TECHNICAL MANUAL

Inducible Dharmacon™ TRIPZ™ Lentiviral shRNA

Product description

The TRIPZ Inducible Lentiviral shRNA Collection was developed in collaboration with Dr. Greg Hannon of Cold Spring Harbor Laboratory (CSHL) and Dr. Steve Elledge of Harvard Medical School. This collection combines the design advantages of microRNA-adapted short hairpin RNA (shRNA) with the pTRIPZ lentiviral inducible vector to create a powerful RNAi trigger capable of producing gene silencing. The vector is engineered to be Tet-On and produces tightly regulated induction of shRNA expression in the presence of doxycycline.

Important safety note

Please follow the safety guidelines for use and production of lentiviral particles as set by your institution's biosafety committee.

- For glycerol stocks of *E. coli* containing lentiviral plasmids, BSL1 guidelines should be followed
- For handling and use of lentiviral products to produce lentiviral particles, BSL2 or BSL2+ guidelines should be followed
- For handling and use of lentiviral particle products, BSL2 or BSL2+ guidelines should be followed

Additional information on the safety features incorporated in the pTRIPZ lentiviral vector and the Trans-Lentiviral Packaging System can be found on page 3.

pTRIPZ Inducible shRNA vectors are not compatible with third generation packaging systems, due to the requirement of the expression of tat, which third generation systems do not contain. We recommend the Dharmacon™ Trans-Lentiviral™ Packaging System for use with our vectors.

Design information

Unique microRNA-30 Based Hairpin Design

Dharmacon shRNA constructs are expressed as human microRNA-30 (miR-30) primary transcripts. This design adds a Drosha processing site to the hairpin construct and has been shown to greatly increase knockdown efficiency (Boden 2004).

The hairpin stem consists of 22 nucleotides of dsRNA and a 19-nucleotide loop from human miR-30. The addition of the miR-30 loop, along with the 125-nucleotide miR-30 flanking sequences, resulted in a 10-fold increase in Drosha and Dicer processing of the expressed hairpins when compared with conventional shRNA designs without microRNA (Silva 2005).

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.

Use of the miR-30 design also allows the use of 'rules-based' 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.

Each shRNA construct has been bioinformatically verified to match NCBI sequence data. To assure 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 Inducible shRNA vector

The pTRIPZ vector is engineered to be Tet-On. This equips the pTRIPZ vector to provide induced expression of an shRNA in the presence of doxycycline. There are two main components on the pTRIPZ vector enabling induction: **1)** the tetracycline response element (TRE), and **2)** 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 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 2004).

TurboRFP in the pTRIPZ inducible shRNA vector

In addition to driving expression of the shRNA, the TRE also drives the expression of a TurboRFP reporter. This induced expression of TurboRFP enables the user to easily observe expression from the TRE promoter, allowing quick assessment of factors such as delivery efficiency and promoter activity.

Tet-on or Tet-off configuration is possible

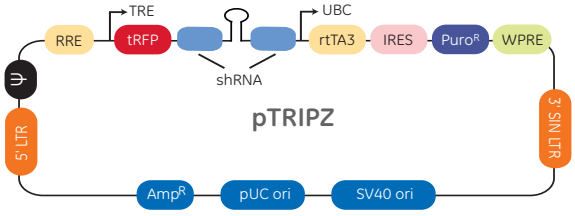
The pTRIPZ vector is versatile in that it can be easily converted to a Tet-Off capable vector using Cre/loxP technology or classical restriction digest. The rtTA3 is flanked by loxP sites allowing in vitro or in vivo excision of the rtTA3 by exposure to Cre recombinase. The rtTA3 is also flanked by a pair of BamHI restriction sites allowing for cleavage and ligation of the vector to remove the rtTA3. Without the rtTA3 present on the vector, a tetracycline transactivator (tTA) can be added to the system in trans; thus creating a Tet-Off system where expression of shRNA and TurboRFP are induced in the absence of doxycycline.

