positive colonies) x 625 (dilution factor) x 40 = 1.38×10^6 TU/ml

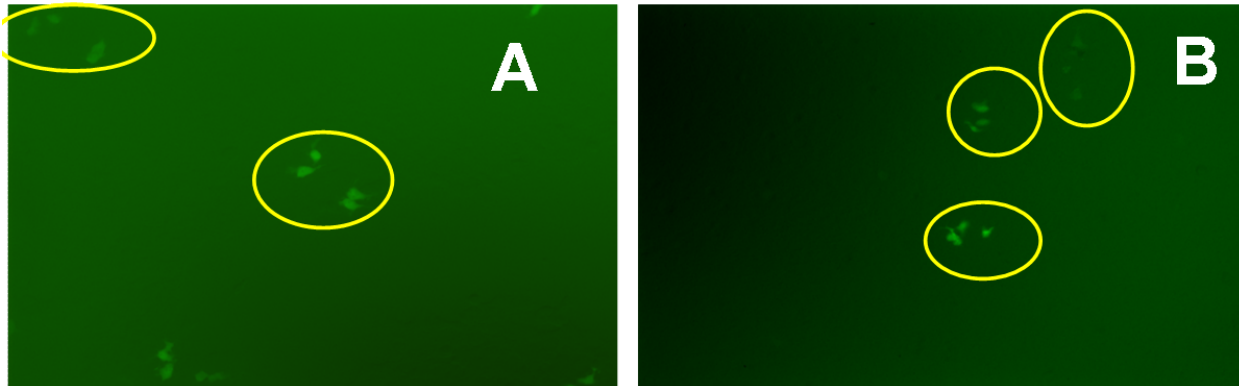


Figure 14. Examples of individual colonies

Once you have generated a lentiviral stock with a suitable titer, you are ready to transduce the lentiviral vector into the mammalian cell line of choice and assay for expression of your recombinant protein.

Multiplicity of Infection (MOI)

To obtain optimal expression of your gene of interest, you will need to transduce the lentiviral vector into your mammalian cell line of choice using a suitable MOI. MOI is defined as the number of transducing units per cell. Although this is cell line dependent, this generally correlates with the number of integration events per cell and as a result, level of expression.

Determining the Optimal MOI

A number of factors can influence determination of an optimal MOI including the nature of your mammalian cell (actively- versus non-dividing), its transduction efficiency, your application of interest, and the nature of your gene of interest. If you are transducing your lentiviral construct into the mammalian cell line of choice for the first time, after you have titered it, we recommend using a range of MOIs (e.g. 0, 0.5, 1, 2, 5, 10, 20) to determine the MOI required to obtain optimal expression for your particular application. It should be noted that to achieve single copy knockdown, an MOI of 0.3 is generally used, as less than 4% of your cells will have more than one insert.

PROTOCOL IX - TRANSDUCTION

Transduction of Target Cells

The protocol below is optimized for transduction of the lentiviral particles into HEK293T, OVCAR8 or MCF7 cells in a 24 well plate using serum free media. If a different culture dish is used, adjust the number of cells, volumes and reagent quantities in proportion to the change in surface area (see Table 13).

It is preferable that transduction be carried out in medium that is serum free and antibiotic free. A reduction in transduction efficiency occurs in the presence of serum, however it is possible to carry out successful transductions with serum present; you will have to optimize the protocol according to your needs.

1. On day 0 plate 5×10^4 cells per well in a 24 well plate. Incubate overnight.

You will be using full medium (i.e. with serum) at this stage.

2. The next day (day 1), remove the medium and add the virus to the MOI you wish to use. Set up all desired experiments and controls in a similar fashion.

Bring the total volume of liquid up so that it just covers the cells efficiently with serum-free media (See Table 15 for guidelines). If you are using concentrated virus you are likely to use very little virus volume and a lot of serum-free media; if you are using unconcentrated virus you will find you need much more virus volume.

Table 15. Suggested volumes of media per surface area per well of adherent cells.

