

Expression Arrest™ pSM2 Retroviral shRNAmir Manual

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PRODUCT DESCRIPTION

The Expression Arrest pSHAG-MAGIC2 (pSM2) retroviral shRNAmir library from Open Biosystems is a whole genome RNAi resource and an excellent choice for transient, stable and *in vivo* RNAi studies. This collection was developed in collaboration with Dr. Greg Hannon (CSHL) and Dr. Steve Elledge (Harvard). The collection has several unique features that make it a very versatile and efficient tool for RNAi studies including large-scale screens (Paddison, Silva et al. 2004).

SHIPPING AND STORAGE

Individual constructs are shipped as bacterial cultures of *E. coli* (DH10βpir116) in LB-Lennox (low salt) broth with 8% glycerol and 25µg/ml chloramphenicol and 25µg/ml kanamycin. Individual constructs are shipped on wet ice. Collections are shipped in 96 well plate format on dry ice. Individual constructs and collections should be stored at -80°C.

Open Biosystems checks all cultures for growth prior to shipment.

TO ALLOW ANY CO₂ THAT MAY HAVE DISSOLVED INTO THE MEDIA FROM THE DRY ICE IN SHIPPING TO DISSIPATE, PLEASE STORE CONSTRUCTS AT -80°C FOR AT LEAST 48 HOURS BEFORE THAWING.

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 22-nt of dsRNA and a 19-nt 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. Increased Drosha and Dicer processing translates into greater siRNA/miRNA production and greater potency for expressed hairpins.

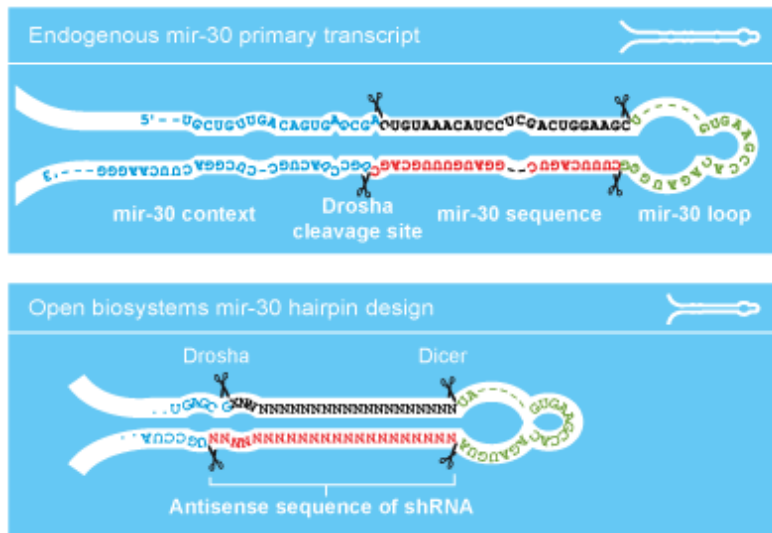


Figure 1. Expression Arrest™ shRNA are expressed as miR30 primary transcripts

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.

Due to the placement of the RNA Polymerase III transcription terminator (four or more thymidines) downstream of the hairpin, each transcript is designed to precisely terminate. RNA Polymerase III terminates on the second thymidine, two uridines remain to create a 2 base overhang. Each shRNA construct has been sequence verified before being cloned into the retroviral vector 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

Expression Arrest shRNAmir are already cloned into the pSM2 retroviral vector (Figure 2-3, Table 1). This vector has a Murine Stem Cell Virus (MSCV) backbone. Features of the vector that make it a versatile tool for RNAi studies include:

- Ability to perform transfections (transient and stable) or transductions using the replication incompetent retrovirus
- Amenable to *in vitro* and *in vivo* applications
- Puromycin drug resistance marker for selecting stable cell lines
- Molecular barcodes enable complex screening in pools

The configuration of pSM2 is shown below. The 5' and 3' flanks are derived from 125 bases surrounding the Human miR30 microRNA.

