Expression Arrest™ TRIPZ™ lentiviral inducible shRNAmir

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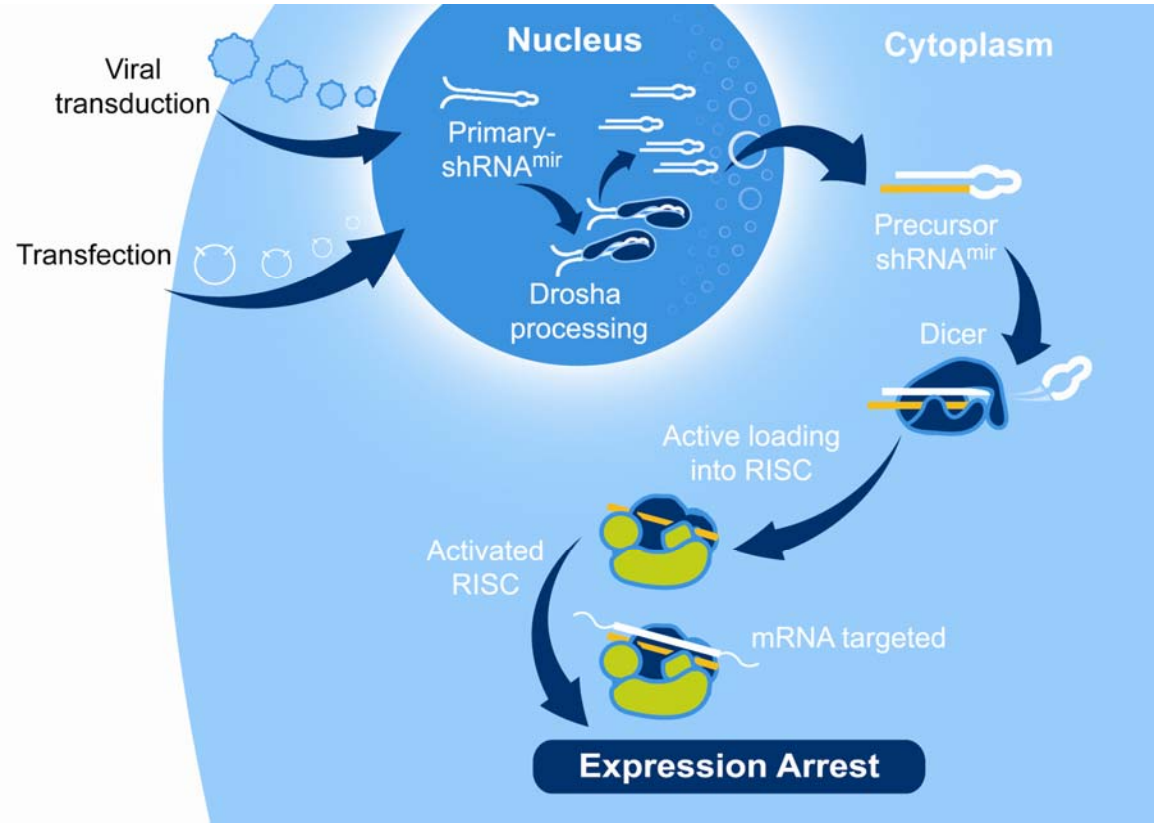

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PRODUCT DESCRIPTION

The TRIPZ lentiviral inducible 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 pTRIPZ lentiviral inducible vector to create a powerful RNAi trigger capable of producing RNAi in most cell types. The vector is engineered to be Tet-On® and produces tightly regulated induction of shRNAmir expression in the presence of doxycycline.

SHIPPING AND STORAGE

Individual constructs are shipped as bacterial cultures of *E. coli* (PrimePlus™) in LB-Lennox (low salt) broth with 8% glycerol, 100µg/ml carbenicillin and 25µg/ml zeocin. 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 CO_2 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.

Important Safety Note:

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

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 *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 *et al.* 2005). Increased Drosha and Dicer processing translates into greater siRNA/miRNA production and greater potency for expressed hairpins.

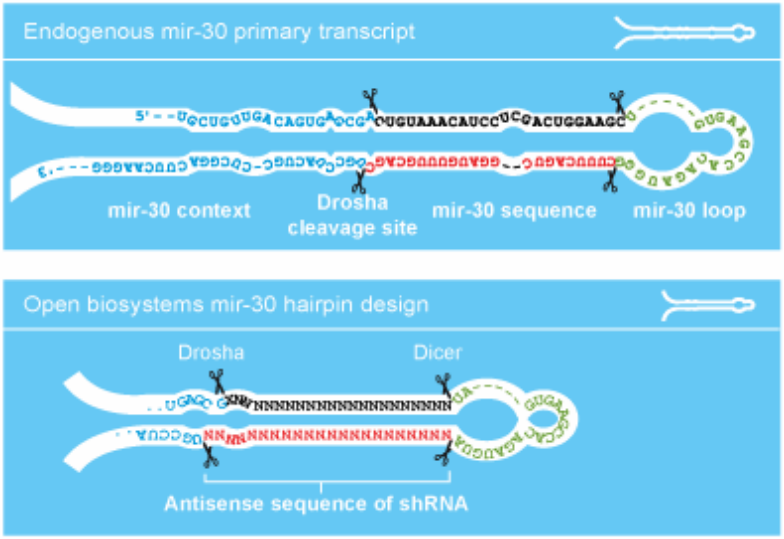


Figure 1. Expression Arrest shRNA are expressed as miR30 primary transcripts

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 before being cloned into the 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.

