

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 shRNAmir construct has been sequence verified after 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 shRNAmir 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.clontech.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, Zhou *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 BamHI 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

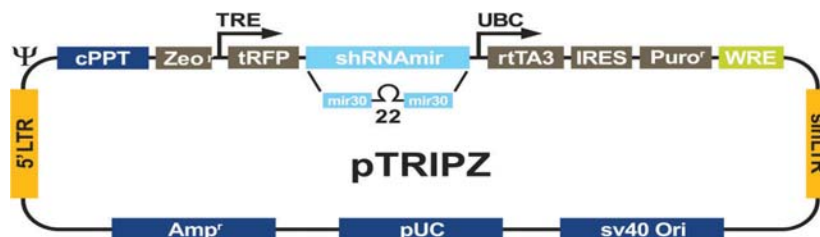


Figure 2. pTRIPZ lentiviral vector

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 shRNA expression |
| IRES-Puro resistance | Mammalian selectable marker |
| Amp resistance | Ampicillin (carbenicillin) bacterial selectable marker. |
| 5'LTR | 5' long terminal repeat |
| pUC ori | High copy replication and maintenance of plasmid in <i>E.coli</i> |
| SIN-LTR | Self inactivating long terminal repeat (Shimada, et al. 1995) |
| RRE | Rev response element |
| Zeo resistance | Bacterial selectable marker |

Vector Map

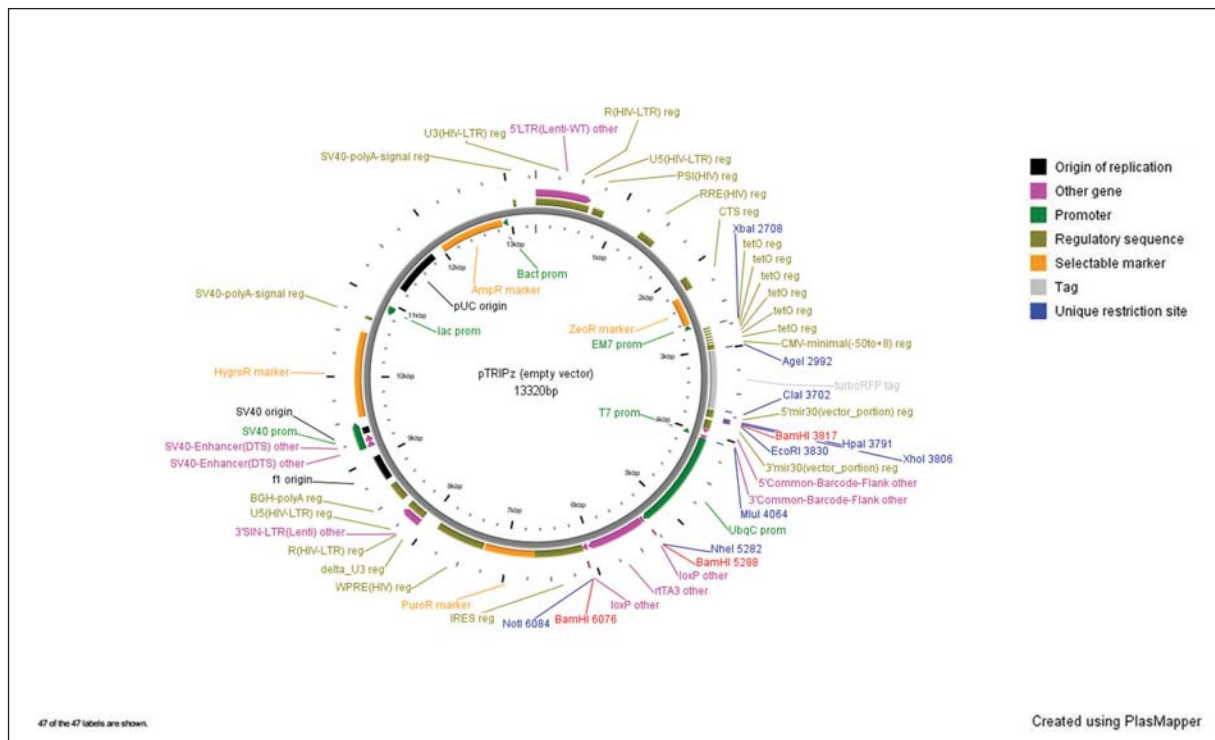


Figure 3. Detailed Vector Map of the pTRIPZ lentiviral vector (without hairpin). The empty vector is 13320 bp in size.

Antibiotic Resistance

pTRIPZ contains 3 antibiotic resistance markers (Table 2).