Vector information

Versatile vector design

Features of the pTRIPZ inducible lentiviral shRNA vector (Figures 1-2) 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 shRNA are part of a single transcript allowing the visual marking of shRNA-expressing cells
- Amenable to *in vitro* and *in vivo* applications
- Inducible RNAi in both dividing and non-dividing cell lines
- Puromycin drug resistance marker for selecting stable cell lines



Vector element	Utility
TRE	Tetracycline-inducible promoter
tRFP	TurboRFP reporter for visual tracking of transduction and shRNA expression
shRNA	microRNA-adapted shRNA (based on miR-30) for gene knockdown
UBC	Human ubiquitin C promoter for constitutive expression of rtTA3 and puromycin resistance genes
rtTA3	Reverse tetracycline-transactivator 3 for tetracycline-dependent induction of the TRE promoter
Puro^R	Puromycin resistance permits antibiotic-selective pressure and propagation of stable integrants
IRES	Internal ribosomal entry site allows expression of rtTA3 and puromycin resistance genes in a single transcript
5' LTR	5' long terminal repeat
3' SIN LTR	3' self-inactivating long terminal repeat for increased lentivirus safety
Ψ	Psi packaging sequence allows viral genome packaging using lentiviral packaging systems
RRE	Rev response element enhances titer by increasing packaging efficiency of full-length viral genomes
WPRE	Woodchuck hepatitis posttranscriptional regulatory element enhances transgene expression in the target cells

Figure 1. Schematic and features of the pTRIPZ Inducible Lentiviral shRNA vector.

Vector map

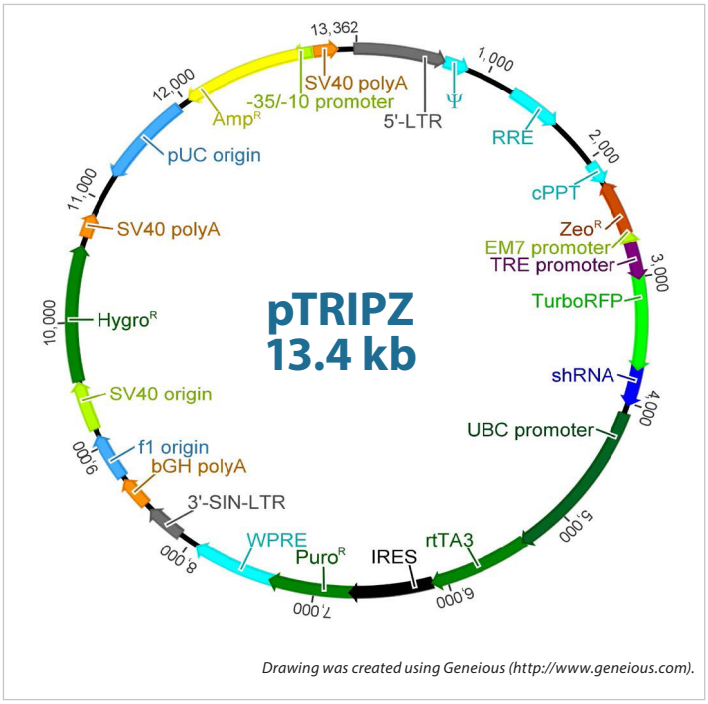


Figure 2. Detailed Vector Map of the pTRIPZ lentiviral vector.
he empty vector is 13,362 bp in size.

Antibiotic resistance

pTRIPZ contains 3 antibiotic resistance markers (Table 1).

Table 1. Antibiotic resistances conveyed by pTRIPZ

Antibiotic	Concentration	Utility
Ampicillin (carbenicillin)	100 µg/mL	Bacterial selection marker (outside LTRs)
Zeocin™	25 µg/mL	Bacterial selection marker (inside LTRs)
Puromycin	Variable	Mammalian selection marker

The pTRIPZ Inducible Lentiviral shRNA library has passed through internal QC processes to ensure high quality and low recombination (Figure 3). Individual clones can be digested with SalI to assess the integrity of the plasmid and rule out recombination. For best results, digest 400 ng of purified plasmid and run the complete digest on a 1% agarose gel alongside undigested plasmid. 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 shRNA may contain a SalI site of its own, generating a fourth band.

Figure 3. Representative TRIPZ Inducible shRNA clones grown for 18 hours at 37 °C and the plasmid isolated. Clones were then digested with SalI and run 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).

Additional safety information

Historically, the greatest safety risk associated with a lentiviral delivery platform stems from the potential generation of recombinant lentiviruses that are capable of autonomous replication. The TRIPZ Lentiviral shRNA platform minimizes these hazards to the greatest degree by combining a disabled viral genome with the proprietary Trans-Lentiviral packaging process. Starting with the HXB2 clone of HIV-1 (GenBank Accession Number K03455), the lentiviral backbone has been modified to eliminate all but the most essential genetic elements necessary for packaging and integration (such as 5' LTR, Psi sequences, polypurine tracts, Rev responsive elements and 3' LTR). The resultant self-inactivating (SIN) vector greatly reduces the probability of producing recombinant particles and limits cellular toxicity often associated with expression of HIV genes.

