TECHNICAL MANUAL

# GIPZ™ Lentiviral shRNA

## Product description

The Dharmacon™ GIPZ™ Lentiviral shRNA Library was developed in collaboration with Dr. Greg Hannon of Cold Spring Harbor Laboratory [CSHL] and Dr. Steve Elledge of Harvard Medical School. This library combines the design advantages of microRNA-adapted shRNA with the pGIPZ lentiviral vector to create a powerful RNA tool capable of producing RNA interference (RNAi) in most cell types including primary and non-dividing cells.

**GIPZ shRNA is available in glycerol stock or viral particle format. If viral particle format is purchased, begin work with Protocol IX – Determining Relative Transduction Efficiency.**

**Important safety note**

Please follow the safety guidelines for use and production of vector-based lentivirus 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 pGIPZ lentiviral vector and the Horizon Trans-Lentiviral Packaging System can be found on page 3.

**Dharmacon™ GIPZ™ 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 Trans-Lentiviral Packaging System for use with our vectors.**

## Design information

**Unique microRNA-30 based hairpin design**

Short hairpin RNA (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 gene silencing efficiency (Boden 2004). The hairpin stem consists of 22 nucleotides (nt) of dsRNA and a 19 nucleotides (nt) loop from human miR-30. Adding the miR-30 loop and 125 nucleotides (nt) of miR-30 flanking sequence on either side of the hairpin results in greater than 10-fold increase in Drosha and Dicer processing of the expressed hairpins when compared with conventional shRNA designs (Silva 2005). Increased Drosha and Dicer processing translates into greater shRNA production and greater potency for expressed hairpins.

Use of the miR-30 design also allowed the use of 'rules-based' designs for target sequence selection. One such rule is the destabilizing of the 5' end of the antisense strand, which results in strand specific incorporation of microRNA/siRNAs into RISC. The proprietary design algorithm targets sequences in coding regions and the 3' UTR with the additional requirement that they contain greater than 3 mismatches to any other sequence in the human or mouse genomes.

Each shRNA construct has been 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.

## Vector information

**Versatile vector design**

Features of the pGIPZ lentiviral vector (Figures 1 and 2) that make it a versatile tool for RNAi studies include:

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

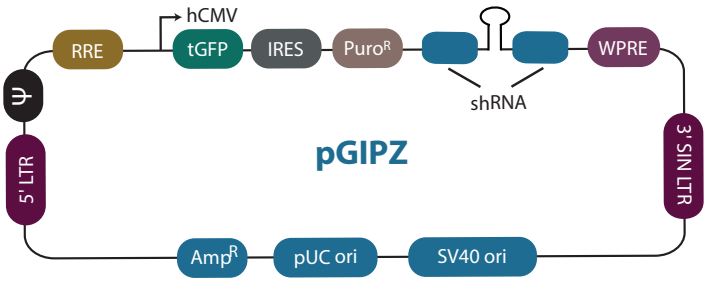
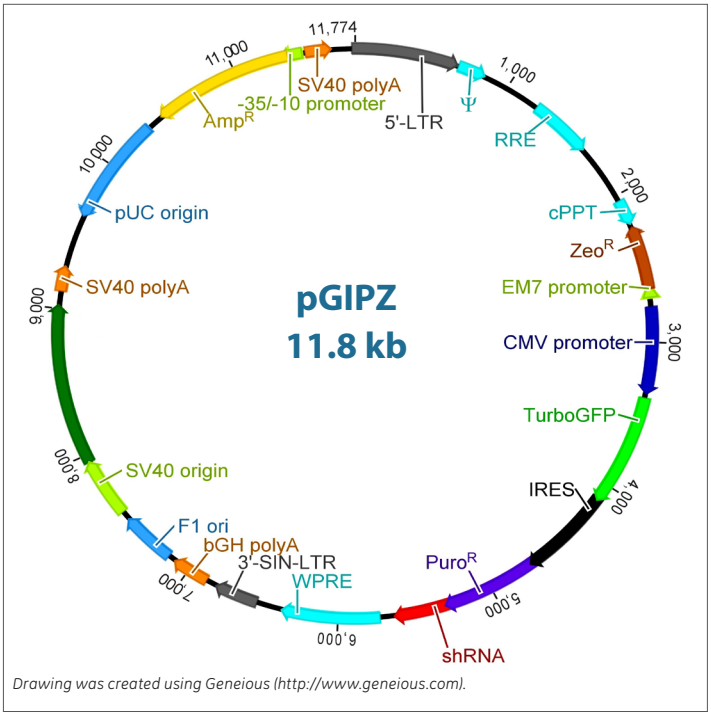