Tet-On® system design of the pTRIPZ™ vector

The pTRIPZ vector is engineered to be Tet-On. The Tet-On technology, equips the pTRIPZ vector to provide for induced expression of a shRNAmir in the presence of doxycycline (www.clonetech.com). There are two main components on the pTRIPZ vector enabling induction: the tetracycline response element (TRE) and the transactivator. The TRE, modified from its natural state to consist of a string of operators fused to the CMV minimal promoter, exhibits reduced basal expression and tighter binding to the second component, the transactivator. The pTRIPZ transactivator, known as the reverse tetracycline transactivator 3 (rtTA3) binds to and activates expression from TRE promoters in the presence of doxycycline. The rtTA3 transactivator is a modified version of the wildtype in two ways. First, unlike the original tetracycline transactivator the rtTA3 is modified to bind to the TRE in the presence of doxycycline rather than in its absence. Secondly, there are three mutations within the transactivator that increase its sensitivity to doxycycline by 25-fold over the initial rtTA without increasing background activity (Das, *et al*. 2004).

Use of TurboRFP™ in the pTRIPZ vector

As an added feature of the pTRIPZ vector, the TRE drives the expression of a TurboRFP reporter in addition to the shRNAmir. This induced expression of TurboRFP enables the user to easily observe expression from the TRE promoter, allowing quick assessment of factors such as: basal expression, viral titer, transduction efficiency/efficacy and overall technical success.

Tet-On or Tet-off® configuration is possible

The pTRIPZ vector is versatile in that it can be easily converted to a Tet-Off capable vector using Cre/loxP technology or classical restriction digest. The rtTA3 is flanked by loxP sites allowing *in vitro* or *in vivo* excision of the rtTA3 by exposure to Cre recombinase. The rtTA3 is also flanked by a pair of *Bam*HI restriction sites allowing for straightforward cleavage and ligation of the vector to remove the rtTA3. Without the rtTA3 present on the vector a tetracycline transactivator (tTA) can be added extraneously to the system allowing it to function as Tet-Off; where expression of shRNAmir and TurboRFP are alternatively induced in the absence of doxycycline. The functionality and versatility of the pTRIPZ vector is thus unsurpassed in the field of RNAi.

VECTOR INFORMATION

Versatile vector design

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

- Ability to use the vector in either a Tet-On or Tet-Off configuration
- TurboRFP and shRNAmir are part of a single transcript allowing the visual marking of shRNAmir expressing cells
- Amenable to *in vitro* and *in vivo* applications
- Inducible RNAi expanded to include both dividing and non-dividing cell lines
- Puromycin drug resistance marker for selecting stable cell lines
- Molecular barcodes enable multiplexed screening in pools

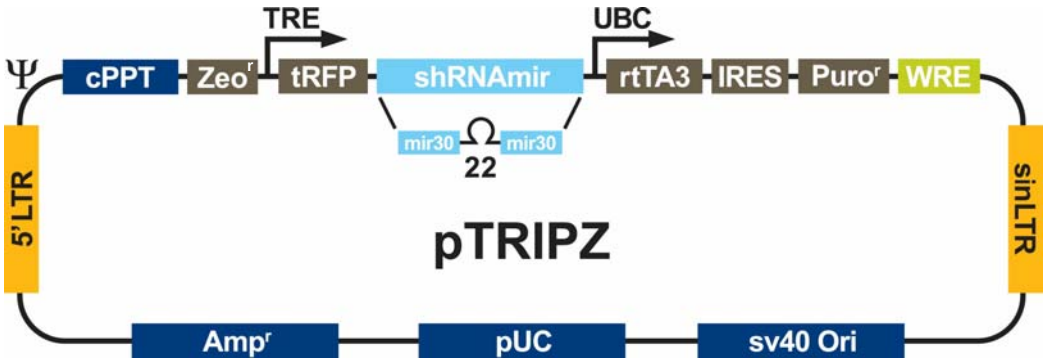


Figure 2. pTRIPZ™ inducible lentiviral vector

Table 1. Features of the pTRIPZ vector

Vector Element	Utility
TRE-minCMV promoter	Tetracycline responsive RNA Polymerase II promoter
UBC promoter	Drives expression of rtTA3 and IRES-puro
rtTA3	Reverse tetracycline transactivator
cPPT	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
WRE	Enhances the stability and translation of transcripts
TurboRFP	Marker to track shRNAmir expression
IRES-Puro	Mammalian selectable marker
AMP	Ampicillin bacterial selectable marker.
5'LTR	5' long terminal repeat
pUC ori	High copy replication and maintenance of plasmid in *E.coli*
SIN-LTR	Self inactivating long terminal repeat
RRE	Rev response element
Zeo	Bacterial selectable marker

ANTIBIOTIC RESISTANCE

pTRIPZ contains 3 antibiotic resistance markers (Table 2).