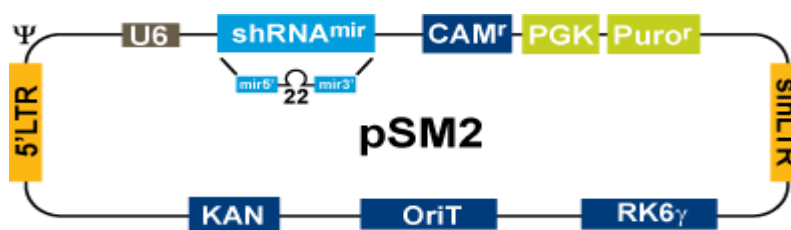


Figure 2. pSM2 Retroviral Vector

Table 1. Features of the pSM2 vector

Vector Element	Utility
U6 promoter	RNA generated with 4 uridine overhangs at each 3' end
Ψ-Retroviral Signaling Sequence	Combined with packaging extract for mammalian cell infection
PGK-Puro	Selection for transfection stability in mammalian cells
Chloramphenicol/Kanamycin	Bacterial selection marker
RK6γ	Conditional origin of replication. Requires the expression of pir1 gene within the bacterial host to propagate
5'LTR	5' long terminal repeat
SIN-LTR	3' Self inactivating long terminal repeat

ANTIBIOTIC RESISTANCE

pSM2 contains 3 antibiotic resistance markers (Table 2).

Table 2. Antibiotic resistances conveyed by pSM2

Antibiotic	Concentration	Utility
Chloramphenicol	25µg/ml	Bacterial selection marker (inside LTR's)
Kanamycin	25µg/ml	Bacterial selection marker (outside LTR's)
Puromycin	variable	Mammalian selectable marker