**Figure 1. pGIPZ lentiviral vector.**

**Table 1. Features of the pGIPZ vector.**

| Vector element | Utility |
|---|---|
| hCMV | Human cytomegalovirus promoter drives strong transgene expression |
| tGFP | TurboGFP reporter for visual tracking of transduction and expression |
| Puro$^R$ | Puromycin resistance permits antibiotic-selective pressure and propagation of stable integrants |
| IRES | Internal ribosomal entry site allows expression of TurboGFP and puromycin resistance genes in a single transcript |
| shRNA | microRNA-adapted shRNA (based on miR-30) for gene knockdown |
| 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 |

## Vector map




*Drawing was created using Geneious (http://www.geneious.com).*

**Figure 2. Detailed vector map of pGIPZ lentiviral vector.**

## Antibiotic resistance

pGIPZ contains three antibiotic resistance markers (Table 2).

**Table 2. Antibiotic resistances conveyed by pGIPZ.**

| 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 |

## Quality control

The GIPZ Lentiviral shRNA Library has passed through internal QC processes to ensure high quality and low recombination (Figures 3 and 4).

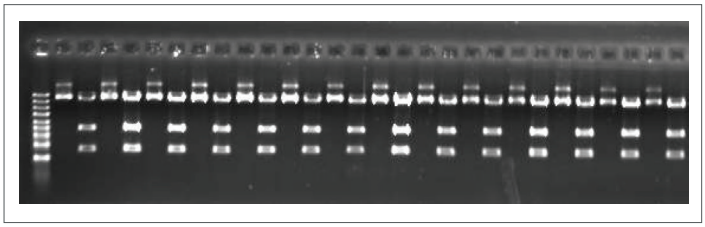
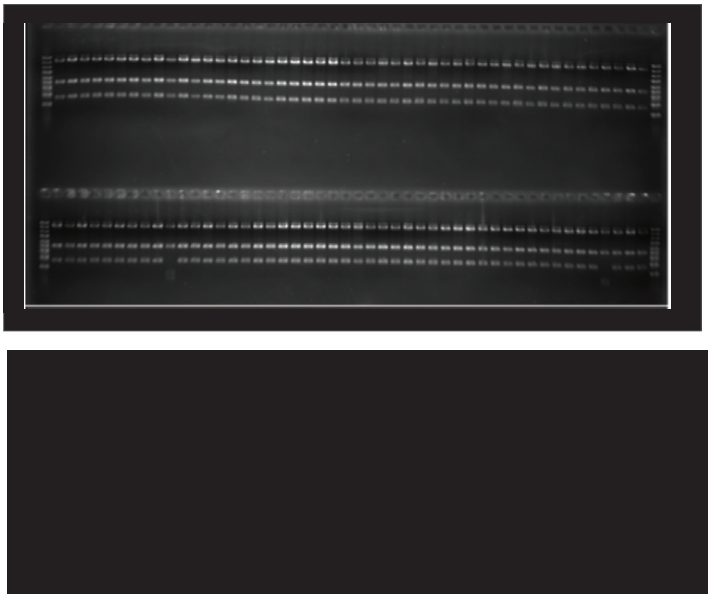


**Figure 3. Representative GIPZ Lentiviral shRNA** clones grown for 16 hours at 30 °C. Plasmid was isolated and normalized to a standard concentration. Clones were then digested with SacII and run on an agarose gel with uncut plasmid. The expected band sizes are 1259 bp, 2502 bp, and 7927 bp. No recombinant 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 viruses that are capable of autonomous replication. The pGIPZ 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 #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 pGIPZ 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 those 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 viruses capable of autonomous replication. Among commercially available lentiviral vector systems, the 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 can be found in (Wu 2000).

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

Any investigator who purchases Horizon 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, Feb 2007 here.
- See also: NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines), September 2009, downloadable here.
- For Biosafety Considerations for Research with Lentiviral Vectors, see.