Tissue Culture Dish	Surface area per well (cm ²)	Suggested total serum free medium volume per well (ml)
100mm	56	5
60mm	20	2
35mm	8	1
6 well	9.4	1
12 well	3.8	0.5
24 well	1.9	0.25
96 well	0.3	0.1

3. Approximately 4-6 hours post-transduction, add an additional 1ml of full media (serum plus pen-strep if you are using it) to your cells and incubate overnight.

We have experienced low toxicity with transduction in the cell lines tested, therefore removal of virus is not required for many cell lines. In our hands higher transduction efficiencies have been achieved if the virus is not removed after 6 hours. However, if toxicity is a problem, aspirate the mixture after 3-6 hours and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.

4. At 48 hours post-transduction examine the cells microscopically for the presence of reporter expression as this will be your first indication as to the efficiency of your transduction.

Note: When visualizing TurboGFP expression, if less than 90% of all cells are green, it is recommended in these cases to utilize puromycin selection in order to reduce background expression of your gene of interest from untransduced cells.

- d. If adding puromycin, use the appropriate concentration as determined based on the above kill curve. Incubate.
- e. Approximately every 2-3 days replace with freshly prepared selective media.
- f. Monitor the cells daily and observe the percentage of surviving cells. At some time point almost all of the cells surviving selection will be harboring the shRNAmir. Optimum effectiveness should be reached in 3-6 days with puromycin.

Please note that the higher the MOI you have chosen the more copies of the shRNAmir and puromycin resistance gene you will have per cell. When selecting on puromycin, it is worth remembering that at higher MOIs, cells containing multiple copies of the resistance gene can withstand higher puromycin concentrations than those at lower MOIs. Adjust the concentration of puromycin to a level that will select for the population of transduced cells you wish to select for, without going below the minimum antibiotic concentration you have established in your kill curve.

5. Once your transduction efficiency is at an acceptable level (with or without puromycin selection), you can proceed to assay cells for reduction in gene or reporter activity by quantitative/real-time PCR (QPCR), western blot or other appropriate functional assay; compare to untreated, reporter alone, non-silencing shRNAmir or other negative controls.

Optimal length of incubation from the start of transfection to analysis is dependent on cell type, gene of interest, and the stability of the mRNA and/or protein being analyzed. QPCR generally gives the best indication of expression knockdown. The use of western blots to determine knock-down is very dependent on quantity and quality of the protein, its half-life, and the sensitivity of the antibody and detection systems used.

PROTOCOL X - QPCR

QPCR Experimental Recommendations:

One of the biggest challenges of any QPCR experiment is to obtain reproducible reliable data. Due to the sensitivity of this multi-step technique care must be taken to ensure results obtained are accurate and trustworthy.

1. Experimental samples should be run in no less than duplicate. It should be noted that with duplicate experiments it will not be possible to assign error bars to indicate consistency from experimental sample to experimental sample. Using triplicate samples or higher will enable error bars to be assigned indicating the level of experimental variation.
2. QPCR should be done in no less than triplicate. Again, it should be noted that with duplicate reactions it will not be possible to assign error bars to indicate the consistency in your QPCR reactions. Using triplicate samples or higher will enable error bars to be assigned indicating the level of variation between QPCR reactions.