Additional safety features can be incorporated by the packaging process itself. Generation of TRIPZ Lentiviral shRNA particles requires a packaging step during which the expression construct containing the silencing sequence is enclosed in a viral capsid. Gene functions that facilitate this process (such as encoded by the structural genes gag, pol, env, etc.) are distributed amongst multiple helper plasmids which do not contain significant regions of homology. This tactic further minimizes the probability of recombination events that might otherwise generate lentiviruses capable of autonomous replication. Among commercially available lentiviral vector systems, the Dharmacon™ Trans-Lentiviral™ Packaging System offers a superior safety profile as the packaging components are separated onto five plasmids. Additionally, expression of gag-pro and tat-rev are under the control of the conditional tetracycline-responsive promoter element (TRE), limiting expression of these viral components strictly to the packaging cell line. A detailed description of the Trans-Lentiviral Packaging System has been published (Wu 2000).

With these safety measures in place, TRIPZ lentiviral shRNA particles can be employed in standard Biosafety Level 2 tissue culture facilities.

Any investigator who purchases Dharmacon viral vector products is responsible for consulting with their institution's health and biosafety group for specific guidelines on the handling of lentiviral vector particles. Further, each investigator is fully responsible for obtaining the required permissions for the acceptance of lentiviral particles into their local geography and institution.

- In the U.S., download the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition, Dec 2009 here.
- See also: NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines), Apr 2016, downloadable here.
- For Biosafety Considerations for Research with Lentiviral Vectors, here.

Protocol I – Replication

For archive replication, grow all TRIPZ Inducible shRNA clones at 37 °C in 2x LB broth (low salt) medium plus 25 µg/mL Zeocin and 100 µg/mL carbenicillin in order to provide maximum stability of the clones.

Replication of plates

Prepare target plates by dispensing ~ 160 µL of 2x LB broth (low salt) medium supplemented with 8% glycerol* and appropriate antibiotic (25 µg/mL Zeocin™ and 100 µg/mL carbenicillin, Table 2).

Prepare source plates

1. Remove foil seals while the source plates are still frozen to minimize 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 wells.
2. Carefully 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. Reseal and 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 to control background recombination products.

Due to the tendency of viral vectors to recombine, we recommend keeping the incubation times as short as possible and avoid subculturing. *Glycerol should be omitted from the medium if you are culturing for plasmid preparation. If making copies of the constructs for long-term storage at -80 °C, 8% glycerol is required.

Table 2. Materials for plate replication.

Item	Vendor	Cat #
LB-Lennox Broth (low salt)	Fisher Scientific	BP1427500
Peptone, granulated, 2 kg - Difco	Fisher Scientific	BP9725-2
Yeast Extract, 500 g, granulated	Fisher Scientific	BP1422-500
NaCl	Fisher Scientific	BP3581
Glycerol	Fisher Scientific	BP2291
Carbenicillin	Fisher Scientific	BP2648-250
Zeocin™	Invivogen	ant-zn-5p
Puromycin	Fisher Scientific	BP2956-100
96-well microplates	Fisher Scientific	12-565-363
Aluminum seals	Fisher Scientific	12-565-475
Disposable replicators	Fisher Scientific	NC9584102

Protocol II – Plasmid preparation

Culture conditions for individual plasmid preparations

For plasmid preparation, grow all TRIPZ Inducible shRNA clones at 37 °C in LB broth (low salt) medium plus 100 µg/mL carbenicillin only.

Most plasmid mini-prep kits recommend a culture volume of 1-10 mL for good yield. For shRNA constructs, 3-5 mL of culture can be used for one plasmid mini-prep generally producing 5-10 µg of plasmid DNA.

Upon receiving your glycerol stock(s) containing the shRNA of interest store at -80 °C until ready to begin.

1. 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.
2. Take a 10 µL inoculum from the glycerol stock into 3-5 mL of 2x LB broth (low salt) medium with 100 µg/mL carbenicillin. Return the glycerol stock(s) to -80 °C.
3. 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.

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

4. Pellet the culture and follow the instructions for your plasmid prep kit to obtain the plasmid DNA. Plasmid DNA can be isolated using Thermo Scientific™ GeneJET™ Plasmid Miniprep Kit (Cat #K0502) or similar.
5. Run 3-5 µL of the plasmid DNA on a 1% agarose gel. The pTRIPZ plasmid with shRNA is 13,320 bp (empty vector) or 13,362 with hairpin shRNA. 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 broth (low salt) medium 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.