VECTOR MAP

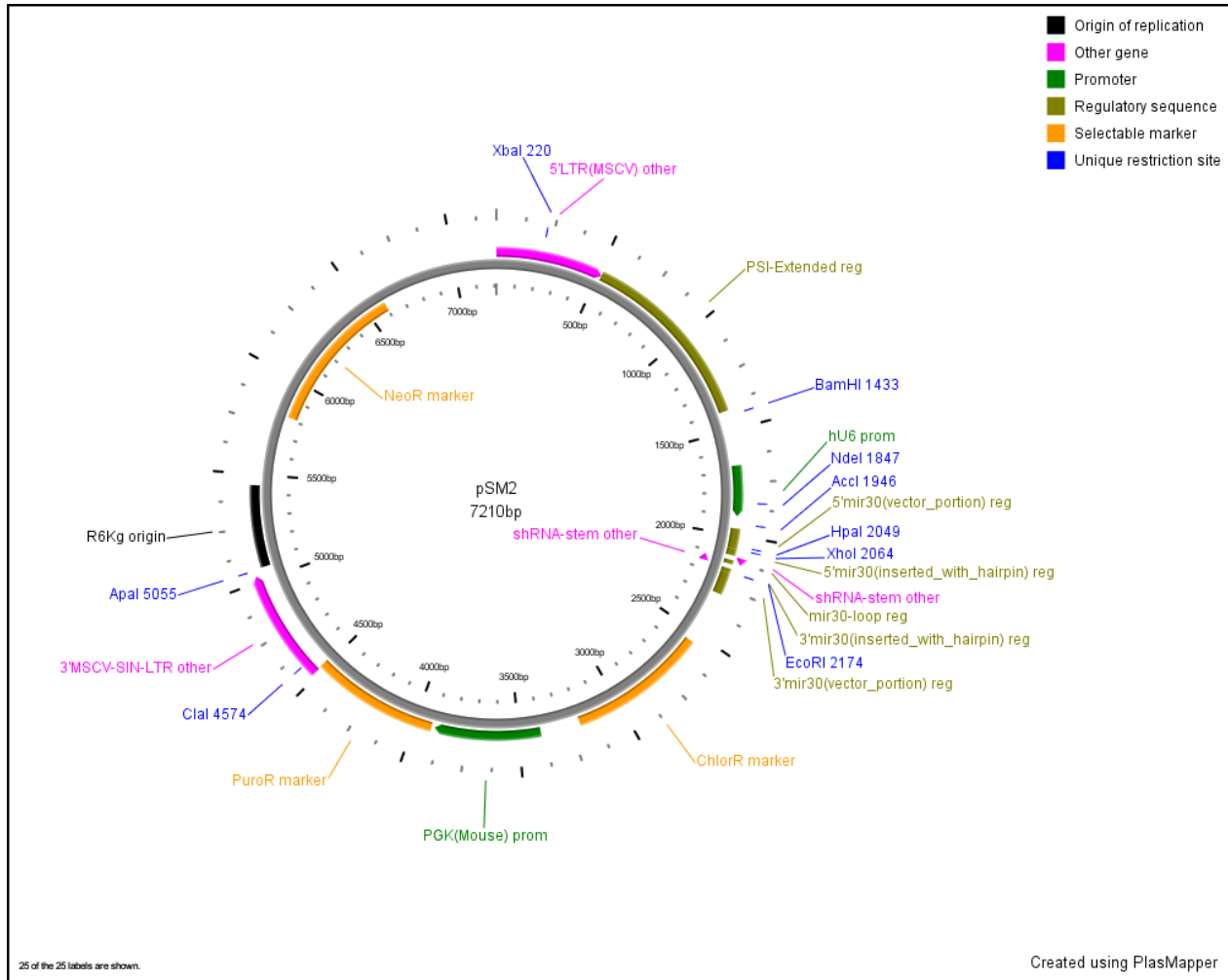


Figure 3. Detailed Vector Map of pSM2

PROTOCOLS

Culturing protocols and maintenance of pSM2

It is well known that viral vectors have a tendency to recombine producing background recombinants. Recombination occurs at the long terminal repeat regions (LTR's). The LTR recombination, which results in loss of most of the plasmid, can confer a growth advantage on the cells. It is therefore critical to maintain careful growth conditions when culturing viral vectors in *E.coli* in order to reduce the number and abundance of background recombinants.

In order to obtain a good yield of cells in a short period of incubation, rich media should be used to culture pSM2 constructs. An incubation period of 14-20 hours at 37°C with aeration is sufficient. It is recommended that the cultures remain frozen at -80°C when not in use. Freeze/thaw cycles do not seem to have any detrimental effect providing the cultures are not incubated at room temperature or higher, for long periods of time.

Gel images of plasmid isolated from cultures grown under the above conditions are shown below in Figure 4.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50

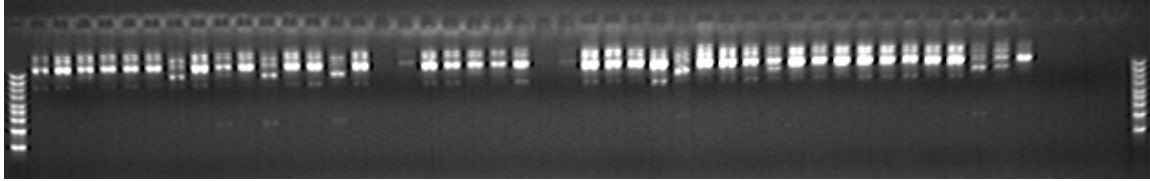


Figure 4. 1.5 ml cultures of 42 different shRNA constructs after 20 hours of incubation at 37°C with shaking (~170rpm). 2X LB media (low-salt) was used for culturing. This vector is a stable retroviral vector and shows minimal recombinants. The pSM2 band usually runs around 7kb although it is not uncommon to see bands around 10kb or even around 5kb. The presence of a faint recombinant band is seen around 1.8kb in lanes 10 and 12. There is also a possible 0.5kb recombinant band (not shown). If the recombinant product(s) is not a significant proportion (over 50%) of your plasmid prep the DNA is still acceptable for transfection since the LTR-LTR recombinant product does not contain the puromycin resistance gene or the shRNA construct.

Background recombination levels associated with pSM2

Although careful growth conditions were maintained when culturing this set, a small percentage of the whole set (~5%) still shows a low level of recombination. The following gel image of uncut samples (Figure 5) is an example of what to expect after plasmid DNA preparation.