3. We have found that normalizing the RNA concentration prior to cDNA synthesis will increase consistency downstream.
4. Make sure the message you are using as your internal control for QPCR is expressed at a level higher than your target genes message.
5. Use only high-quality calibrated pipettes, in conjunction with well fitting barrier tips.
6. When pipetting, take the time to visually inspect the fluid in the tip(s) for accuracy and lack of bubbles, especially when using a multi-channel pipette.
7. Be sure to spin your QPCR plate prior to loading in the machine in order to collect the sample at the bottom of the well as well as eliminate any bubbles that may have developed.
8. With regard to knockdown experiments using shRNA, it is vitally important that you greatly reduce if not eliminate entirely those cells which are not transduced or transfected from the population (i.e. those cells that are not expressing the fluorescent marker). This can be done in several ways: increase the efficiency of your transfection, use a higher multiplicity of infection (MOI) for your transduction, or utilize the puromycin selection marker and drug select against those cells that do not contain the shRNA.
9. Always utilize the non-silencing control as a reference for target gene expression, as opposed to an untreated sample. The non-silencing treated samples will most accurately reproduce the conditions in your experimental samples. The non-silencing best controls for changes in QPCR internal control gene expression.
10. You may also use an untreated sample to indicate substantial changes in target gene expression as seen in the non-silencing control due to generic consequences of viral infection/transfection reagents etc. However, it should be noted that small changes in expression levels between an untreated sample and the non-silencing control are to be expected.
11. Ct values greater than 35 should be avoided as they tend to be more variable. Samples with such high Ct values should be repeated at higher cDNA concentrations and with a lower expressing QPCR internal control (such as TBP).
12. Ct values less than 11 for the QPCR internal control should be avoided as it is difficult to determine a proper background subtraction using these values. If this occurs, use Ct values from both your internal control as well as your experimental target to determine an optimum cDNA concentration.
13. It may be necessary to change internal controls if conditions in steps 11 and 12 cannot be simultaneously met.

CONTROLS AND VALIDATION

RNAi *in vitro* shRNA mir starter kits

The use of vector-based RNAi for gene silencing is a powerful and versatile tool. Successful gene silencing *in vitro* is dependent on several variables including 1) The target cell line being studied 2) Transfection and transduction efficiency 3) Abundance of the mRNA or protein of interest in the target cell line 4) Half life of the protein 5) Robust experimental protocols. For all these reasons it is very important to run controlled experiments where the transfection and transduction efficiencies are as high as possible and measurable.

Controls are a critical part of a gene silencing experiment. They enable accurate representation of knockdown data and provide confidence in the specificity of the response. Changes in the mRNA or protein levels in cells treated with negative or non-silencing controls reflect non-specific responses in cells and can be used as a baseline against which specific knockdown can be measured. Positive controls are useful to demonstrate that your experimental system is functional and your shRNA construct is successfully activating the RNAi pathway.

Controls

The pGIPZ™ EG5 and GAPDH lentiviral shRNAmir vectors have been validated as positive controls for RNAi experiments performed using the pGIPZ shRNAmir-containing lentiviral vectors. These shRNAmir have been tested in transduction based experiments and have shown efficient knockdown at both mRNA and protein levels. The EG5 control has been validated to knockdown human EG5 by means of QPCR and *in situ* hybridization of cells in tissue culture. The GAPDH control has been validated to knockdown human and mouse GAPDH by QPCR. The pGIPZ non-silencing lentiviral shRNAmir vector has been validated as negative control for RNAi experiments performed using the pGIPZ shRNAmir-containing lentiviral vectors.

Transduction based validation studies

HEK293T cells were trypsinized from a healthy, growing culture, seeded into 24 well plates at $5-8 \times 10^4$ cells per well and allowed to adhere for 24 hours in DMEM with 10% FCS.

DMEM containing serum was replaced with 200 μ l serum free media and lentiviral particles containing GAPDH or EG5 shRNAmir, non-silencing or non-transduced controls were added to the appropriate wells at three different multiplicity of infections (MOI) and incubated for 6 hours. DMEM containing serum was then added and the transduced cells were further incubated for a total of 48 hours

RNA Extraction and Validation

At 48 hours post-transduction, transduced cells were lysed and total RNA was extracted using the Qiagen RNeasy Kit (catalog no. 74104). The RNA was converted to cDNA using the ABI-High Capacity cDNA RT Kit (catalog no. 4368813), using 500ng total RNA in a 100 μ l reaction. A 1/100 dilution of the cDNA was used in QPCR. Each gene was validated in triplicate, standardized to a 18s endogenous control and compared to non-silencing or non-transduced experimental controls. Knockdown was calculated as the percentage remaining gene expression normalized to the relevant non-silencing control (Figures 15, 16 and 17). The non-silencing control was shown to not knockdown endogenous genes (Figure 15).