We use the above 96-well bio-block plasmid preparation protocol in conjunction with a Qiagen™ Turbo™ Kit (Cat #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 Thermo Scientific™ FastDigest™ SalI (Cat# FD0644) for diagnostic quality control of TRIPZ Inducible Lentiviral shRNA vectors.

1. Using filtered pipette tips and sterile conditions, add the components for a FastDigest SalI restriction digest (as listed in Table 3) to a sterile PCR thin-wall tube. Mix gently by pipetting.
2. Incubate at 37 °C for 5 minutes to digest.
3. Load the gel with 20 µL of each of the digested samples (using SalI) 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 (Figure 3). For a TRIPZ Inducible shRNA vector with a hairpin the 7104 bp band will be slightly larger. You should also be aware of the possibility that any particular shRNA may contain a SalI site of its own, generating a fourth band.

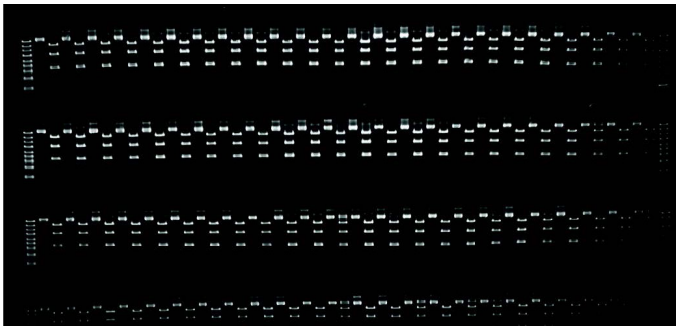


Figure 3. Representative TRIPZ Inducible shRNA clones grown for 18 hours at 37 °C and the plasmid isolated. Clones were then digested with SalI and run on an agarose gel. The expected band sizes are 7104 bp, 4028 bp, 2188 bp. No recombinant products are visible. 10 kb molecular weight ladder (10 kb, 7 kb, 5 kb, 4 kb, 3 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb).

Table 3. Restriction Digest Components.

Component	Amount
Water, Nuclease-free	X µL
10x FastDigest Buffer	2 µL
DNA sample (up to 1 µg) in water	X µL
Recommended FastDigest Enzyme(s)	1 µL
Final Volume	20 µL

Protocol IV – Cloning

Moving shRNA constructs from pGIPZ to pTRIPZ

1. Isolate plasmid from your GIPZ shRNA clone according to Protocol II – Plasmid Preparation.
2. Set up a restriction digest of the prepared plasmid using MluI and XhoI restriction enzymes according to Table 3, adding 1µL of each enzyme to the tube. Incubate reaction at 37 ˚C for 5 minutes.

You will need a large amount of DNA digested in order to visualize the small 345 bp band on an agarose gel. If it is difficult to see this band you will need to scale up the digest for more than 5 µg of DNA.

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 MluI and XhoI 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 shRNA of interest and purify using the Thermo Scientific™ GeneJET™ Gel Extraction Kit (Cat #K0691) column according to the kit directions. Elute in 50 µL nuclease-free water.
5. Quantify the insert fragment.
6. Prepare the pTRIPZ empty vector for ligation to the shRNA insert by digesting 3 µg of plasmid with restriction enzymes MluI and XhoI as above.
7. 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.
8. Gel isolate the 13,061 bp band using the GeneJET Gel Extraction Kit (Cat #K0691) according to the kit directions. 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.
9. Quantify the amount of isolated cut vector per µL.
10. Ligate 7.4 ng of the shRNA insert and 250 ng of cut pTRIPZ vector.

This yields a molar ratio of 1 vector to 1 insert. Prepare a no-insert ligation control in parallel.

11. Transform 5 µL of the diluted ligation mix into competent E.coli cells. 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.
12. 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.
13. Sequence verify clones. The pTRIPZ sequencing primer is as follows: 5′-GGAAAGAATCAAGGAGG-3′

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

Protocol V – Puromycin selection

Puromycin kill curve and puromycin selection

In order to generate stable cell lines expressing the transgene of interest, it is important to determine the minimum amount of antibiotic required to kill non-transfected cells. A simple procedure to test this is as follows:

Day 1: Using the same cell type and relative cell densities to be used in subsequent transfection procedures, plate cells and culture overnight.

Day 2: Replace complete growth medium with growth medium supplemented with a range of antibiotic concentrations, for example puromycin (0-15 µg/mL), including untreated control cells with no antibiotic added.

Day 4: Refresh medium and assess viability.