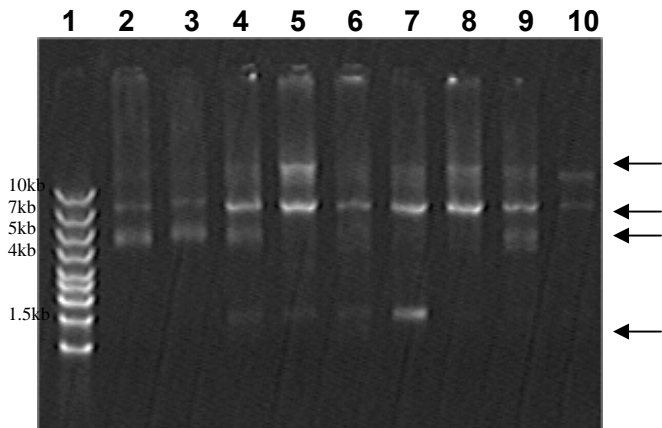


Figure 5. Lane 1– 10kb marker (10kb, 7kb, 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb), Lanes 2 to 10– 10µl of plasmid prep product of nine different shRNA constructs. A 1µl inoculum of 9 different shRNA constructs was cultured in 1ml of 2XLB medium (low-salt) in a bioblock with aeration by shaking at 200 RPM at 37°C for 16 hours. Plasmids were isolated and run uncut on a 0.9% agarose-TAE gel. The uncut pSM2 plasmid can run in a variety of conformations. The first three arrows from the top point to various forms of the correct pSM2 plasmid, which when digested with restriction enzymes produces the correct band size. The last arrow from the top points to a recombinant product (~1.8kb). There is also a possible 0.5kb recombinant band (not shown). Samples on lanes 4, 5, 6 and 7 show varying levels of recombination. Samples 2, 3, 8, 9 and 10 show minimal to no recombination.

PROTOCOL I - REPLICATION

Table 3. Materials for plate replication

Item	Vendor	Cat. No.
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-4760
Chloramphenicol	VWR	EM-3130
Kanamycin	VWR	80058-286
Puromycin	Cellgro	61-385-RA
96 well microplates	Nunc	260860
Aluminum seals	Nunc	276014
Disposable replicators	Genetix	X5054
Disposable replicators	Scinomix	SCI-5010-OS

Culture conditions for replication of shRNA constructs

Prepare media with glycerol and the appropriate antibiotics. Use minimal inoculums (0.5-1 μ l in 1ml LB) or use replicating pins for 96 well microtiter plates. Incubate with shaking (or without shaking for microtiter plates) at 37°C for 16-20 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.

You should grow all pSM2 constructs in both chloramphenicol (25 μ g/ml) and kanamycin (25 μ g/ml). You can grow the constructs in media containing only chloramphenicol (50 μ l/ml), but you CANNOT only select with kanamycin. The kanamycin resistance marker is located outside of the hairpin with respect to the LTRs, and if you select only with kanamycin you could select for a recombinant containing a kanamycin resistance marker and an origin of replication, but no hairpin.

2X-LB broth (low-salt) media preparation

Peptone	20g/l
Yeast Extract	10g/l
NaCl	5g/l
Chloramphenicol	25 μ g/ml
Kanamycin	25 μ g/ml
*Glycerol	8% for long term storage

Note: LB media can be used instead of 2XLB

**Glycerol should 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.*

Replication of plates

Prepare target plates by dispensing ~160 μ l of LB media supplemented with 8% glycerol and appropriate antibiotic (25 μ g/ml of chloramphenicol and 25 μ g/ml of kanamycin).

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 glycerol stock for each plasmid preparation.

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 kimwipe soaked in EtOH.

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 plate 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 37°C incubator for 14-20 hours.

PROTOCOL II - PLASMID PREP

Culture conditions for individual plasmid preparations

Most plasmid mini-prep kits recommend a culture volume of 1–10ml for good yield.

For shRNA constructs, 5ml of culture can be used for one mini-prep generally producing from 5–20µg of plasmid DNA.

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 or the colony glycerol stock for each plasmid preparation.

1. Upon receiving your glycerol stock(s) containing the shRNA 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. Using a sterile loop or a pipette tip, streak the shRNA culture onto a LB agar plate containing 25µg/ml chloramphenicol and 25µg/ml kanamycin. Incubate the plate overnight at 37°C. Return the glycerol stock(s) to –80°C.
4. The following day, pick 1 to 3 colonies from the agar plate and inoculate 6ml of the 2XLB plus 25µg/ml chloramphenicol and 25µg/ml kanamycin. Incubate at 37°C for 16-20 hours with vigorous shaking (300rpm).
5. The following day remove 1ml of the culture and place in a sterile 2ml sterile microcentrifuge tube. Place this tube at 4°C until the plasmid DNA from the remaining culture has been analyzed. Pellet the remaining 5ml culture and begin preparation of plasmid DNA. We recommend preparing Ultra-pure DNA to ensure both high-purity and low endotoxin levels (Qiagen Catalog no. 12123) as required for transfection into eukaryotic cells.

If you wish to continue at a later time cell pellets can be kept frozen at –20°C overnight.