Table 2. Antibiotic resistances conveyed by pSM2

| Antibiotic | Concentration | Utility |
|----------------------------|---------------|---|
| Ampicillin (carbenicillin) | 100 µg/ml | Bacterial selection marker (outside LTRs) |
| Zeocin | 2.5µg/ml | Bacterial selection marker (inside LTRs) |
| Puromycin | Variable | Mammalian selectable marker |

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 *Sa*I and run out on an agarose gel. The expected band sizes are **7104 bp, 4028 bp, 2188 bp**. No recombinant products are visible. 10 kb molecular weight ladder (10 kb, 7 kb, 5 kb, 4 kb, 3 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb)

The pTRIPZ vector appears stable without showing any recombination.

Protocol I - Replication

Table 3. Materials for plate replication

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

For archive replication, grow all pTRIPZ clones at 37°C in LB-Lennox (low salt) media plus 25 µg/ml zeocin and 100 µg/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 ~160 µl of LB-Lennox (low salt) media supplemented with 8% glycerol* and appropriate antibiotic (25 µg/ml zeocin and 100 µg/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 37°C incubator 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 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.*

Protocol II - Plasmid Preparation

Culture Conditions For Individual Plasmid Preparations

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

Most plasmid mini-prep kits recommend a culture volume of 1–10 ml for good yield. For shRNAmir constructs, 5 ml of culture can be used for one plasmid mini-prep generally producing 5–10 µg 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 10 µl inoculum from the glycerol stock into 3-5 ml of 2X LB (low salt) with 100 µg/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 3-5 ml culture as a starter inoculum. Allow the starter culture to incubate for 8 hours at 37°C then dilute it 1:500 to 1:1000 into a larger culture volume. Incubate at 37°C for 18-19 hours with vigorous shaking.*
5. Pellet the 3-5 ml culture and begin preparation of plasmid DNA.
6. Run 3-5 µl of the plasmid DNA on a 1% agarose gel. pTRIPZ with shRNAmir is 13320 bp.

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

Culture Conditions For 96-Well Bio-Block Plasmid Preparation

Inoculate a 96-well bio-block containing 1 ml per well of 2X-LB (low salt) media with 100 µg/ml carbenicillin with 1 µl of the glycerol stock culture. Incubate at 37°C with shaking (~170-200 rpm). 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: We use the above 96-well bio-block plasmid preparation protocol in conjunction with a Qiagen Turbo kit (Catalog #27191). We use 2 bio-blocks combined, do not perform the optional wash and elute the DNA in water.

Protocol III - Cloning

Moving shRNA_{mir} Constructs From pSM2 to pTRIPZ

1. Order the pSM2 vector already expressing the shRNA_{mir} of interest.
2. Order the following PCR primers:
pSM2 forward - 5' aagcccttgtagaccacctaagcct 3'
pSM2 reverse - 5' actggtgaaactcaccaggatt 3'
3. Order a KOD Hotstart Polymerase kit from Novagen (Catalog # 71086-5 for 20U)
4. Resuspend the PCR primers at a stock concentration of 50 pmol/μl in sterile DEPC water. Dilute the stock 1:10 for a working concentration of 5pmol/μl in sterile DEPC water.
5. Set up the following PCR reaction at room temperature (Table 4). Add the components in the order listed. The following is for one 50 ul reaction. To do more reactions simply multiply the master mix components by the desired number of reactions plus 10%. We recommend doing 4 reactions to ensure enough fragment will be available for cloning.

Table 4. PCR reaction

| Component | Volume In μl |
|--|--------------|
| H ₂ O (DEPC) | 25 |
| 5M Betaine | 5 |
| 10X PCR buffer for KOD Hotstart Polymerase | 5 |
| dNTP's (2 mM each) | 5 |
| MgSO ₄ 25 mM | 2 |
| pSM2 Forward Primer (5 pmol/μl) | 3 |
| pSM2 Reverse Primer (5 pmol/μl) | 3 |
| KOD Hotstart Polymerase (1 U) | 1 |
| Total volume | 49 |
| Template (1 ul of glycerol stock from your pSM2 clone of interest) | 1 |

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

Table 5. PCR program

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

7. Put the four PCR reactions through a Wizard SV Gel and PCR Clean-up System column according to the kit directions (Promega Catalog #A9281 for 50 preps), with the exception of eluting with 110 μl of the provided nuclease free water. All four reactions can be run on a single SV Gel and PCR Clean-up column.
8. Set up the following restriction digest using the Clean-up column eluent (Table 6) and incubate at 37°C for 3 hours.

Table 6. Restriction digest

| Component | Volume In μl |
|-------------------------------|--------------|
| PCR eluent | 100 |
| 10X Restriction Enzyme Buffer | 20 |
| <i>Mlu</i> I | 2 |
| <i>Xho</i> I | 2 |
| Sterile Water | 76 |
| Total volume | 200 |

9. Run the entire digest on a 1.2%-1.5% agarose gel. Three bands should be seen (789 bp, 683 bp, 345b p) (Figures 5 and 6). Three bands will appear only if both *Mlu*I and *Xho*I have cut. Therefore the digest is diagnostic of the enzyme cuts in the following fashion (Table 7).