Table 2. Antibiotic resistances conveyed by pTRIPZ

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

VECTOR MAP

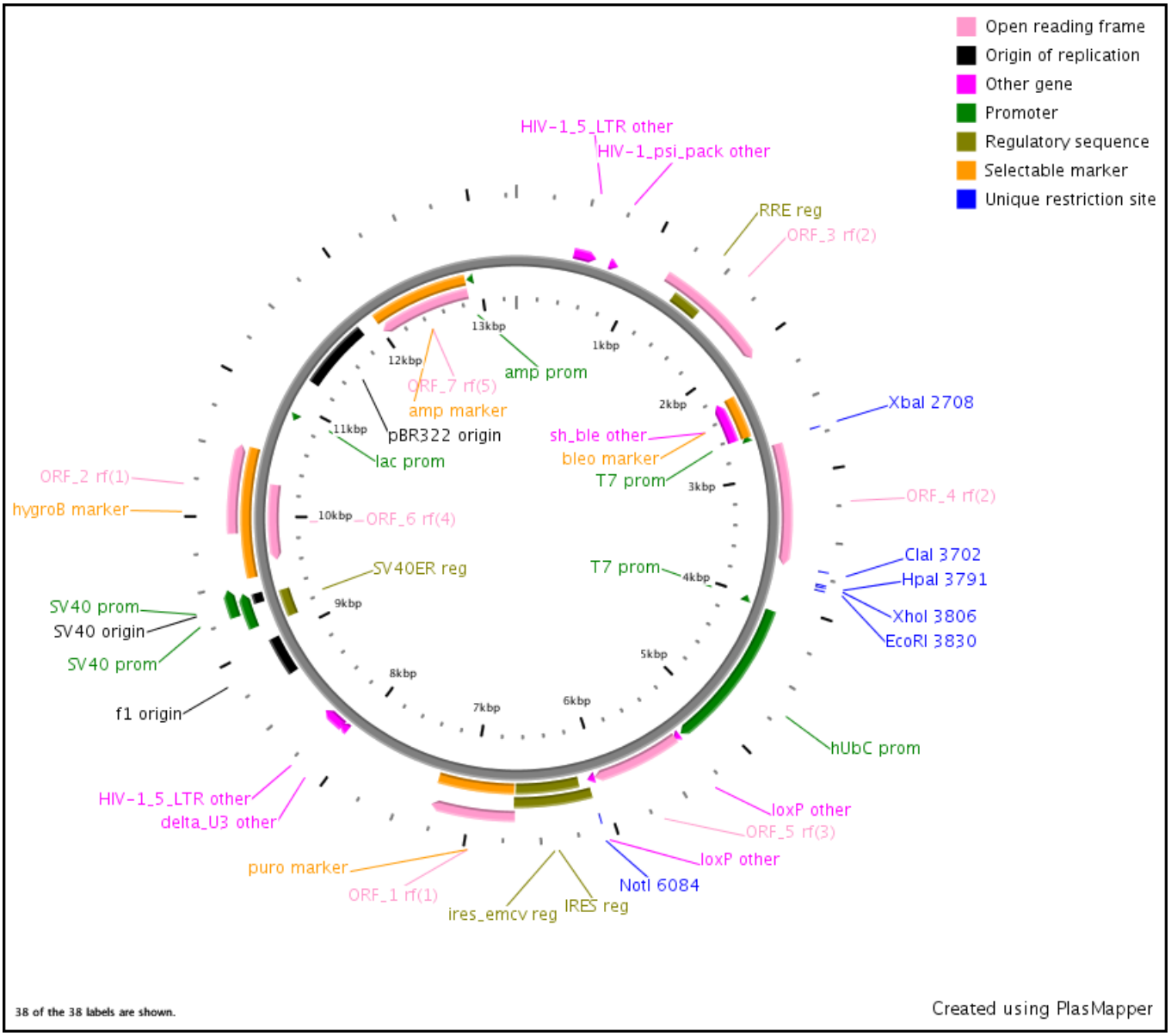


Figure 3. Detailed Vector Map of the pTRIPZ™ lentiviral vector (without hairpin). The empty vector is 13320 bp in size.

PROTOCOLS

Culturing protocols and maintenance of pTRIPZ™

The pTRIPZ lentiviral shRNAmir library has passed through internal QC processes to ensure high quality and low recombination (Figure 4).

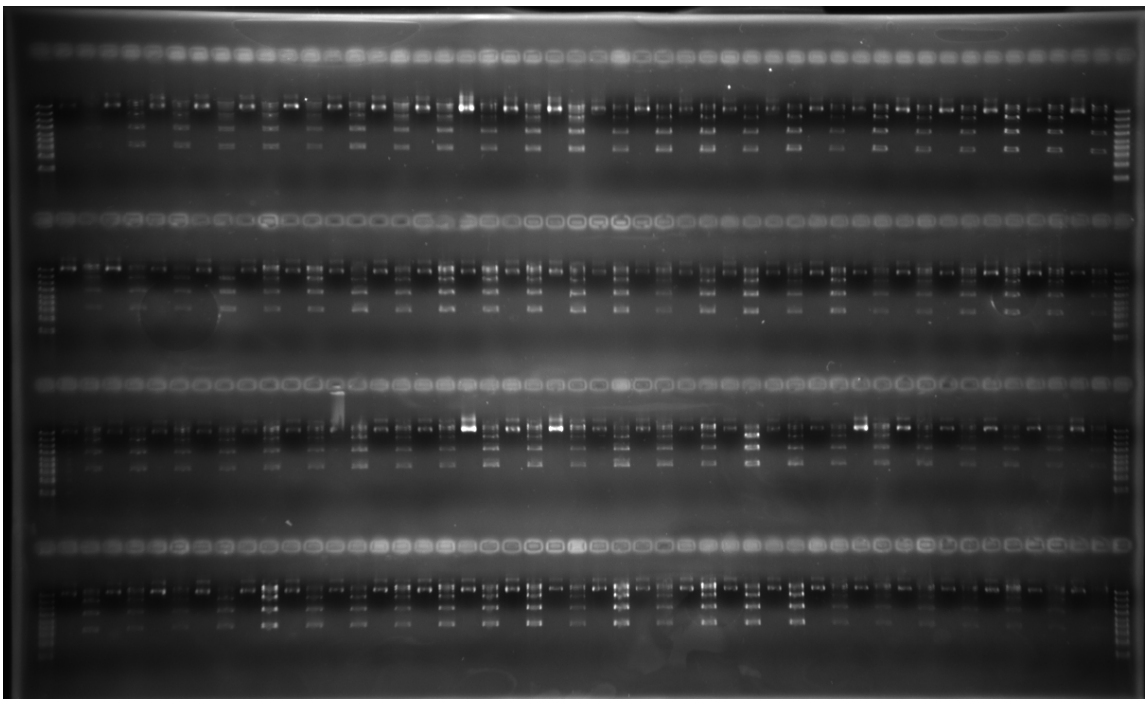


Figure 4. Representative shRNAmir containing pTRIPZ lentiviral clones grown for 18 hours at 37°C and the plasmid isolated. Clones were then digested with *Sal*I and run out on a gel. The expected band sizes are **7104bp, 4028bp, 2188bp.** No recombinant products are visible. 10kb molecular weight ladder (10kb, 7kb, 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb)

The pTRIPZ vector appears stable without showing any recombination.