- Replace medium with fresh medium supplemented with the appropriate concentration of puromycin every 2-3 days depending on the growth of cells.
- Examine cells daily and identify the minimal concentration of antibiotic that efficiently kills all non-transfected cells between 4-6 days following addition of antibiotic.

Protocol VI – Transfection

General protocol for transfection of adherent and suspension cells in a 24-well plate

Quantities and volumes should be scaled-up according to the number of cells/wells to be transfected

1. In each well, seed ~ 5×10^4 adherent cells or ~ 5×10^5 suspension cells in 0.5 mL of growth medium 24 hours prior to transfection.

The recommended confluency for adherent cells on the day of transfection is 70-90%. Suspension cells should be in logarithmic growth phase at the time of transfection.

2. Dilute 1 µg of DNA in 50 µL of serum-free DMEM or other serum-free growth medium.
3. Gently mix DharmaFECT kb reagent and add 3 µL of it to the diluted DNA. Mix immediately by pipetting.
4. Incubate 10 minutes at room temperature. Remove medium from well and replace with 0.45 mL fresh growth medium.

Prepare immediately prior to transfection. We recommend starting with 1 µg of DNA and 3 µL of DharmaFECT kb per well in a 24-well plate (see scale-up Table 4). Subsequent optimization may further increase transfection efficiency depending on the cell line and transgene used.

5. Gently add 50 µL of the DharmaFECT kb/DNA mixture to each well.
6. Gently rock the plate to achieve even distribution of the complexes.
7. Incubate at 37 °C in a CO_2 incubator.
8. Analyze transgene expression 24-48 hours later. For stable transfection, cells should be grown in selective medium for 10-15 days.

Table 4. Scale-up ratios for transfection of adherent and suspension cells with DharmaFECT kb transfection reagent.

Tissue culture vessel	Growth area, cm^2/well	Volume of medium, mL	Adherent (suspension) cells to seed the day before transfection*	Amount of DNA		Volume of DharmaFECT kb, µL	
				µg**	µL***	Recommended	Range
96-well plate	0.3	0.1	$0.5\text{-}1.2 \times 10^4$ (2.0×10^4)	0.2	10	0.6	0.4-1.0
48-well plate	0.7	0.25	$1.0\text{-}3.0 \times 10^4$ (5.0×10^4)	0.5	25	1.5	0.8-2.2
24-well plate	2.0	0.5	$2.0\text{-}6.0 \times 10^4$ (1.0×10^5)	1.0	50	3.0	2.0-5.0
12-well plate	4.0	1.0	$0.4\text{-}1.2 \times 10^5$ (2.0×10^5)	2.0	100	6.0	3.9-9.0
6-well plate	9.5	2.0	$0.8\text{-}2.4 \times 10^5$ (4.0×10^5)	4.0	200	9.0	6.0-12.0
60 mm plate	20	3.0	$2.0\text{-}6.3 \times 10^5$ (1.0×10^6)	6.0	300	18.0	12.0-24.0

* These numbers were determined using HEK293T and U2OS cells. Actual values depend on the cell type.
** Amount of DNA and DharmaFECT kb transfection reagent used may require optimization.
*** The volume of DNA should be 1/10 of the volume of the culture medium used for dilution of the DNA.

Induction of TurboRFP/shRNA expression In transfected cells using doxycycline

Optimization within a range of 0.1-2 µg/mL doxycycline is recommended for transfected cells as amounts of doxycycline will vary due to the transfectablility of the cell line, amounts of DNA used to transfect, as well as other variables.

After 24 hours of incubation post-transfection, add medium 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 of transfection efficiency. Then assay cells for reduction in gene expression by reverse transcription quantitative real-time qPCR (RT-qPCR), western blot, or other appropriate functional assay; compare to untreated, non-silencing shRNA or other negative controls.

The half-life of doxycycline in culture is 24 hours. It is therefore advisable to refresh the doxycycline-containing medium 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. RT-qPCR generally gives the best indication of expression knock-down. The use of western blots to determine knockdown is very dependent on quantity and quality of the protein sample, its half-life, and the sensitivity and specificity 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/DharmaFECT kb reagent mixture should be added to cells after incubation for complex formation, to a total volume of 250 µL serum-free medium with antibiotics.

Transfection optimization using Dharmacon DharmaFECT kb transfection reagent

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 DharmaFECT kb reagent and DNA amount indicated in Table 4, 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 shRNA constructs, we recommend the Trans-Lentiviral shRNA Packaging System (Cat #TLP5912, TLP5913, TLP5914, or TLP5917). The Trans-Lentiviral Packaging System allows creation of a replication-incompetent (Shimada 1995), HIV-1-based lentiviral particles 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 shRNA/ORF Packaging System generates a replication-incompetent particle based on the trans-lentiviral system developed by Kappes (Kappes and Wu 2001). For protocols and information on packaging TRIPZ Inducible shRNA with our Trans-Lentiviral shRNA/ORF Packaging System Kit, please see the product manual available here.