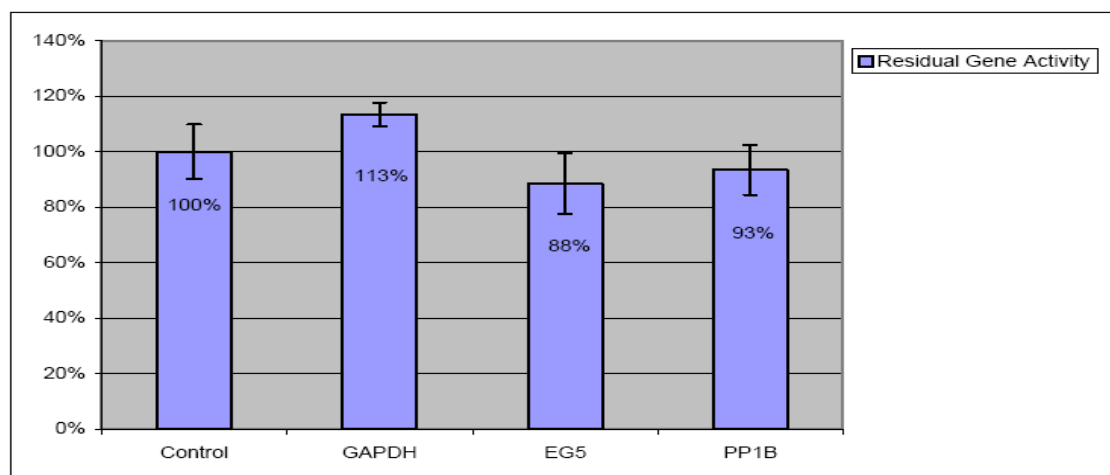


Figure 15. Non-silencing lentiviral shRNAmir control does not knockdown endogenous genes. The above data represents the baseline amount of GAPDH, EG5 or PP1B mRNA set at 100% in the control. The relative amounts of each of these mRNAs are then represented after treatment with non-silencing shRNAmir. Thus the non-silencing shRNAmir has no significant effect on endogenous gene expression.

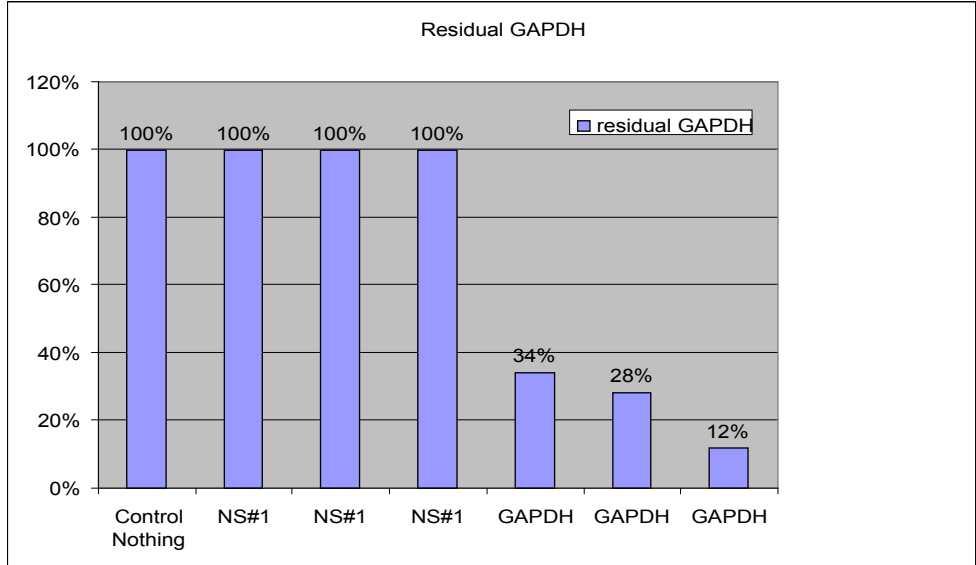


Figure 16. HEK293T cells were transduced with lentiviral particles expressing GAPDH or non-silencing shRNAmir at variable MOIs ranging from 9-48. The graph depicts the residual levels of GAPDH relative to its non-silencing control.