PROTOCOL I – PLATE 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	VWR	EM-3130
Zeocin	Invivogen	ant-zn-5p
96 well microplates	VWR	62407-174
Aluminum seals	VWR	73520-056
Disposable replicators	Genetix	X5054
Disposable replicators	Scinomix	SCI-5010-OS

For archive replication, grow all pTRIPZ™ 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 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 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 PREP
Culture conditions for individual plasmid preparations
For plasmid preparation, grow all pTRIPZ clones at 37°C in 2X-LB broth (low salt) media plus 100µg/ml carbenicillin only for rapid growth.

2X-LB broth (low salt) media prepration

Peptone	20g/l
Yeast Extract	10g/l
NaCl	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.
4. Incubate at 37°C for 18-19 hours with vigorous shaking.
   *If a larger culture volume is desired, use the 3-5ml overnight culture as a starter inoculum. 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. pTRIPZ™ without shRNAmir is 13320bp.

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.

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 - RESTRICTION DIGEST

The following is a sample protocol for restriction enzyme digestion using S*al*I for diagnostic quality control of pTRIPZ lentiviral vectors.

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

Component	Amount
Sterile, nuclease-free water	Xµl
Restriction enzyme 10X buffer	1µl
DNA sample (400ng) in water	400ng
Restriction enzyme 10U (NEB)	0.5µl
Final volume	20µl

2. Mix gently by pipetting.
3. Incubate in a thermalcycler at 37°C for 2 hours to digest
4. Load the gel with 20µl of each of the digested samples (using *Sal*I) on a 1% agarose gel. Run uncut sample alongside the digested samples.

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 (cm2)	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 x 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 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 µl)
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 the reporter	50	0	50
Control for knockdown efficiency	0	50	450-500	50
Non-silencing control	500 – scrambled 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, RFP, 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 150µl of serum free medium to each of the tubes containing transfection complexes and mix gently. Add the 250µl DNA/Arrest-In complex mixture to the cells and incubate for 3-6 hours in a CO_2 incubator at 37°C.

Your total volume will be 250µl at this stage.

4. Following the 3-6 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 CO_2 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 3-6 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. It is important to wait at least 48 hours post transfection 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 1-10µg/ml. You should use the lowest concentration that kills 100% of the cells in 3-5 days from the start of puromycin selection.

Induction of TurboRFP/shRNAmir Expression in Transfected Cells using Doxycycline
Optimization within a range of 0.1µg/ml - 2µg/ml doxycycline is recommended for transfected cells as amounts of doxycycline necessary will vary due to the transfectablility of the cell line, amounts of DNA used to transfect as well as other variables.

After 24-72 hours of incubation, examine the cells microscopically for the presence of TurboRFP expression as this will be your first indication as to the efficiency of your transfection. Then assay cells for reduction in gene activity by quantitative/real-time RT-PCR, western blot or other appropriate functional assay; compare to untreated, 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 knock-down. The use of western blots to determine knock-down is very dependent on quantity and quality of the protein sample, 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 4 and 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 - PACKAGING LENTIVIRUS

For packaging our pTRIPZ™ inducible lentiviral shRNA constructs, we recommend the Trans-Lentiviral™ shRNA Packaging System (TLP4614, TLP4615). The Trans-Lentiviral shRNA Packaging System allows creation of a replication-incompetent, HIV-1-based lentivirus which can be used to deliver and express your gene or shRNA of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes, *et al.* (2001). For protocols and information on packaging pTRIPZ with our Trans-Lentiviral shRNA Packaging System, please see the product insert available at the following link:
http://www.openbiosystems.com/Viral%20Packaging/TransLentiviral%20Packaging%20Syst/

PROTOCOL VI - TRANSDUCTION

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 293T cell line as an alternative.

Note: If you have generated a lentiviral stock of the expression control (e.g. pTRIPZ™ 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 x 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 Dilution Media (DMEM containing 0.5% FBS and 8µg/ml polybrene). Utilize the plate as shown in Figure 5 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.

   Polybrene is a cation that is often pre-incubated with the virus particles to give it a net positive charge, which helps counteract the negatively-charged cell surface membrane. Polybrene – (Sequabrene™, Sigma Catalog no. S-2667).

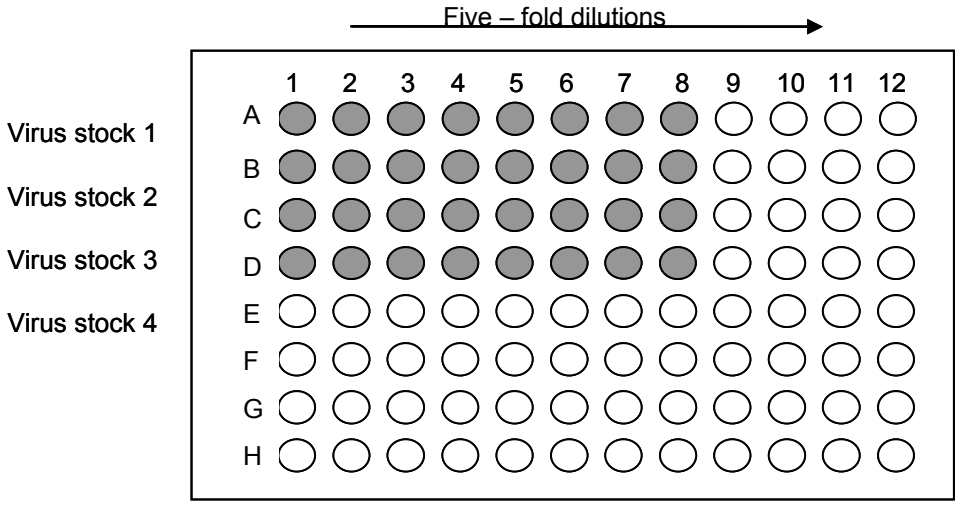


Figure 5. Five-fold serial dilutions of virus stock.