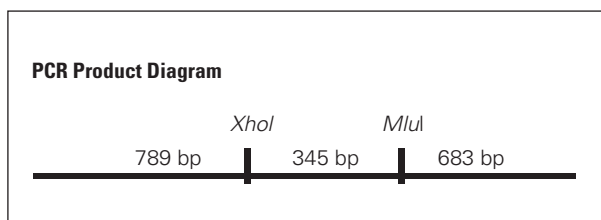


Figure 6. PCR product diagram

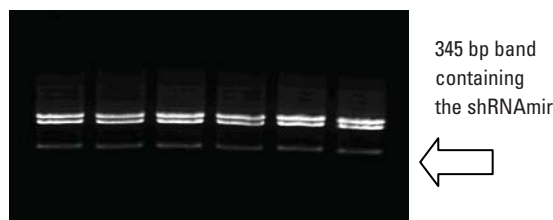


Figure 7. *MluI* and *XhoI* digest of PCR eluent with the expected digestion pattern (789 bp, 683 bp, 345 bp)

Table 7. Possible digestion patterns

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

- Excise the 345 bp band containing the shRNAmir of interest and purify on a Wizard SV Gel and PCR Clean-up System column according to the kit directions (Promega Catalog #A9281 for 50 preps). Elute in 50 μ l nuclease-free water.
- Quantitate the insert fragment.
- Prepare the pTRIPZ empty vector for ligation to the shRNAmir insert. Set up the following restriction digest (Table 8).

Table 8. Restriction digest

| Component | Volume In μ l |
|---------------------------------------|-------------------|
| pTRIPZ empty vector (250 ng/ μ l) | 12 |
| 10X Restriction Enzyme Buffer | 10 |
| <i>MluI</i> | 2 |
| <i>XhoI</i> | 2 |
| Sterile Water | 74 |
| Total volume | 100 |

- Mix the solution by pipetting and then gently spin the reaction for approximately 10 seconds to collect all the solution in the bottom of the tube. This will aid in decreasing contamination of uncut vector in your vector prep to follow. Incubate at 37°C for 3 hours.
- Run the entire digest on a 0.8% agarose gel. Make sure to run the gel through no less than 3 cm length of agarose. This will also aid in decreasing contamination of uncut vector in your vector prep.
- Gel isolate the 13061 bp band using a Wizard SV Gel and PCR Clean-up System column according to the kit directions (Promega catalog #A9281 for 50 preps). Elute in 50 μ l nuclease free water. You will likely not see a band representing the excised portion of the vector as it is too small.
- Quantitate the amount of cut vector per μ l you have isolated.
- Set up the following ligation reactions (Table 9):

Table 9. Ligation reactions

| Component | No Insert Control | shRNAmir Ligation |
|---|-------------------|-------------------|
| shRNAmir insert cut with <i>MluI</i> and <i>XhoI</i> (total 7.4 ng) | XXXXXXXXXX | ___ μ l |
| pTRIPZ vector cut with <i>MluI</i> and <i>XhoI</i> (total 250 ng) | ___ μ l | ___ μ l |
| DEPC water | ___ μ l | ___ μ l |
| 10X ligase buffer | 2.0 μ l | 2.0 μ l |
| Ligase | 0.5 μ l | 0.5 μ l |
| Total volume | 20.0 μ l | 20.0 μ l |

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

18. Ligate for 3 hours at room temperature. Dilute the ligation mix by adding 160 μ l DEPC water.
19. Transform 5 μ l of the diluted ligation mix into PrimePlus competent *E.coli* (Thermo Scientific Open Biosystems Catalog #MBC4246). Follow the transformation protocol for the competent cells. Plate the transformed cells onto agar plates containing 100 μ g/ml carbenicillin and 25 μ g/ml zeocin. Be sure to transform the same volume of ligation mix and plate the same volume of cells for both the control and the experimental sample. Plating 100 μ l, 50 μ l, and 10 μ l aliquots is recommended.
20. Incubate plates at 37°C overnight. Count colonies and determine the ratio of colonies on the control plate versus the experimental plates. Determine the number of colonies to sequence verify.
21. Sequence verify clones. The pTRIPZ sequencing primer is as follows:
5'- GGAAAGAATCAAGGAGG-3'
Note: This primer runs in the forward direction. The melting temperature of this 17 mer=46.7°C.

Moving shRNAmir Constructs From pGIPZ to pTRIPZ

1. Isolate plasmid from your pGIPZ clone according to Protocol II - Plasmid Preparation.
Culture conditions for individual plasmid preparations.
2. Set up the following restriction digest using the plasmid DNA prepared (Table 10) and incubate at 37°C for 3 hours.
Note: You will need a large amount of DNA digested in order to visualize the small 345 bp band on an agarose gel. If you are struggling to see this band you may need to digest more than 5 μ g of DNA.