- Run 3-5µl of the plasmid DNA on a 1% agarose gel. The uncut pSM2 shRNA constructs run at about 7-10kb (although many other possible conformations of the uncut pSM2 plasmid are possible). The most common products of a recombination event will run at ~1.5-1.8kb or 0.5kb. If recombination is present at a significant amount then return to the plate and pick another colony and repeat plasmid preparation. A small amount of recombination is acceptable during transfection since the LTR-LTR recombinant product does not contain the puromycin resistance gene or the shRNA.
- Prepare an 8% glycerol stock culture using the 1ml of culture you removed prior to plasmid preparation. This culture can be used for future plasmid preparations but it is still recommended you streak isolate and work from a fresh colony. Store at -80°C.

Culture conditions for 96 well plasmid preparation

Inoculate 96 well bio-block containing 1ml per well of 2X-LB media with 1µl of the culture. Incubate at 37°C with shaking (~170-200RPM). We have observed that incubation times from 16 hours produces good plasmid yield. For plasmid preparation, follow the kit protocols recommended by the manufacturer.

PROTOCOL III - RESTRICTION DIGEST

Restriction Digests of pSM2

You may wish to restriction digest a sample of your plasmid DNA following plasmid DNA preparation. The following is a protocol for dual restriction enzyme digestion using *EcoRI* and *XhoI* for quality control of pSM2 vectors (shRNA library and controls). The protocol for *HindIII/XbaI* double digest is exactly the same except replace the *EcoRI* Buffer with the 10X Buffer 2 and exchange the enzymes used. The *HindIII/XbaI* double digest is the best diagnostic digest to perform to check for recombination of the pSM2 vector.

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

Sterile, nuclease-free water	14.8µl
Restriction enzyme <i>EcoRI</i> 10X buffer	2µl
BSA (10X, 10mg/ml)	0.2µl
DNA sample 1µg, in water or TE buffer	1µl
Restriction enzyme <i>EcoRI</i> , 20U	1µl
Restriction enzyme <i>XhoI</i> , 20U	1µl
Final volume	20µl

- Mix gently by pipetting.
- Incubate in a thermalcycler at 37°C for 2.5 hours to digest then at 70°C for 20 minutes to kill the enzyme.
- Add 4µl of 6X Loading Dye (or another appropriate DNA loading buffer), and proceed to gel analysis.
- Load the gel with 20µl of each of the digested samples (a *EcoRI/XhoI* and *HindIII/XbaI*) on a 1% agarose gel. Also run 1µl (1µg) of the uncut sample combined with 16µl of water and 3µl of 6x dye alongside the digested samples.

6. The *EcoRI/XhoI* digest will release the 97bp hairpin insert and leave an approximately 7kb band. The *HindIII/XbaI* digests should have 3 bands: 3690bp, 2260bp and 1253bp (although a possible 4th band at 500bp is acceptable).

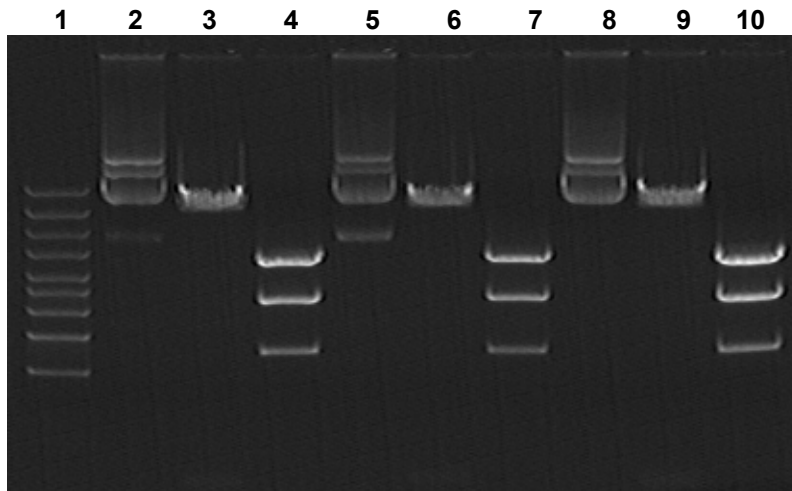


Figure 6. The 1% agarose gel above contains a 10kb ladder followed by undigested sample and restriction digests of the non-silencing shRNA control (lanes 2,3,4), the eGFP shRNA control (lanes 5,6,7) and the FFLuc shRNA control (lanes 8,9,10). For each sample the lanes are as follows: undigested sample, an *EcoRI/XhoI* digest, then the *XbaI/HindIII* digest.