## Replication of individual clones

Once the clone has been streak isolated and the identity of the strain has been confirmed** by Sanger sequencing (See: What is the sequencing primer for pGIPZ?), we recommend making a stock of the pure culture. Grow the pure culture in LB broth + appropriate antibiotic (See protocol below: Protocol 1 - replication). Vortex the culture to evenly mix the glycerol throughout the culture. The culture can be stored indefinitely at –80 °C.

**Testing of 3-5 colonies is recommended.

## Protocol I – replication

For archive replication, grow GIPZ shRNA clones at 30 °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. Prepare medium with 8% glycerol** and the appropriate antibiotics.

2x LB broth (low-salt) medium preparation

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

Appropriate antibiotic(s) at recommended concentration(s). Glycerol 8% for long-term storage.

***1x LB medium can be used instead of 2x LB broth medium.**

****Glycerol can 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 3. Materials for plate replication.**

| Item | Vendor | Cat # |
|---|---|---|
| 2x LB 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 | Invitrogen | 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 |

**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).

**Prepare source plates**

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

**Replicate**

1. Gently place a disposable replicator in the thawed source plate and lightly move the replicator around inside the well to mix the culture. Make sure to scrape the bottom of the plate of the well.
2. Gently remove the replicator from the source plate and gently place in the target plate and mix in the same manner to transfer cells.
3. Dispose of the replicator.
4. Place the lids back on the source plates and target plates.
5. Repeat steps 1-4 until all plates have been replicated.
6. Return the source plates to the -80 °C freezer.
7. Place the inoculated target plates in a 30 °C incubator 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.

**Due to the tendency of viral vectors to recombine, we recommend keeping the incubation times as short as possible and avoid subculturing. Return to your glycerol stock of your pure culture (see Replication of individual clones) for each plasmid preparation.**

## Protocol II – Plasmid preparation

**Culture conditions for individual plasmid preparations**

For plasmid preparation, grow all GIPZ shRNA clones at 37 °C in 2x LB broth (low salt) medium plus 100 µg/mL carbenicillin only.

1. Upon receiving your glycerol stock(s) containing the shRNA of interest, confirm the clone identity (See: Replication of individual clones) and store immediately 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 broth (low salt) medium with 100 µg/mL carbenicillin. Return the glycerol stock(s) to –80 °C.

**If a large culture volume is desired, incubate the 3-5 mL culture for 8 hours at 37 ˚C with shaking and use as a starter inoculum.**

Dilute the starter culture 1:500–1:1000 into the larger volume.

4. Incubate at 37 °C for 18–19 hours with vigorous shaking.

5. Pellet the culture and begin preparation of plasmid DNA. Plasmid DNA can be isolated using Thermo Scientific™ GeneJET™ Plasmid Miniprep Kit (Cat #K0502) or similar.
6. Run 0.2–1 µg of the plasmid DNA on a 1% agarose gel. pGIPZ with shRNA is 11774 bp.

**Due to the tendency of viral vectors to recombine, we recommend keeping the incubation times as short as possible and avoid subculturing. Return to your original glycerol stock of your pure culture (see Replication of individual clones) 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.

**For the library collection, 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 molecular grade water.**

## Protocol III – restriction digest

The following is a sample protocol for restriction enzyme digestion using Thermo Scientific™ FastDigest™ Restriction Enzymes KpnI (Cat #FD0524), SalI (Cat #FD0644), XhoI (Cat #FD0694) and/or NotI (Cat #FD0594) and Thermo Scientific™ Restriction Enzyme™ SalI (Cat #ER0205) for diagnostic quality control of pGIPZ lentiviral vectors.

1. Add the following components (Table 4), in the order stated, to a sterile PCR thin-wall tube.
2. Mix gently by pipetting.
3. Incubate in a thermal cycler at 37 °C for 5 minutes for FastDigest enzymes or as suggested by the manufacturer.
4. Load the gel with 10 µL of each of the digested samples, KpnI, SacII, SalI, XhoI and/or NotI on a 1% agarose gel. Run uncut sample alongside the digested samples. (Figure 5).