Table 10. Restriction digest

| Component | Volume In μ l |
|--|-------------------|
| pGIPZ clone plasmid DNA – no less than 5 μ g | _____ μ l |
| 10X Restriction Enzyme Buffer | 20 |
| <i>Mlu</i> I | 2 |
| <i>Xho</i> I | 2 |
| Sterile Water | _____ μ l |
| Total volume | 200 |

3. Run the entire digest on a 1.2%-1.5% agarose gel. Two bands should be seen (345 bp and a large band near 11.4 Kb). Two bands will appear only if both *Mlu*I and *Xho*I have cut. If one or the other of these enzymes does not cut you will not see the 345 bp band but only a band at ~11.7 Kb.
4. Excise the 345 bp band containing the shRNAmir of interest and purify on a Wizard SV Gel and PCR Clean-up System column according to the kit directions (Promega Catalog #A9281 for 50 preps). Elute in 50 μ l nuclease free water.
5. Quantitate the insert fragment.
6. Prepare the pTRIPZ empty vector for ligation to the shRNAmir insert. Set up the following restriction digest (Table 11).

Table 11. Restriction digest

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

7. Mix the solution by pipetting and then gently spin the reaction for approximately 10 seconds to collect all the solution in the bottom of the tube. This will aid in decreasing contamination of uncut vector in your vector preparation to follow. Incubate at 37°C for 3 hours.
8. Run the entire digest on a 0.8% agarose gel. Make sure to run the gel through no less than 3 cm length of agarose. This will also aid in decreasing contamination of uncut vector in your vector preparation.

9. Gel isolate the 13061 bp band using a Wizard SV Gel and PCR Clean-up System column according to the kit directions (Promega Catalog #A9281 for 50 preps). Elute in 50 µl nuclease-free water. You will likely not see a band representing the excised portion of the vector as it is too small.
10. Quantitate the amount of cut vector per µl you have isolated.
11. Set up the following ligation reactions (Table 12):

Table 12. Ligation reactions

| Component | No Insert Control | shRNAmir Ligation |
|---|-------------------|-------------------|
| shRNAmir insert cut with <i>Mlu</i> I and <i>Xho</i> I (total 7.4 ng) | XXXXXXXXXX | ___ µl |
| pTRIPZ vector cut with <i>Mlu</i> I and <i>Xho</i> I (total 250 ng) | ___ µl | ___ µl |
| DEPC water | ___ µl | ___ µl |
| 10X ligase buffer | 2.0 µl | 2.0 µl |
| Ligase | 0.5 µl | 0.5 µl |
| Total volume | 20.0 µl | 20.0 µl |

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

12. Ligate for 3 hours at room temperature. Dilute the ligation mix by adding 160 µl DEPC water.
13. Transform 5 µl of the diluted ligation mix into PrimePlus competent *E. coli* (Thermo Scientific Open Biosystems Catalog #MBC4246). Follow the transformation protocol for the competent cells. Plate the transformed cells onto agar plates containing 100 µg/ml carbenicillin and 25 µg/ml zeocin. Be sure to transform the same volume of ligation mix and plate the same volume of cells for both the control and the experimental sample. Plating 100 µl, 50 µl, and 10 µl aliquots is recommended.
14. Incubate plates at 37°C overnight. Count colonies and determine the ratio of colonies on the control plate versus the experimental plates. Determine the number of colonies to sequence verify.
15. Sequence verify clones. The pTRIPZ sequencing primer is as follows:
5'- GGAAAGAATCAAGGAGG-3'

Note: This primer runs in the forward direction. The melting temperature of this 17 mer=46.7°C.

Protocol IV - Restriction Digest

The following is a sample protocol for restriction enzyme digestion using *Sal*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 in Table 13, to a sterile PCR thin-wall tube.

Table 13. PCR Components

| Component | Amount |
|-------------------------------|---------|
| Sterile, nuclease-free water | X µl |
| Restriction enzyme 10X buffer | 1.0 µl |
| DNA sample (400 ng) in water | ___ µl |
| <i>Sal</i> I 10 U (NEB) | 0.5 µl |
| Final volume | 20.0 µl |

2. Mix gently by pipetting.
3. Incubate in a thermocycler at 37°C for 2 hours to digest.
4. Load the gel with 20 µl of each of the digested samples (using *Sal*I) on a 1% agarose gel. Run uncut sample alongside the digested samples. Expected band sizes for the empty vector are: 7104 bp, 4028 bp, 2188 bp. For a pTRIPZ vector with a hairpin the 7104 bp band will be slightly larger. You should also be aware of the possibility that any particular shRNAmir may contain a *Sal*I site of its own, generating a fourth band.