3. To each well add 80µl of Dilution Media.

4. 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.

5. 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.

6. 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.

7. 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.

8. Label 24 well plate as shown in Figure 8 using one row for each virus stock to be tested.

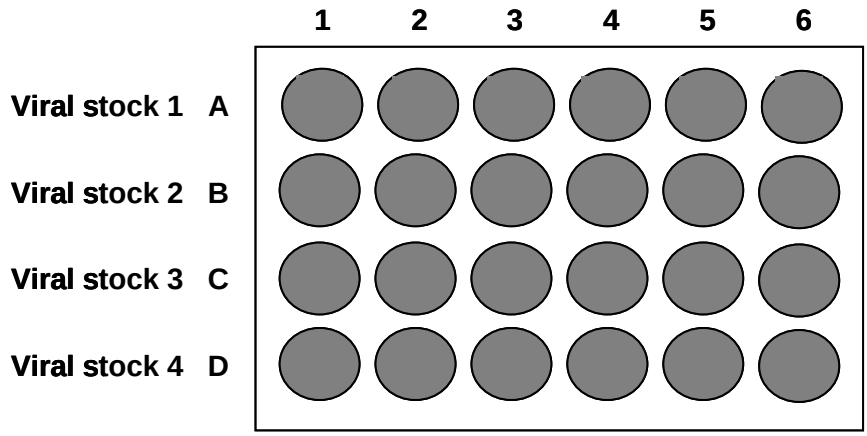


Figure 6. Twenty four well tissue culture plate, seeded with TLA-HEK293T™ cells, used to titer the virus.

9. Remove culture media from the cells in the 24 well plate.

10. Add 150µl of Transduction Media (same as Dilution Media without polybrene) to each well.

11. Transduce cells by adding 25µl of diluted virus from the original 96 well plate (in Figure 7) to a well on the 24 well destination plate (in Figure 8) containing the cells.

    For example, transfer 25µl from well A2 of the 96 well plate into well A1 in the 24 well plate (Table 6).

Table 6. 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 gently rinse cells with PBS

14. Add 1ml of DMEM (10% FBS, 1% Pen-Strep) containing 0.5µg/ml of doxycycline.

15. Culture cells for 72 hours.

16. Count the TurboRFP expressing cells or colonies of cells (Figure 9).

Count each multi-cell colony as 1 transduced cell, as the cells will be dividing over the 72 hour culture period. Figure 7 illustrates this principle of counting.

17. Transducing units per ml (TU/ml) can be determined using the following formula:
# of TurboRFP positive colonies counted x dilution factor x 40 = #TU/ml

Example: 55 TurboRFP positive colonies counted in well A3.
55 (TurboRFP positive colonies) x 3125 (dilution factor) x 40 = 6.88×10^6 TU/ml

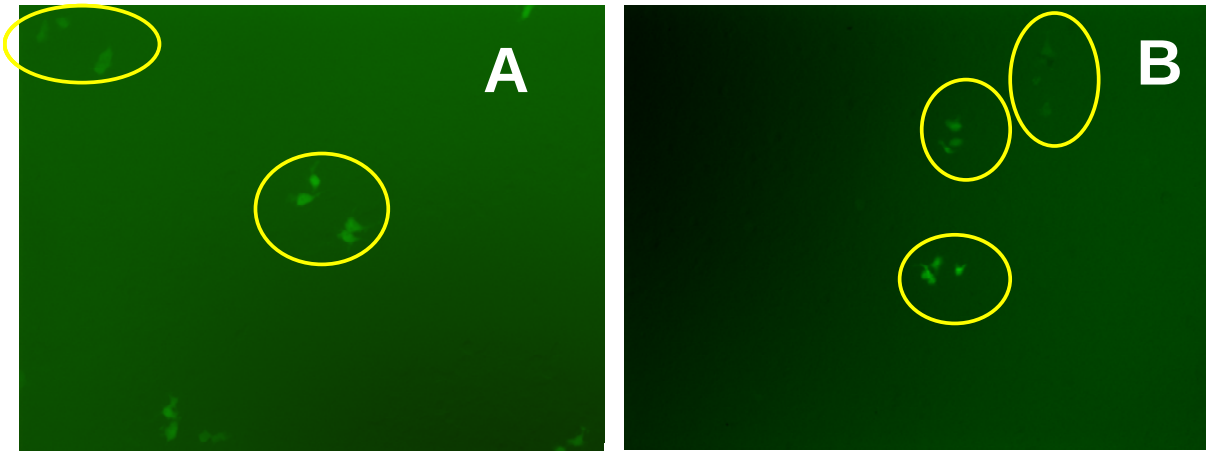


Figure 7. Examples of individual colonies

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 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.

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 - 8 x 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 1- 4 days under puromycin selection.
7. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 1 - 4 days from the start of antibiotic selection.

Transduction of Target Cells
1. On day 0 plate 5 - 8 x 10^4 cells per well in a 24 well plate. Incubate overnight.