Protocol VIII – Titering

Follow the procedure below to determine the titer of your lentiviral stock using your mammalian cell line of choice. This protocol uses the HEK293T packaging cell line that is available as part of our Trans-Lentiviral shRNA Packaging Kit (Cat #TLP5917).

If you have generated a lentiviral stock of the expression control (such as pTRIPZ Inducible shRNA Non-silencing control), we recommend titering this stock as well.

1. The day before transduction, seed a 24-well tissue culture plate with 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.
2. Make dilutions of the viral stock in a round bottom 96-well plate using serum-free medium. Utilize the plate as shown (Figure 4) using one row for each viral stock to be tested. The goal is to produce a series of five-fold dilutions to reach a final dilution of 390,625 fold.

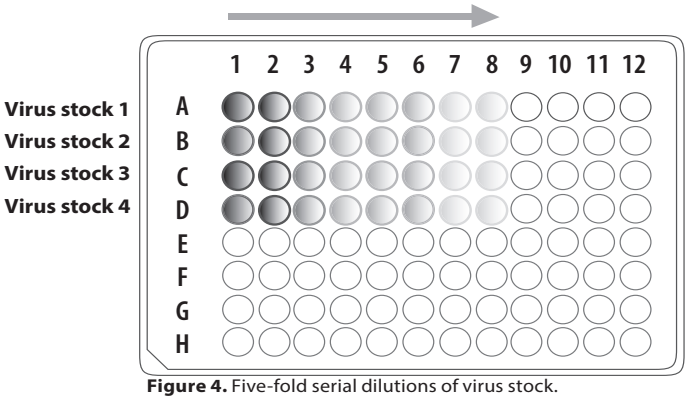


Figure 4. Five-fold serial dilutions of virus stock.

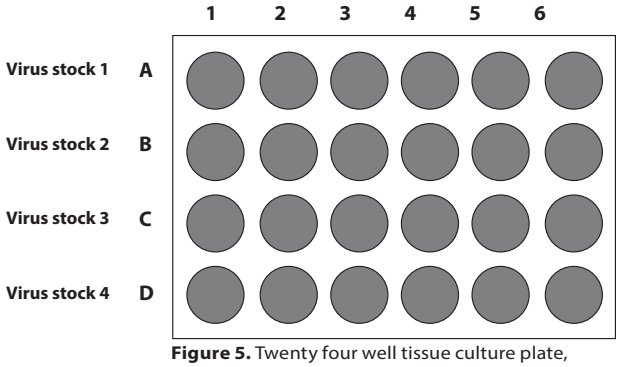


Figure 5. Twenty four well tissue culture plate, seeded with HEK293T cells, used to titer the virus.

3. Add 80 µL of serum-free medium to each well.
4. Add 20 µL of thawed viral stock to each corresponding well in column 1 (five-fold dilution). Pipette contents of well up and down 10-15 times. Discard pipette tips.
5. With new pipette tips, transfer 20 µL from each well of column 1 to the corresponding well in column 2. Pipette contents of well up and down 10-15 times. Discard pipette tips.
6. Repeat transfers of 20 µL from columns 2 through 8, pipetting up and down 10-15 times and changing pipette tips between each dilution.

Note: It is strongly recommended that you use a high quality multichannel pipettor when performing multiple dilutions.

7. Label the 24-well plate as shown in Figure 5 using one row for each viral stock to be tested.
8. Remove culture medium from the cells in the 24-well plate.
9. Add 225 µL of serum-free medium to each well.
10. Transduce cells by adding 25 µL of diluted lentiviral particles from the original 96-well plate (Figure 4) to a well on the 24-well destination plate (Figure 5) containing the cells. For example, transfer 25µL from well A2 of the 96-well plate into well A1 in the 24-well plate (Table 5).
11. Incubate transduced cultures at 37 °C for 4 hours.
12. Remove transduction mix from cultures and gently rinse cells with PBS.
13. Add 1 mL of DMEM (10% FBS, 1% pen-strep) containing 1 µg/mL of doxycycline.
14. Culture cells for 72 hours.
15. Count the TurboRFP expressing cells or colonies of cells (Figure 6).
Note: Count each multi-cell colony as 1 transduced cell, as the cells will be dividing over the 72 hour culture period.
16. Transducing units per mL (TU/mL) can be determined using the following formula:
# of TurboRFP positive colonies counted × dilution factor × 40 = # TU/mL.
Example: 55 TurboRFP positive colonies counted in well A3.
5 (TurboGFP positive colonies) × 625 (dilution factor) × 40 = 1.38×10^6 TU/mL.