Note: pSM2 must be transformed into PIR1 competent bacteria. The pSM2 plasmid harbors a conditional bacterial origin of replication, which requires the expression of the “pir1” gene to be rendered functional.

PROTOCOL IV - 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 4).

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 4. 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 (µg)*	Arrest-In (µg)**
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.

The principle is to prepare the shRNA plasmid DNA and transfection reagent dilutions in an equal amount of serum free medium in two separate tubes. These two mixtures (i.e. the DNA and the Arrest-In) will be added to each other and incubated for 20 minutes prior to addition to the cells. This enables the DNA/Arrest-In complexes to form.

- 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.

- d. Set up all desired experiments and controls in a similar fashion as outlined in Table 5. It is also advisable to set up an Arrest-In only control.

Table 5. Quantities of DNA for transfection experiments.

Type of transfection experiment	shRNA Plasmid DNA (ng)	Reporter* (ng)	Carrier DNA** (ng)	Serum free medium (final volume in μ l)
shRNA plasmid DNA	500 – hairpin to gene of interest	0	0	50
Transfection efficiency	0	500	0	50
Knockdown efficiency of reporter	450-500 – hairpin to reporter	50	0	50
Control for knockdown efficiency	0	50	450-500	50
Non-silencing control	500 – scramble hairpin	0	0	50

*It is not necessary to transfect a reporter into cells if you are using a construct which already has a reporter for convenient estimation of transfection efficiency. Recommended reporters for other vectors include GFP, luciferase, and/or β -gal (X-gal staining and/or ONPG assays).

**Carrier DNA is required to increase the total DNA quantity for the formation of adequate DNA/Arrest-In complexes. Recommended carriers are pUC19 or pBluescript plasmids.

3. Aspirate the growth medium from the cells. Add an additional 150 μ l of serum free medium to each of the tubes containing transfection complexes and mix gently. Add the 250 μ l DNA/Arrest-In complex mixture to the cells and incubate for 3-6 hours in a CO₂ incubator at 37°C.

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

4. Following the 5-6 hour incubation, add an equal volume of growth medium (250 μ l) 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. If selecting for stably transfected cells (optional), transfer the cells to medium containing puromycin for selection (protocol V). It is important to wait at least 48 hours before beginning selection.

The working concentration of puromycin needed varies between cell lines. We recommend you determine the optimal concentration of puromycin required to kill your host cell line prior to selection for stable shRNA transfectants. Typically, the working concentration ranges from 1-10 μ g/ml. You should use the lowest concentration that kills 100% of the cells in 3-5 days from the start of puromycin selection.

6. 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 shRNA 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. Quantitative/real-time RT-PCR generally gives the best indication of expression knockdown. The use of western blots to determine knockdown is very dependent on quantity and quality of the protein, its half-life, and the sensitivity of the antibody and detection systems used.

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).

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 5 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.

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 - 15µg/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.

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 transfection reagent to DNA ratio, DNA concentrations and cell confluency. We recommend that you initially begin with $5-8 \times 10^4$ cells/well of a 24 well plate, and with the Arrest-In™ and DNA amount indicated in Table 2.

Additional Factors Influencing Successful Transfection:

1. **Concentration and purity of nucleic acids** – Determine the concentration of your DNA using 260nm absorbance. Avoid cytotoxic effects by using pure preparations of nucleic acids.
2. **Transfection in serum containing or serum-free medium** – Our studies indicate that Arrest-In/DNA complexes should always 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 transfection medium can be replaced with normal growth medium if high toxicity is observed.
3. **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 these additives be initially excluded until optimized conditions are achieved, then these components can be added, and the cells can be monitored for any changes in the transfection results.
4. **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.

PROTOCOL VI - TRANSDUCTION

Transduction of Target Cells

The protocol below is optimized for transduction of the retroviral 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 6).

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 12 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 6. 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.

- a. At 48 hours post-transduction add 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.

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.

4. Once your transduction efficiency is at an acceptable level 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 VII - 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.

VALIDATION OF CONTROLS

Control hairpin sequence design

All pSM2 shRNAmir controls were designed using the same unique microRNA based design rules as used in the shRNA library and subsequently cloned into the *XhoI* and *EcoRI* restriction sites of the retroviral pSM2 vector.