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 $5-8 \times 10^4$ cells per well in a 24-well plate in enough wells to carry out your puromycin dilutions. Incubate overnight.
2. Prepare media specifically for your cells containing a range of antibiotic, for example: 0 - 15 $\mu\text{g/ml}$ puromycin.
3. The next day (day 1) replace the growth media with the media containing the dilutions of the antibiotic into the appropriate wells.
4. Incubate at 37°C.
5. Approximately every 2–3 days replace with freshly prepared selective media.
6. Monitor the cells daily and observe the percentage of surviving cells. Optimum effectiveness should be reached in 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.

Protocol VI - Transfection

The protocol below is optimized for transfection of the shRNA plasmid DNA into HEK293T cells in a 24-well plate using serum-free media. If a different culture dish is used, adjust the number of cells, volumes and reagent quantities in proportion to the change in surface area (Table 14).

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 Thermo Scientific Open Biosystems 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 Open Biosystems Arrest-In™ mixtures as they will be added to the cells.

Table 14. 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 | 20.0 | 2.0 | 4.0 | 21.0 |
| 35 mm | 8.0 | 1.0 | 2.0 | 10.0 |
| 6-well | 9.4 | 1.0 | 2.0 | 10.0 |
| 12-well | 3.8 | 0.5 | 1.0 | 5.0 |
| 24-well | 1.9 | 0.25 | 0.5 | 2.5 |
| 96-well | 0.3 | 0.1 | 0.1 - 0.2 | 0.5 - 1.0 |

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

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

1. The day before transfection (day 0), plate the cells at a density of 5×10^4 cells per well of a 24-well plate.
Full medium (i.e. with serum and antibiotics) will be used at this stage.
2. On the day of transfection, form the DNA/Arrest-In transfection complexes.
 - a. For each well to be transfected, dilute 500 ng shRNA plasmid DNA into 50 μl (total volume) of serum-free medium in a microfuge tube.
 - b. For each well to be transfected, dilute 2.5 μg (2.5 μl) of Arrest-In into 50 μl (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 100 µl at this stage.

3. Aspirate the growth medium from the cells. Add an additional 150 µl of serum-free medium to each of the tubes containing transfection complexes and mix gently. Add the 250 µl 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 µl at this stage (150 µl serum-free medium + 100 µl DNA:Arrest-In mixture).

4. Following the 5-6 hour incubation, add an equal volume of growth medium (250 µl) 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 experience, 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 24 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 µg/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 0.1 µg/ml - 2 µg/ml doxycycline is recommended for transfected cells as amounts of doxycycline necessary will vary due to the transfectability of the cell line, amounts of DNA used to transfect as well as other variables.

After 24 hours of incubation post-transfection, add media containing the appropriate amount of doxycycline. Examine the cells microscopically for the presence of TurboRFP expression 24-48 hours after doxycycline addition, 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 shRNAmir or other negative controls.

Note: The half-life of doxycycline in culture is 24 hours. It is therefore advisable to refresh the doxycycline containing media on the cells regularly to avoid fluctuation in the transcription levels from the inducible promoter.

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 QPCR 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 250 µl serum-free medium or to a total volume of 250 µl 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 14 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 VII - Packaging Lentivirus

The pTRIPZ vector is *tat* dependant, so you must use a packaging system that expresses the *tat* gene. For packaging our lentiviral shRNAmir constructs, we recommend the Thermo Scientific Open Biosystems TransLenti Viral shRNA Packaging System (TLP4614, TLP4615). The Open Biosystems TransLenti Viral™ shRNA Packaging System allows creation of a replication-incompetent (Shimada, *et al.* 1995), HIV-1-based lentivirus which can be used to deliver and express your gene or shRNAmir of interest in either dividing or non-dividing mammalian

cells. The TransLenti Viral shRNA Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes (Kappes and Wu 2001). For protocols and information on packaging pTRIPZ with our TransLenti Viral shRNA Packaging System, please see the product manual available at www.openbiosystems.com.

Protocol VIII - Titering

Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of choice. This protocol uses the Thermo Scientific Open Biosystems TLA-HEK293T cell line (Catalog #HCL4517) that is available as part of our TransLenti Viral shRNA Packaging System. You can use a standard HEK293T cell line as an alternative.

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

- The day before transduction, seed a 24-well tissue culture plate with TLA-HEK293T cells at 5×10^4 cells per well in DMEM (10% FBS, 1% pen-strep).
The following day, the well should be no more than 40-50% confluent.
- Make dilutions of the viral stock in a round bottom 96-well plate using serum-free media. Utilize the plate as shown in Figure 7 using one row for each virus stock to be tested. The goal is to produce a series of 5-fold dilutions to reach a final dilution of 390625-fold.