Knockdown of the EG5 (KIF11) gene allowed evaluation of phenotypic evidence of RNAi as well as its molecular manifestation (Figures 17 and 18).

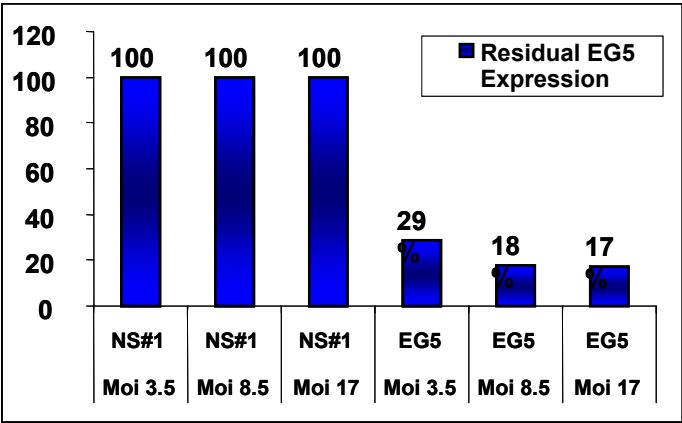


Figure 17. HEK293T cells were transduced with lentiviral particles expressing EG5 or non-silencing shRNAmir at MOIs of 3.5, 8.5 and 17. The graph depicts the residual levels of EG5 relative to its non-silencing control.

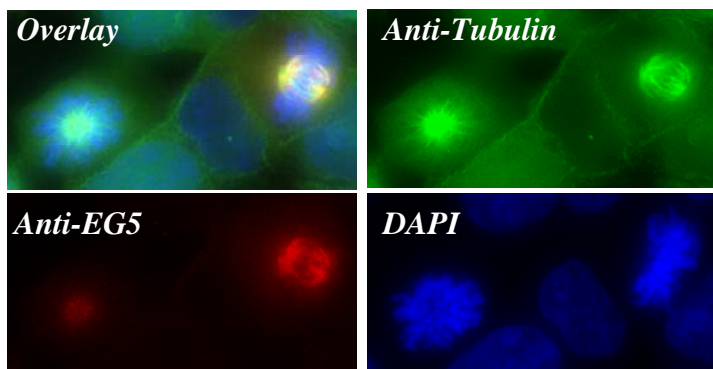


Figure 18. The characteristic phenotype observed by the targeting of the EG5 (KIF11) gene results in the formation of half spindles, mitotic arrest and monoastral microtubular arrays (green, see the cell on the left). By contrast, normal cells show bipolar spindles and microtubule networks in mitosis and in interphase (see the cell on the right). The comparative expression of EG5 (red) between the cell on the left and the right shows the extensive knockdown of EG5 in the cell displaying the phenotype (left). The cells were visualized at 100x magnification using a Leica DMIRB fluorescence microscope. HEK293T cells were stained for tubulin (anti-tubulin, green), DNA (DAPI, blue) and EG5 (anti-EG5, red).

RELATED REAGENTS

Table 16. Related Reagents

Reagent	Vendor	Catalog number
GAPDH verified positive control*	Open Biosystems	RHS4371
EG5 verified positive control*	Open Biosystems	RHS4480
Non-silencing verified negative control*	Open Biosystems	RHS4346
Arrest-In™ transfection reagent 0.5ml-10ml*	Open Biosystems	ATR1740-1743
pGIPZ™ empty vector	Open Biosystems	RHS4349
Trans-Lentiviral GIPZ Packaging System	Open Biosystems	TLP4614
Trans-Lentiviral GIPZ Packaging System (contains cell line)	Open Biosystems	TLP4615

*these items also available in the lentiviral RNAi/shRNAmir starter kit (catalog no. RHS4287)

FAQS

For answers to questions that are not addressed here, please email technical support at info@openbiosystems.com with your question, your sales order or purchase order number and the catalog number or clone ID of the construct or collection with which you are having trouble.