The Firefly Luciferase pSM2 shRNAmir is a positive control designed against pGL3 Firefly Luciferase (Promega Catalog no. E1741). The eGFP pSM2 shRNAmir is a positive control designed against the enhanced GFP reporter (Invitrogen Catalog no. v355-20; GenBank accession number: pEGFP U76561). The non silencing pSM2 shRNAmir is a negative control containing a target sequence that does not match any known mammalian genes.

Cos-1 cells were plated in a 24-well plate at a density of 2.5×10^4 one day prior to transfection. The following day shRNA constructs were transfected into the cells using Arrest-In™ reagent. Co-transfections were performed using a 10:1 ratio of shRNA to the reporter, and CMV-βgal was used as a transfection control. At 48 hours post-transfection the activity was measured and the results were normalized to the non-silencing shRNA (Figure 7 and 8).

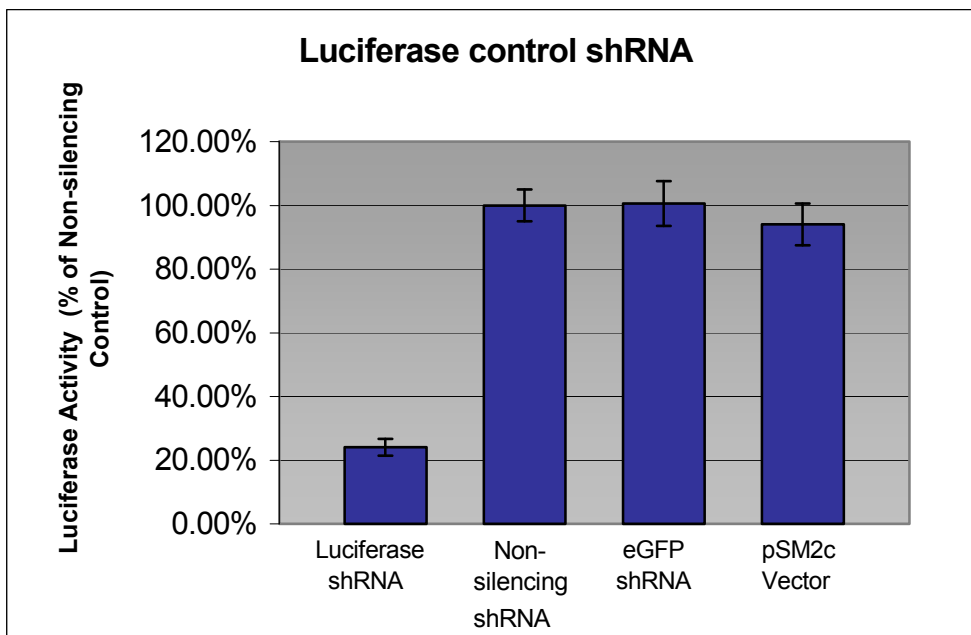


Figure 7: The data is shown as percent suppression of luciferase activity normalized to the non-silencing control. The firefly luciferase shRNA decreased luciferase activity by nearly 80% relative to the non-silencing control whereas the eGFP shRNA used as a negative control did not suppress activity.