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 gene silencing.

Table 5. 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.

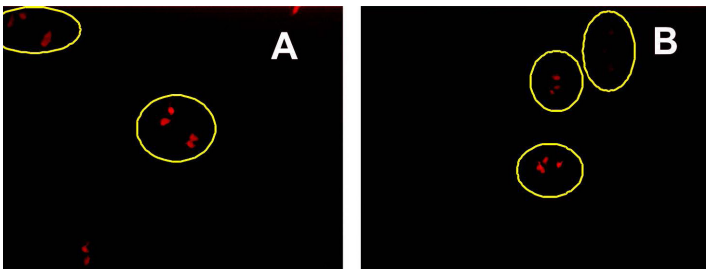


Figure 6. Examples of individual colonies.

Multiplicity of Infection (MOI)

To obtain optimal silencing 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.

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 the lentiviral particles, we recommend using a range of MOIs (for example, 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, OVCAR-8 or MCF7 cells in a 24-well plate using serum-free medium. 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 6). It is strongly recommended that you optimize transduction conditions to suit your target cell line for the highest transduction efficiency possible.

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 need to optimize the protocol according to your needs.

Table 6. Suggested volumes of medium 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

1. On day 0, plate 5-8 × 10^4 cells per well for complete medium (including serum) in a 24-well plate. Incubate overnight.
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 medium (See Table 6 for guidelines). If you are using concentrated virus you are likely to use very little virus volume and a lot of serum-free medium; if you are using unconcentrated virus you will find you need much more virus volume.*
3. Approximately 6-8 hours post-transduction, add 1 mL of complete medium (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 medium with complete medium containing puromycin into the appropriate wells. When 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 medium.
   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 shRNA. Optimum effectiveness should be reached in 3-10 days with puromycin.

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."

Protocol X – Induction of TurboRFP/shRNA expression

Induction of TurboRFP and shRNA in transduced cells using doxycycline

1. Remove medium and gently rinse cells with PBS.
2. Add medium containing doxycycline at a concentration of 0.5 µg/mL. TurboRFP will become visible within 24 hours and will be at full intensity by 72 hours.

Doxycycline concentrations ranging from 0.1 µg/mL to 2 µg/mL can be used. We recommend you optimize the doxycycline concentration according to your experimental needs. Cells transduced with pTRIPZ Inducible shRNA at single copy are capable of producing visible TurboRFP at concentrations as low as 0.1 µg/mL. Increasing TurboRFP signal will be seen with both increasing doxycycline concentrations as well as increased TRIPZ shRNA copy number.

3. Maintain cells in doxycycline for the duration of the experiment or as desired. The half-life of doxycycline in culture is 24 hours. It is therefore advisable to refresh the doxycycline containing medium on the cells regularly to avoid fluctuation in the transcription levels from the inducible promoter.

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

4. Proceed to extract RNA for knockdown evaluation RT-qPCR.

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 and shRNA Expression off after doxycycline induction

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

Wash the cells in PBS before splitting and use medium 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 medium without doxycycline, incubate for 3 hours.
3. Wash the cells with PBS one to three times.
4. Add medium 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.