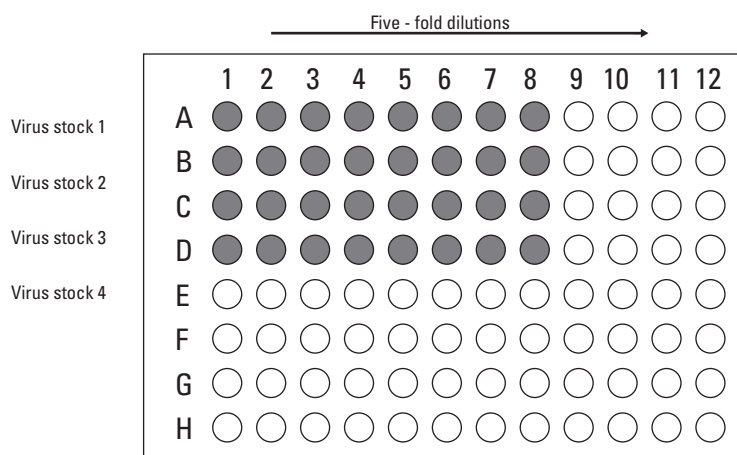


Figure 7. Five-fold serial dilutions of virus stock.

- Add 80 μ l of serum-free media to each well.
- Add 20 μ l of thawed virus stock to each corresponding well in column 1 (5-fold dilution).
Pipette contents of well up and down 10-15 times. Discard pipette tip.
- With new pipette tips, transfer 20 μ l from each well of column 1 to the corresponding well in column 2.
Pipette 10-15 times and discard pipette tips.
- With new pipette tips, transfer 20 μ l from each well of column 2 to the corresponding well in column 3.
Pipette 10-15 times and discard pipette tip.
- Repeat transfers of 20 μ l 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.

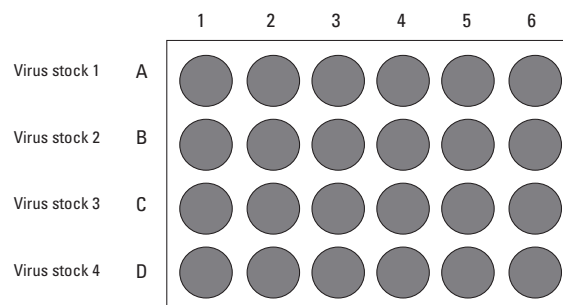


Figure 8. Twenty four well tissue culture plate, seeded with TLA-HEK293 cells, used to titer the virus.

- Label 24-well plate as shown in Figure 8 using one row for each virus stock to be tested.
- Remove culture media from the cells in the 24-well plate.
- Add 225 μ l of serum-free media to each well.

11. Transduce cells by adding 25 µl of diluted virus from the original 96 well plate (Figure 7) to a well on the 24-well destination plate (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 15).

Table 15. 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 | | 25 µl | 5 * |
| A2 | A1 | 25 µl | 25 |
| A3 | A2 | 25 µl | 125 |
| A4 | A3 | 25 µl | 625 |
| A5 | A4 | 25 µl | 3125 |
| A6 | A5 | 25 µl | 15625 |
| A7 | A6 | 25 µl | 78125 |
| A8 | | 25 µl | 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 1 ml of DMEM (10% FBS, 1% pen-strep) containing 1 µg/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 48 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 (TurboGFP positive colonies) x 625 (dilution factor) x 40 = 1.38x10⁶ TU/ml

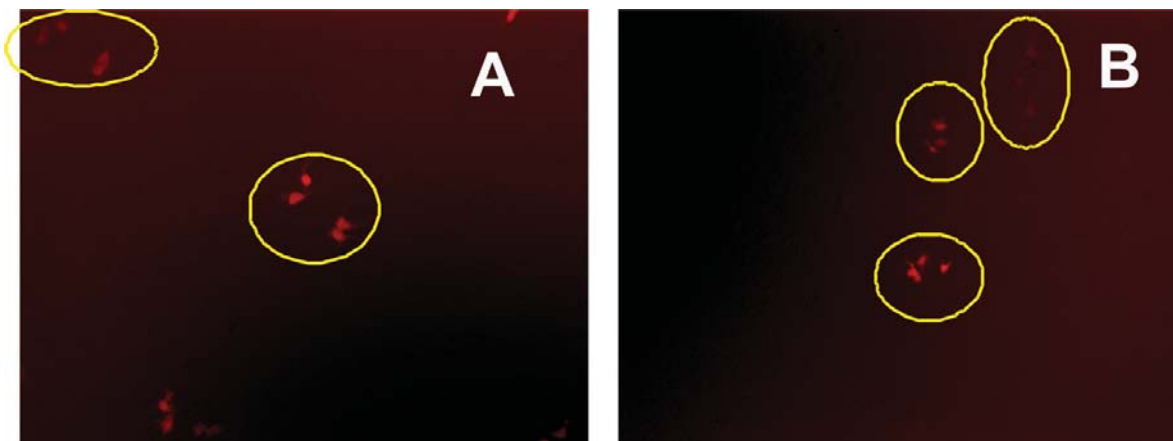


Figure 9. Examples of individual colonies

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

Multiplicity of Infection (MOI)

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

Determining the Optimal MOI

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

Protocol IX - Transduction

Transduction of Target Cells

The protocol below is optimized for transduction of the lentiviral particles into HEK293T, OVCAR8 or MCF7 cells in a 24-well plate using serum-free media. If a different culture dish is used, adjust the number of cells, volumes and reagent quantities in proportion to the change in surface area (see Table 16).