2. Prepare media specifically for your cells (adding the appropriate concentration of puromycin as determined based on the above "kill curve" if planning to utilize puromycin selection).

   Note: When using MOIs between 3-5, not all cells will be transduced (see Table 7, Figure 8). It is recommended in these cases to utilize puromycin selection in order to reduce background expression of your gene of interest from untransduced cells.

3. The next day (day 1), remove the medium and add the virus to the MOI you wish to use.

   Bring the total volume of liquid up so that it just covers the cells efficiently with serum-free media. 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.

4. Approximately 6-8 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.

5. At 48 hours post transduction, replace the full growth media with full growth media containing the puromycin into the appropriate wells. Incubate.

6. Approximately every 2-3 days replace with freshly prepared selective media.

7. Monitor the cells daily and observe the percentage of surviving cells as well as the level and total percentage of TurboRFP expression. At some time point almost all of the cells surviving selection will be expressing TurboRFP. Optimum effectiveness should be reached in 3-10 days with puromycin.

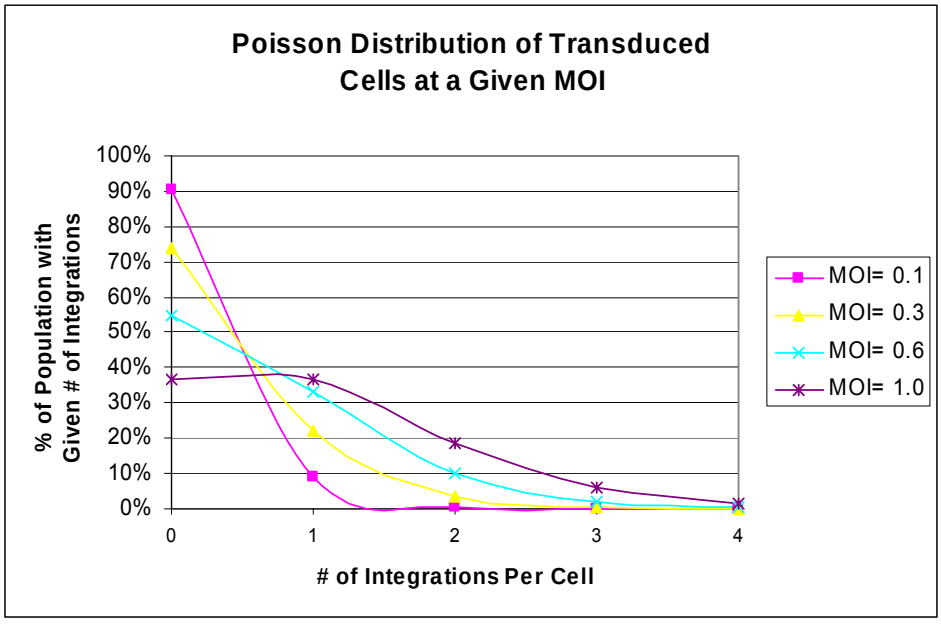
Please note that the higher the MOI you have chosen the more copies of the shRNA and puromycin resistance gene you will have per cell. When selecting on puromycin, it is worth remembering that at higher MOIs, cells containing multiple copies of the resistance gene can withstand higher puromycin concentrations 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".

8. Proceed to extract RNA for knock down evaluation by quantitative/real-time RT-PCR.

Table 7. A Poisson distribution showing the expected number shRNA integrants per cell at different MOIs. At a specific MOI the number of cells having 0, 1, 2, 3, or 4 viral integrants per cell is depicted in the table below. For example at a MOI of 0.3 less than 4% of the cells have more than one copy of the virus.

MOI	0	1	2	3	4
0.1	0.90	0.09	0.00	0.00	0.00
0.2	0.82	0.16	0.02	0.00	0.00
0.3	**0.74**	**0.22**	**0.03**	**0.00**	**0.00**
0.4	0.67	0.27	0.05	0.01	0.00
0.5	0.61	0.30	0.08	0.01	0.00
0.6	0.55	0.33	0.10	0.02	0.00
0.7	0.50	0.35	0.12	0.03	0.00
0.8	0.45	0.36	0.14	0.04	0.01
0.9	0.41	0.37	0.16	0.05	0.01
1	0.37	0.37	0.18	0.06	0.02

A



B

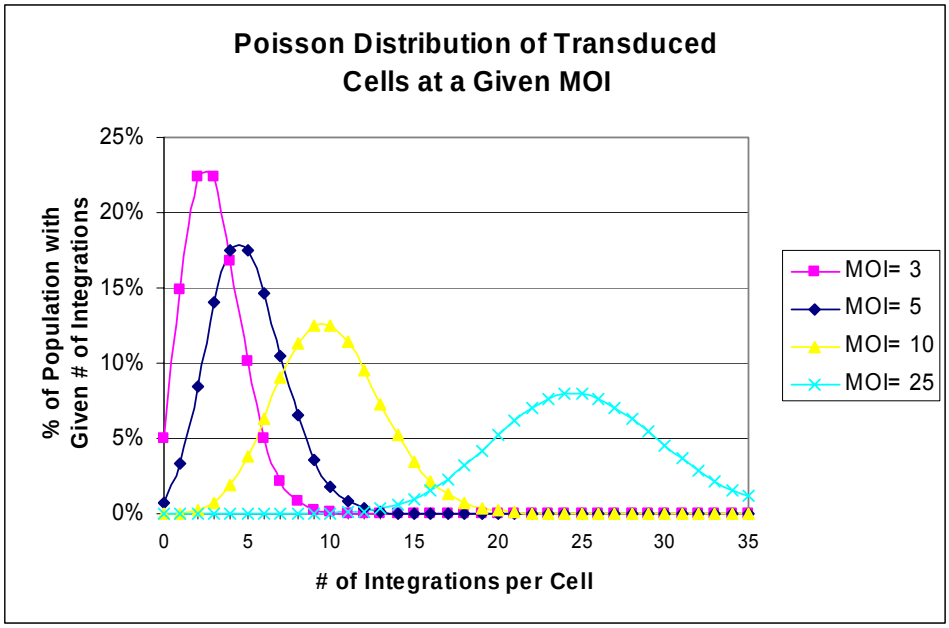


Figure 8. Graphs depicting the distribution of the number of integrants per cell at MOIs of 0.1, 0.3, 0.6, 1 (A), 3, 5, 10, 25 and 25 (B).