What clones are part of my collection?

A CD containing the data for this collection will be shipped with each collection. This file contains the location and accession number for each construct in the collection.

This data file can be downloaded from the lentiviral pGIPZ™ product page:

<http://www.openbiosystems.com/RNAi/shRNAmirLibraries/GIPZLentiviralshRNAmir/>

Where can I find the sequence of an individual shRNAmir construct?

If you are looking for the sequence an individual shRNAmir construct, you can use the gene search. Just enter the catalog number or clone ID of your hairpin into the gene search on the Open Biosystems website, hit submit and then click on the query result. If you then click on the oligo ID (the V2 number) and then click on the word “sequence” in the details grid, the hairpin sequence is listed with the target, mir-30 context and loop sequences annotated. If you are

looking for the sequence of several shRNAmir constructs, you can access this information in the data file of the collection. This data file can be downloaded from the lentiviral pGIPZ product page:

<http://www.openbiosystems.com/RNAi/shRNAmirLibraries/GIPZLentiviralshRNAmir/>

Which antibiotic should I use?

You should grow all pGIPZ constructs in both 25µg/ml zeocin and 100µg/ml carbenicillin for archive replication. You should grow the constructs in media containing **only 100µg/ml carbenicillin** for plasmid preparation.

What packaging cell line should I use for making lentivirus?

The pGIPZ vector is *tat* dependant, so you must use a packaging system that expresses the *tat* gene. For packaging our lentiviral shRNAmir constructs, we recommend the Trans-Lentiviral™ shRNA Packaging System (TLP4614, TLP4615). The Trans-Lentiviral shRNA Packaging System allows creation of a replication-incompetent (Shimada, et al. 1995), HIV-1-based lentivirus which can be used to deliver and express your gene or shRNAmir of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral shRNA Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes (Kappes and Wu 2001). For protocols and information on packaging pGIPZ™ with our Trans-Lentiviral shRNA Packaging System, please see the product manual available at the following link:

http://www.openbiosystems.com/collateral/rnai/pi/Trans-Lentiviral_GIPZ_Packaging_System.pdf

Can I use any 2nd generation packaging system with the GIPZ vector?

The pGIPZ vector is *tat* dependant, so you must use a packaging system that expresses the *tat* gene.

What does the number 40 refer to in the formula for the calculation of titer?

The titer units are given in transducing units (TU) per ml, so the number 40 is used to convert the 25µl used in the titration ("volume of diluted virus used", Table 3) to one milliliter.

What is the sequencing primer for GIPZ?

The pGIPZ sequencing primer is 5'- GCATTAAAGCAGCGTATC -3'

Notes: The binding site lies from base 5820-5842 and runs in the reverse complement direction. The melting temperature of this 18mer=52.7°C.

How can I make a stable cell line?

In order to generate stable cell lines, it is important to determine the minimum amount of puromycin required to kill non-transfected/transduced cells. This can be done by generating a puromycin kill curve. After you have determined the appropriate concentration of puromycin to use, you can transfect or transduce your cells with the shRNA construct and culture with puromycin in order to select for those cells that have a stable integrant. Cells not containing a stable integrant will not be selected for.

Where do you purchase puromycin?

We purchase puromycin from Cellgro™ (catalog no. 61-385-RA).

How many transfections are available in each volume size of Arrest-In™?

The number of transfections that can be performed depends on the size of the culture dish used and the volume size of Arrest-In purchased. Refer to Table 17 below for the approximate number of transfections.

Table 17. Number of transfections depending on culture dish size and volume of Arrest-In™ purchased.