Table 16. 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) |
|---------------------|--|--|
| 100 mm | 56.0 | 5.0 |
| 60 mm | 20.0 | 2.0 |
| 35 mm | 8.0 | 1.0 |
| 6-well | 9.4 | 1.0 |
| 12-well | 3.8 | 0.5 |
| 24-well | 1.9 | 0.25 |
| 96-well | 0.3 | 0.1 |

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 $5 - 8 \times 10^4$ 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.
Bring the total volume of liquid up so that it just covers the cells efficiently with serum-free media (See Table 16 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.
3. Approximately 6-8 hours post-transduction, add an additional 1 ml 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 experience, higher transduction efficiencies have been achieved if the virus is not removed after 6 hours. However, if toxicity is a problem, aspirate the mixture after 3 - 6 hours and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth
4. At 48 hours post-transduction replace the current full growth media with full growth media containing puromycin (if required) into the appropriate wells. If adding puromycin, use the appropriate concentration as determined based on the above “kill curve”. Incubate.
 - a. Approximately every 2-3 days replace with freshly prepared selective media.
 - b. 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 3-10 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”.

Protocol X - 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 0.5 $\mu\text{g/ml}$. TurboRFP will become visible within 24 hours and will be at full intensity by 72 hours.
Note: Doxycycline concentrations ranging from 0.1 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$ 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 0.1 $\mu\text{g/ml}$. 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.
4. Proceed to extract RNA for knockdown evaluation by quantitative/real-time RT-PCR.
The optimal length of incubation from the start of induction to analysis is dependent on cell type, your gene of interest and the stability of the mRNA and/or protein being analyzed.

Turning TurboRFP Off After Doxycycline Induction

1. Split and replat 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.

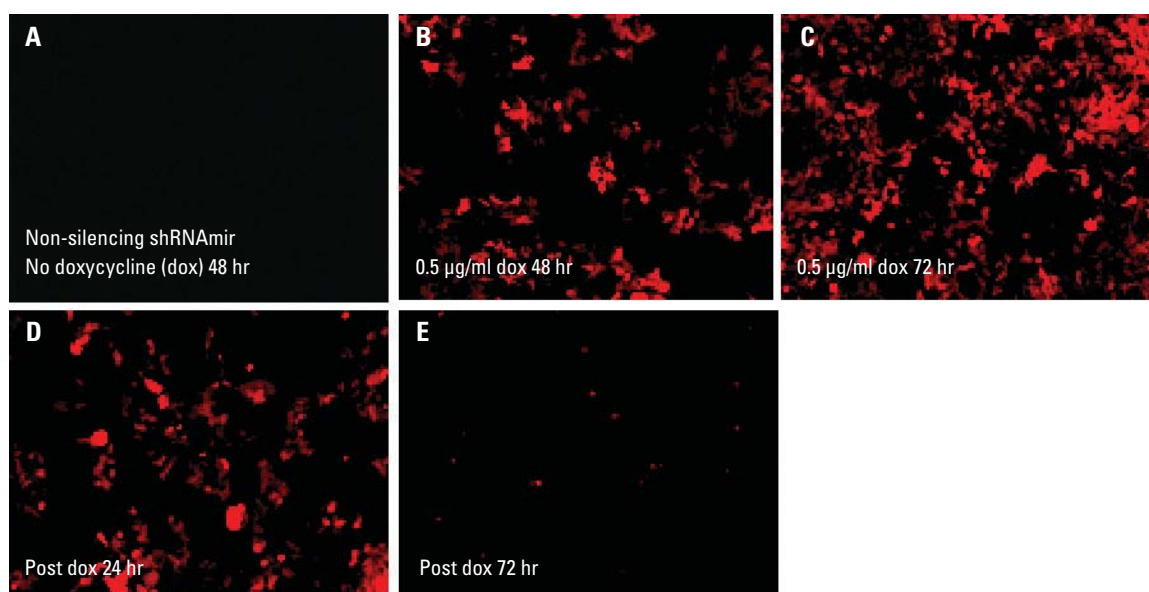


Figure 10. Induction of shRNAmir (tracked by TurboRFP) with doxycycline is tightly regulated. HEK293T cells were transduced at an MOI of 0.3, puromycin selected (2 $\mu\text{l/ml}$) for 96 hours after which 0.5 $\mu\text{g/ml}$ of doxycycline was added to the cells and TurboRFP expression was assessed at 48-72 hours (B,C). Post-doxycycline samples (D, E) were photographed at times indicated. At 72 hours after doxycycline removal TurboRFP expression is reduced to background levels.