PROTOCOL VII – INDUCTION OF TURBORFP/SHRNAMIR EXPRESSION

Induction of TurboRFP in Transduced Cells using Doxycycline

1. Remove media and gently rinse cells with PBS.

2. Add media containing doxycycline at a concentration of 0.5µg/ml. TurboRFP will become visible within 24 hours and will be at full intensity by 72 hours.

   Note: Doxycycline concentrations ranging from 0.1µg/ml to 0.2µg/ml can be used. We recommend you optimize the doxycycline concentration according to your experimental needs. Cells transduced with TRIPZ at single copy are capable of producing visible TurboRFP at concentrations as low as 0.1µg/ml. Increasing TurboRFP signal will be seen with both increasing doxycycline concentrations as well as increased TRIPZ copy number.

3. Maintain cells on doxycycline for the duration of the experiment or as desired.

   Note: Induction of TurboRFP can be conducted exclusive of, in conjunction with or sequential to puromycin selection.

Turning TurboRFP Off After Doxycycline Induction

1. Split and replate the cells into a fresh plate/well at a ratio suitable for your experimental purposes.

   Note: Wash the cells in PBS before splitting and use media that does NOT contain doxycycline during the split. Doxycycline is inclined to adhere to the cells so precautions should be taken to make sure that no residual doxycycline gets carried over.

2. After splitting the cells into fresh media without doxycycline, incubate for 3 hours.

3. Wash the cells with PBS one to three times.

4. Add media without doxycycline. The TurboRFP protein will be turned over in approximately 72 hours to the point it will no longer be visible under the microscope.

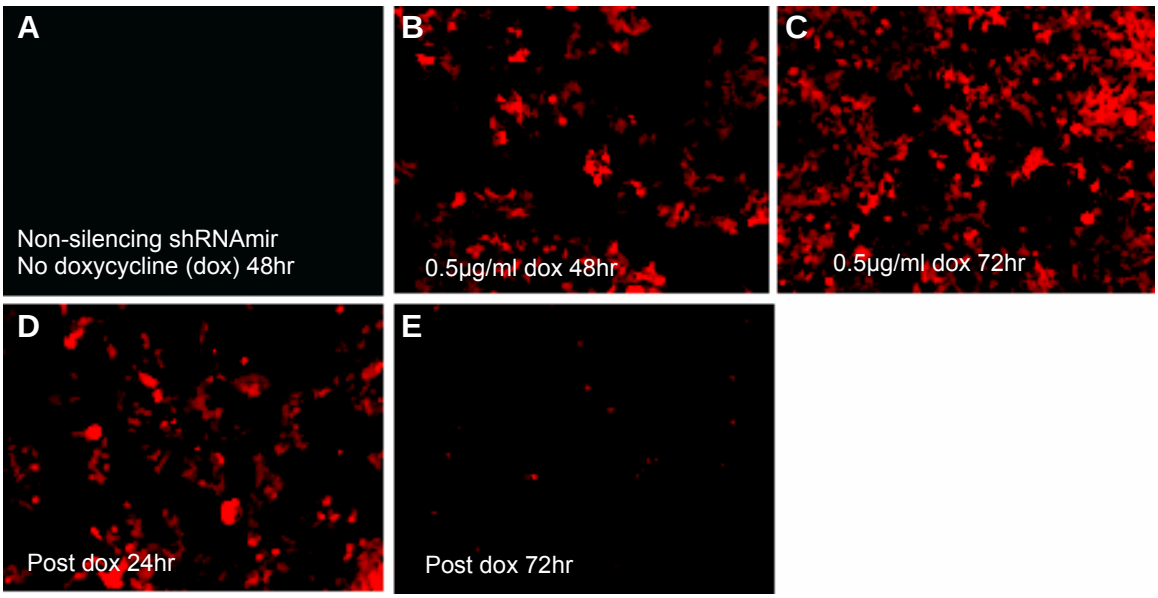


Figure 9. Induction of shRNAmir (tracked by TurboRFP) with doxycycline is tightly regulated. HEK293T cells were transduced at an MOI of 0.3, puromycin selected (2µl/ml) for 96 hours after which 0.5µg/ml of doxycycline was added to the cells and TurboRFP expression was assessed at 48-72hrs (B,C). Post-doxycycline samples (D,E) were photographed at times indicated. At 72 hrs after doxycycline removal TurboRFP expression is reduced to background levels.