Tissue Culture Dish	Surface area per well (cm ²)	Arrest-In (1mg/ml) (µg)*	0.5ml qty (rxns)**	1.0ml qty (rxns)**	5.0ml qty (rxns)**	10ml qty (rxns)**
60 mm	20	21	47-50	100	500	1000
35 mm	8	10	100	200	1000	2000
6 well	9.4	10	100	200	1000	2000
12 well	3.8	5	200	400	2000	4000
24 well	1.9	2.5	400	800	4000	8000
96 well	0.3	0.5-1	1000	2000	10000	20000

**Recommended starting amounts of Arrest-In reagent as defined in Table 1.

**Approximate number of transfections based on recommended starting amount of Arrest-In. Individual results may vary depending on amounts of Arrest-In used.

TROUBLESHOOTING

For help with transfection or transduction of your lentiviral constructs, please email technical support at info@openbiosystems.com with the answers to the questions below, your sales order or purchase order number and the catalog number or clone ID of the construct with which you are having trouble.

1. Are you using direct transfection or transduction into your cell line?
2. What did the uncut and restriction digested DNA look like on a gel?
3. What was the transfection efficiency if you used direct transfection? What transfection reagent was used?
4. Were positive and negative knockdown controls used (i.e. our GAPDH or EG5 validated positive controls and the validated non-silencing negative control)?
5. What were the results of the controlled experiments?
6. How was knockdown measured (i.e. quantitative real-time RT-PCR or western blot)?
7. What is the abundance and the half-life of the protein? Does the protein have many isoforms?
8. What packaging cell line was used if you are using infection rather than transfection?
9. What was your viral titer?
10. What was your MOI?
11. Did you maintain the cells on puromycin after transfection or transduction?
12. How much time elapsed from transfection/transduction to puromycin selection?

If transfection into your cell line is unsuccessful, you may need to consider the following list of factors influencing successful transfection:

1. Concentration and purity of plasmid DNA and nucleic acids-determine the concentration and purity of your DNA using 260/280nm absorbance. Avoid cytotoxic effects by using pure preparations of nucleic acids.
2. Insufficient mixing of transfection reagent or transfection complexes.
3. Transfection in serum containing or serum free media-our studies indicate that Arrest-In™/DNA complexes should preferably be formed in the absence of serum. In the cell lines tested we found that the highest transfection efficiencies can be obtained if the cells are

exposed to the transfection complexes in serum free conditions followed by the addition of medium containing twice the amount of normal serum to the complex medium 3-6 hours post transfection (leaving the complexes on the cells). However, the serum free transfection medium can be replaced with normal growth medium if high toxicity is observed.

4. Presence of antibiotics in transfection medium-the presence of antibiotics can adversely affect the transfection efficiency and lead to increased toxicity levels in some cell types. It is recommended that antibiotics be excluded until transfection has mostly occurred (3-6 hours) and then be added together with the full medium.
5. Cell history, density, and passage number-it is very important to use healthy cells that are regularly passaged and in growth phase. The highest transfection efficiencies are achieved if cells are plated the day before, however, adequate time should be given to allow the cells to recover from the passaging (generally >12 hours). Plate cells at a consistent density to minimize experimental variation. If transfection efficiencies are low or reduction occurs over time, thawing a new batch of cells or using cells with a lower passage number may improve the results.

If transduction into your cell line is unsuccessful, you may need to consider the following list of factors influencing successful transduction:

1. Transduction efficiency is integrally related to the quality and the quantity of the virus you have produced. Factors to bear in mind when transducing include MOI (related to accurate titer), the presence of serum in the media, the use of polybrene in the media, length of expose to virus, and viral toxicity to your particular cells.
2. High quality transfer vector DNA and the appropriate and efficient viral packaging are required to make high quality virus able to transduce cells effectively. See also suggestions 3-6 for factors influencing successful transfection (above).
3. All cell lines are not equally permissible to transduction by lentivirus. You may consider testing additional cell lines to find one more suitable for your experiments.

If Arrest-In™ seems to be toxic to a particular cell line, try reducing the DNA:Arrest-In ratio.

REFERENCES

Cited references as well as suggested reading:

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