Protocol XI - 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 shRNAmir, 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 florescent 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.

Table 17. Related Reagents

| Reagent | Vendor | Catalog # |
|--|-----------------------------------|------------------|
| GAPDH verified positive control* | Thermo Scientific Open Biosystems | RHS4744 |
| Non-silencing verified negative control* | Thermo Scientific Open Biosystems | RHS4743 |
| Arrest-In Transfection Reagent 0.5 ml-10 mls* | Thermo Scientific Open Biosystems | ATR1740-1743 |
| pTRIPZ empty vector | Thermo Scientific Open Biosystems | RHS4750 |
| TransLenti Viral shRNA Packaging System | Thermo Scientific Open Biosystems | TLP4614 |
| TransLenti Viral shRNA Packaging System (contains cell line) | Thermo Scientific Open Biosystems | TLP4615 |
| TransLenti Viral shRNA Bulk Packaging System | Thermo Scientific Open Biosystems | TLP4691, TLP4692 |

*these items also available in the lentiviral RNAintro shRNAmir starter kit (catalog number RHS4741)

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 at www.openbiosystems.com.

Where Can I Find The Sequence Of An Individual shRNAmir Construct?

If you are looking for the sequence an individual shRNAmir construct, you can use the gene search. Just enter the catalog number or clone ID of your hairpin into the gene search on the Thermo Scientific Open Biosystems website, hit submit and then click on the query result. If you then click on the oligo ID (the V2 number) and then click on the word “sequence” in the details grid, the hairpin sequence is listed with the target, mir-30 context and loop sequences annotated. If you are looking for the sequence of several shRNAmir constructs, you can access this information in the data file of the collection. This data file can be downloaded from the Lentiviral pTRIPZ product page at www.openbiosystems.com.

Which Antibiotic Should I Use?

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

What Packaging Cell Line Should I Use For Making Lentivirus?

For packaging our lentiviral shRNAmir constructs, we recommend the TransLenti Viral shRNA Packaging System (TLP4614, TLP4615). The TransLenti Viral shRNA Packaging System allows creation of a replication-incompetent (Shimada, *et al.* 1995), HIV-1-based lentivirus which can be used to deliver and express your gene or shRNAmir of interest in either dividing or non-dividing mammalian cells. The TransLenti Viral shRNA Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes (Kappes and Wu 2001). For protocols and information on packaging pTRIPZ with our TransLenti Viral shRNA Packaging System, please see the product manual available at www.openbiosystems.com.

Can I Use Any 2nd Generation Packaging System With The TRIPZ Vector?

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

What Does The Number 40 Refer To In The Formula For The Calculation Of Titer?

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

What Is The Sequencing Primer For TRIPZ?

The pTRIPZ sequencing primer is 5'- GGAAAGAATCAAGGAGG -3'

Note: This primer runs in the forward direction. The melting temperature of this 17mer=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. After you have determined the appropriate concentration of puromycin to use, you can transfect or transduce your cells with the shRNA construct and culture with puromycin in order to select for those cells that have a stable integrant. Cells not containing a stable integrant will not be selected for.

Where Do You Purchase Puromycin?

We purchase puromycin from Cellgro (Catalog #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 18 below for the approximate number of transfections.

Table 18. 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 (1 mg/ml) (µg)* | 0.5 ml qty (rxns)** | 1.0 ml qty (rxns)** | 5.0 ml qty (rxns)** | 10 ml qty (rxns)** |
|---------------------|--|---------------------------|---------------------|---------------------|---------------------|--------------------|
| 60 mm | 20.0 | 21.0 | 47-50 | 100 | 500 | 1000 |
| 35 mm | 8.0 | 10.0 | 100 | 200 | 1000 | 2000 |
| 6-well | 9.4 | 10.0 | 100 | 200 | 1000 | 2000 |
| 12-well | 3.8 | 5.0 | 200 | 400 | 2000 | 4000 |
| 24-well | 1.9 | 2.5 | 400 | 800 | 4000 | 8000 |
| 96-well | 0.3 | 0.5-1.0 | 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 is the abundance and the half-life of the protein? Does the protein have many isoforms?
9. What packaging cell line was used if you are using infection rather than transfection?
10. What was your viral titer?
11. What was your MOI?
12. Did you maintain the cells on puromycin after transfection or transduction?
13. 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 260 nm 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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FAQS/Troubleshooting

For answers to questions that are not addressed here, please email technical support at openbiosystems@thermofisher.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.

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