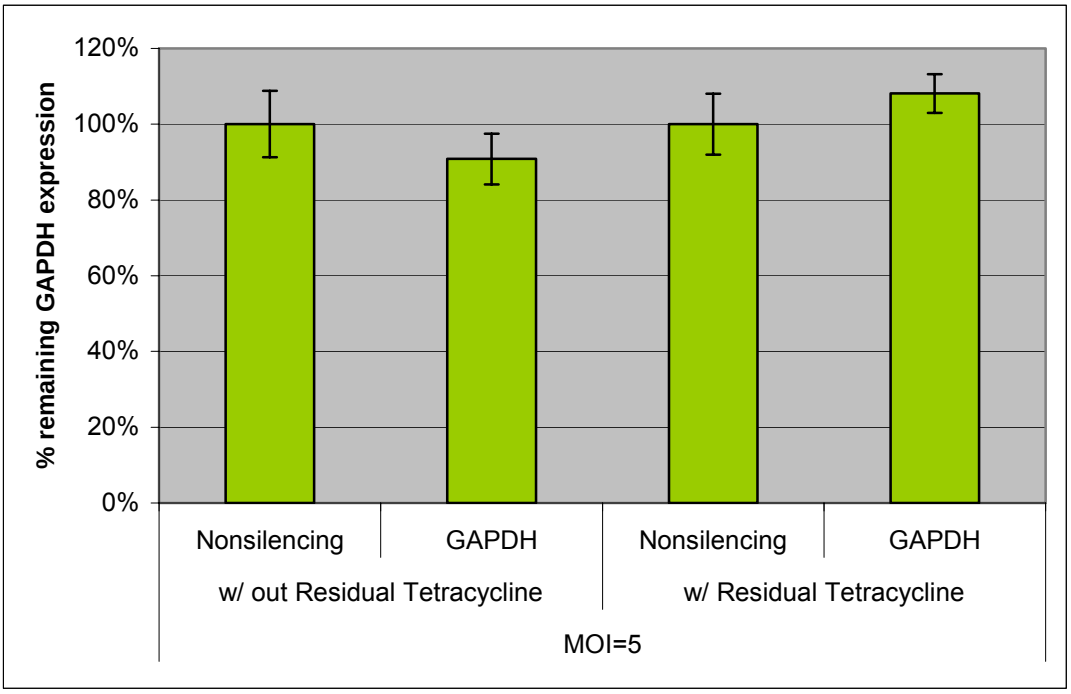


Figure 10. Residual tetracycline in media does not result in leaky expression. The pTRIPZ™ vector does not express shRNAmir to GAPDH at levels capable of producing significant knockdown, in the absence of doxycyline (whether in media with residual tetracycline or in special tetracycline free media). Each bar represents four tissue culture replicates all triplicated in quantitative/real-time RT-PCR for a total of 12 data points each. Statistical analysis was performed and no statistically significant differences were found between nonsilencing and GAPDH shRNAmir samples. Transduction at an MOI =5 was performed in HEK293T cells after which the cells were selected with 2ug/ml Puromycin for 96hrs.

RELATED REAGENTS

Table 8. Related Reagents

Reagent	Vendor	Catalog number
GAPDH verified positive control	Open Biosystems	RHS4744
Non-silencing verified negative control	Open Biosystems	RHS4743
Arrest-In™ transfection reagent 0.5ml-10mls	Open Biosystems	ATR1740-1743
Trans-Lentiviral™ shRNA Packaging System	Open Biosystems	TLP4614
Trans-Lentiviral shRNA Packaging System (contains cell line)	Open Biosystems	TLP4615

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 pTRIPZ product page:

http://www.openbiosystems.com/RNAi/shRNAmirLibraries/TRIPZLentiviralshRNAmir/

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 lentiviral pTRIPZ product page:
http://www.openbiosystems.com/RNAi/shRNAmirLibraries/TRIPZLentiviralshRNAmir/

Which antibiotic should I use?
You should grow all pTRIPZ™ constructs in both zeocin (25µg/ml) and ampicillin (carbenicillin) (100µg/ml) for archive replication. You can grow the constructs in media containing only zeocin (25µl/ml), but you CANNOT only select with ampicillin (carbenicillin). The ampicillin (carbenicillin) resistance marker is located outside of the hairpin with respect to the LTRs, and if you select only with ampicillin you could select for a recombinant containing an ampicillin resistance marker and an origin of replication, but no hairpin. You should grow the constructs in media containing only ampicillin (carbenicillin) (100µg/ml) for plasmid preparation.

What packaging cell line should I use for making lentivirus?
For packaging our inducible lentiviral shRNA constructs, we recommend our Trans-Lentiviral™ Packaging System. The Trans-Lentiviral Packaging System allows creation of a replication-incompetent, HIV-1-based lentivirus which can be used to deliver and express your gene or shRNA of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes, *et al.* (2001)
TLP4614 - Trans-Lentiviral shRNA Packaging System (does not contain a packaging cell line)
TLP4615 - Trans-Lentiviral shRNA Packaging System (contains the TLA-HEK293T™ cell line)

Can I use any 2nd generation packaging system with the pTRIPZ vector?
The pTRIPZ vector is *tat* dependant, so you must use a packaging system that expresses the *tat* gene.

What is the sequencing primer for pTRIPZ?
The pTRIPZ sequencing primer is 5'- GGAAAGAATCAAGGAGG -3'
Notes: The binding site lies at base 1005 and runs in the forward direction. The melting temperature of this 17-mer= 46.7˚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. The purpose of a kill curve is to find out the minimum amount of puromycin that will kill your cells, so that you can use the minimum amount of puromycin to test for resistant colonies (so as not to kill all of your cells). We suggest that you perform serial dilutions until you find the lowest amount of puromycin that will kill your cells, starting with 0.5µg/ml. 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 9 below for the approximate number of transfections.

Table 9. 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 validated positive control and the validated non-silencing negative control)?
5. What were the results of the controlled experiments?
6. How much doxycycline is being used?
7. How was knockdown measured (i.e. quantitative/real-time RT-PCR or western blot)?
8. What time points were assayed?
9. What is the abundance and the half-life of the protein? Does the protein have any isoforms?
10. What packaging cell line was used if you are using infection rather than transfection?
11. What was your viral titer?
12. What was your MOI?
13. Did you maintain the cells on puromycin after transfection or transduction?
14. 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 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 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. 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 Arrest-In seems to be toxic to a particular cell line, try reducing the DNA:Arrest-In ratio.

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