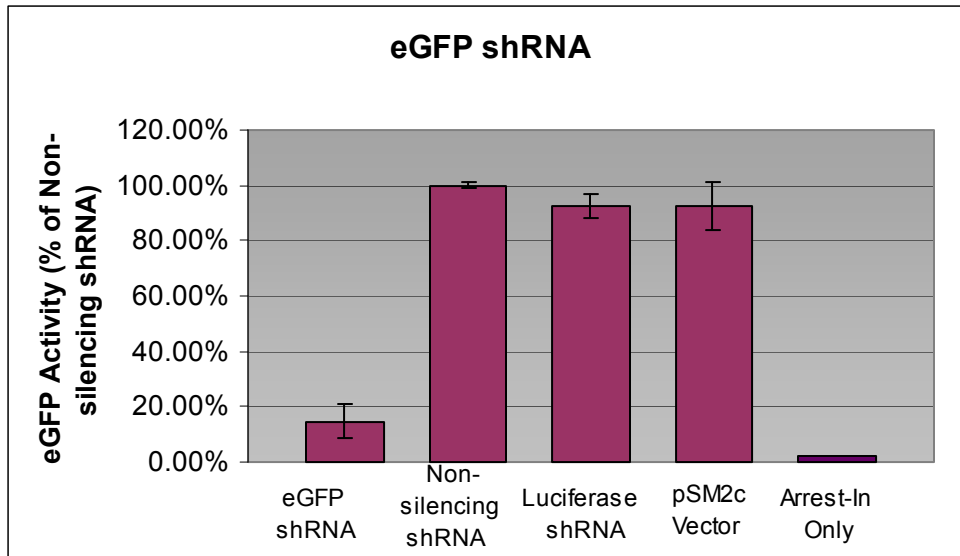


Figure 8: The data is shown as eGFP activity normalized to the non-silencing control. The eGFP shRNA illustrated a high level of silencing relative to the non-silencing shRNA whereas the Luciferase shRNA as a negative control did not affect the activity.

RELATED REAGENTS

Table 7. Related reagents

Reagent	Vendor	Catalog number
Luciferase verified positive control*	Open Biosystems	RHS1705
eGFP verified positive control*	Open Biosystems	RHS1706
Non-silencing verified negative control*	Open Biosystems	RHS1707
B-gal verified transfection control*	Open Biosystems	RHS4335
Arrest-In™ Transfection reagent 0.5ml-10mls*	Open Biosystems	ATR1740-1743
pSM2 empty vector	Open Biosystems	RHS1704
LinX retroviral packaging cell line	Open Biosystems	LNK1500
Phoenix retroviral packaging cell line	Orbigen	RVK-10001
*these items also available as RNAi shRNAmir starter kits	Open Biosystems	RHS3600-luciferase RHS3601-eGFP

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 retroviral pSM2 product page:

<http://www.openbiosystems.com/RNAi/shRNAmirLibraries/pSM2Retroviral/>

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, 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 retroviral pSM2 product page:

<http://www.openbiosystems.com/RNAi/shRNAmirLibraries/pSM2Retroviral/>

Where can I find the sequence of the pSM2 vector?

A pdf of the pSM2 vector map and sequence is located at the following link:

<http://www.openbiosystems.com/collateral/rnai/technical/pSM2%20sequence.pdf>

What is the sequencing primer for pSM2?

The sequencing primer to sequence shRNA in the pSM2 vector is the U6 primer listed below:
5' TGT GGA AAG GAC GAA ACA CC

Which antibiotic should I use?

You should grow all pSM2 constructs in both chloramphenicol (25µg/ml) and kanamycin (25µg/ml). You can grow the constructs in media containing only chloramphenicol (50µl/ml), but you CANNOT only select with kanamycin. The kanamycin resistance marker is located outside of the hairpin with respect to the LTRs, and if you select only with kanamycin you could select for a recombinant containing a kanamycin resistance marker and an origin of replication, but no hairpin.

What packaging cell line should I use for retroviral shRNAmir?

We recommend using a retroviral packaging system (i.e. Phoenix (Orbigen) or AmphoPack (Clontech)) packaging cell lines for packaging retrovirus.

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 be selected against.

TROUBLESHOOTING

For help with transfection or transduction of your retroviral 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.

Below is the list of questions we use to troubleshoot pSM2 retroviral transfection/transduction:

- 1.) Are you using direct transfection or infection into your cell line?
- 2.) What did the DNA look like on a gel? When cut with *EcoRI* and *XhoI* what were the band sizes? With *HindIII* and *XbaI*?
- 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 eGFP construct or luciferase construct and the non-silencing construct)?
- 5.) How was knockdown measured (i.e. real time PCR or western blot)?
- 6.) What is the abundance and what is the half-life of the protein? Does the protein have many isoforms?
- 7.) What packaging cell line was used if you are using infection rather than transfection?
- 8.) What was your viral titer if you are using infection rather than transfection?
- 9.) Did you maintain the cells on puromycin after transfection or transduction?
- 10.) How many individual colonies did you see during initial selection on puromycin?
- 11.) How much time elapsed from transfection/infection to puromycin selection?
- 12.) Are you assaying individual colonies or pools? We recommend that you clone out selected colonies and assay them for knockdown instead of a pool. Not all integrations are in active regions of the genome and so not all puromycin resistant clones will actively express the hairpin.

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 of your DNA using 260nm 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 5-6 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 (5-6 hours) and then be added together with the full medium.
5. High protein expression levels—some proteins when expressed at high levels can be cytotoxic; this effect can also be cell line specific.
6. 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.
3. See also suggestions 3-6 for factors influencing successful transfection (above).

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

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