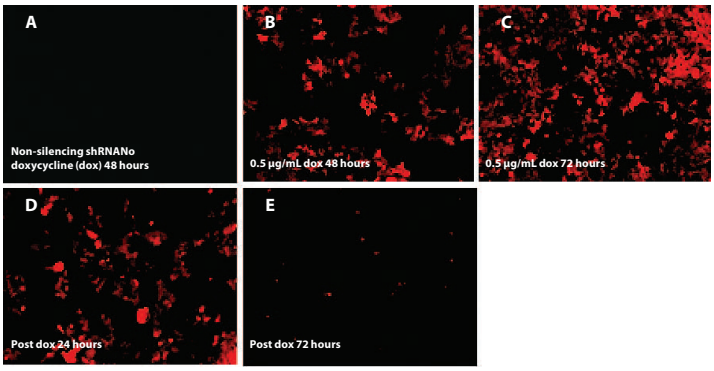


Figure 7. Induction of TRIPZ shRNA (tracked by TurboRFP) with doxycycline is tightly regulated. HEK293T cells were transduced at an MOI of 0.3, puromycin selected (2 µL/mL) for 96 hours after which 0.5 µg/mL of doxycycline was added to the cells and TurboRFP expression was assessed at 48-72 hours (B,C). 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 and 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. We have found that normalizing the RNA concentration prior to cDNA synthesis will increase consistency downstream.
3. Make sure the mRNA you are using as your internal control for qPCR is expressed at a level higher than your target mRNA.
4. Use only high-quality calibrated pipettes, in conjunction with well fitting barrier tips.
5. 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.
6. Be sure to spin your qPCR plate prior to loading in the instrument in order to collect the sample at the bottom of the well, as well as eliminate any bubbles that may have developed.
7. With regard to knockdown experiments using shRNA, it is important that you greatly reduce, if not eliminate entirely, those cells which are not transduced or transfected from the population (cells that are not expressing the fluorescent reporter). 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.
8. Always utilize the non-silencing control as a reference for target gene expression, as opposed to an untreated sample. The non-silencing control treated samples will most accurately reproduce the conditions in your experimental samples.
9. 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.
10. Cq values greater than 35 should be avoided as they tend to be more variable. Samples with such high Cq values should be repeated at higher cDNA concentrations and with a lower expressing qPCR internal control.
11. Cq 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 Cq values from both your internal control as well as your experimental target to determine an optimum cDNA concentration.
12. It may be necessary to change internal controls if conditions in steps 10 and 11 cannot be simultaneously met.

Table 7. Related Reagents.

Related reagents	Cat #
TRIPZ Human GAPDH Inducible Lentiviral shRNA Positive Control	RHS4744
TRIPZ Inducible Lentiviral Non-silencing shRNA Control	RHS4743
TRIPZ Inducible Lentiviral Empty Vector shRNA Control	RHS4750
Trans-Lentiviral shRNA Packaging System	TLP5912, TLP5913, TLP5914
Trans-Lentiviral shRNA Packaging System with HEK293T Cells	TLP5917

Frequently Asked Questions (FAQs)

What clones are part of my collection?

A USB 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.

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

If you are looking for the sequence of an individual shRNA construct, you can search for the clone on our website (dharmacon.horizondiscovery.com). Enter the catalog number or clone ID of your construct into the search at the top of the page. You should see your product in the Catalog Number section of the results. Click on the plus sign to expand the details for this clone and select the Sequence tab.

Which antibiotic should I use?

You should grow all TRIPZ 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 shRNA constructs, we recommend the Trans-Lentiviral shRNA Packaging Kit (see Table 7). The Trans-Lentiviral Packaging Kit allows creation of a replication-incompetent (Shimada 1995), lentiviral particles 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 Kit generates replication-incompetent particles based on the trans-lentiviral system developed by Kappes (Kappes and Wu 2001). For protocols and information on packaging TRIPZ with our Trans-Lentiviral shRNA Packaging Kit, please see the product manual available at here.

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

Packaging on the TRIPZ shRNA into lentiviral particles is tat-dependent, 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 viral particles" Table 5) to one milliliter.

What is the sequencing primer for use with TRIPZ shRNA?
The TRIPZ sequencing primer is 5'-GGAAAGAATCAAGGAGG-3'.
Note: This primer runs in the forward direction.

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 (see Protocol V – Puromycin Selection). 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 to select for those cells that have a stable integrant.

If transfection in to 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. 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.
4. Cell history, density, and passage number — it is very important to use healthy cells that are regularly passaged and in growth phase. The highest transfection efficiencies are achieved if cells are plated the day before, however, adequate time should be given to allow the cells to recover from the passaging (generally > 12 hours). Plate cells at a consistent density to minimize experimental variation. If transfection efficiencies are low or reduction occurs over time, thawing a new batch of cells or using cells with a lower passage number may improve the results.

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 lentiviral particles you have produced. Factors to consider when transducing include MOI (related to accurate titer in the target cell line), the presence of serum in the medium, the use of Polybrene™ in the media, length of exposure to lentiviral particles, 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 3-4 for factors influencing successful transfection (above).

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FAQs/troubleshooting

For answers to questions that are not addressed here, please email technical support at ts.dharmacon@horizondiscovery.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.

Label licenses

The shRNA and gene expression Products, use and applications, are covered by pending and issued patents. Certain Label licenses govern the use of the products, these can be found at dharmacon-licensing-statements. It is each Buyer's responsibility to determine which intellectual property rights held by third parties may restrict the use of Products for a particular application. Please review the Label Licenses governing all use of the shRNA and gene expression Products.

If you have any questions

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