**Table 4. Restriction digest components.**

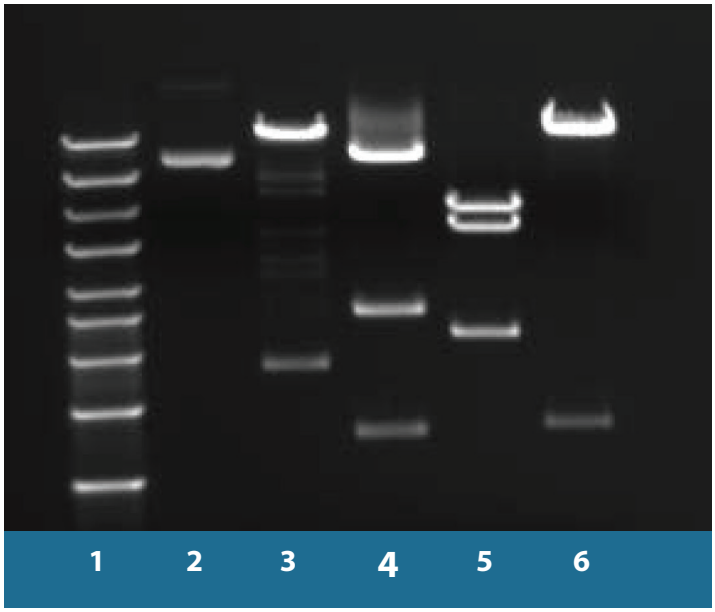
| Component | Amount |
|---|---|
| Water, nuclease-free (Cat #R0581) | X µL |
| 10x FastDigest™ buffer | 2 µL |
| DNA sample (up to 1 µg) in water | X µL |
| FastDigest enzyme | 1 µL |
| Final Volume | 20 µL |

## Protocol IV – Transfection

Quantities and volumes should be scaled-up according to the number of cells/wells to be transfected (Table 5). This example is for 24-well plate format.

1. In each well, seed ~ $5 \times 10^4$ adherent cells or ~ $5 \times 10^5$ 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.**




**Lane 1** - 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)
**Lane 2** - Uncut pGIPZ vector
**Lane 3** - KpnI digested pGIPZ produces two bands at 1917 bp and 9857 bp.
**Lane 4** - SacII digest produces three bands at 1345 bp, 2502 bp and 7927 bp
**Lane 5** - SalI produces three bands at 2188 bp, 4465 bp and 5121 bp
**Lane 6** - XhoI, NotI double digest produces two bands at 1291 bp & 10,483 bp

2. Dilute 1 µg of DNA in 50 µL of DMEM or other serum-free growth medium.
3. Gently mix DharmaFECT kb transfection reagent and add 3 µL to the diluted DNA. Mix immediately by pipetting.
4. Incubate 10 minutes at room temperature. Remove medium from wells 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 reagent per well in a 24-well plate (see scale-up Table 5). Subsequent optimization may further increase transfection efficiency depending on the cell line and transgene used.**

5. Gently add 50 µL of the DharmaFECT kb reagent/DNA mixture to each well.

**The transfection efficiency with DharmaFECT kb transfection reagent (Horizon, Cat #T-2006-01) is equally high in the presence of serum. This is not the case with other transfection reagents.**

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 (see Protocol V – Puromycin Selection).

**Table 3. Scale-up ratios for transfection of adherent and suspensioncells 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 | 0.5-1.2 × $10^4$ (2.0 × $10^4$) | 0.2 | 10 | 0.6 | 0.4-1.0 |
| 48-well plate | 0.7 | 0.25 | 1.0-3.0 × $10^4$ (5.0 × $10^4$) | 0.5 | 25 | 1.5 | 0.8-2.2 |
| 24-well plate | 2.0 | 0.5 | 2.0-6.0 × $10^4$ (1.0 × $10^5$) | 1.0 | 50 | 3.0 | 2.0-5.0 |
| 12-well plate | 4.0 | 1.0 | 0.4-1.2 × $10^5$ (2.0 × $10^5$) | 2.0 | 100 | 6.0 | 3.9-9.0 |
| 6-well plate | 9.5 | 2.0 | 0.8-2.4 × $10^5$ (4.0 × $10^5$) | 4.0 | 200 | 9.0 | 6.0-12.0 |
| 60 mm plate | 20 | 3.0 | 2.0-6.3 × $10^5$ (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.

## Protocol V – Puromycin selection

**Determining antibiotic dose response (Kill curve)**

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:

1. Day 1: Using the same cell type and relative cell densities to be used in subsequent transfection or transduction procedures, plate cells and culture overnight.
2. Day 2: Replace complete growth medium with growth medium supplemented with a range of puromycin concentrations (0-15 µg/mL), including untreated control cells with no antibiotic added.
3. Day 4: Refresh medium and assess viability.
4. Replace medium with fresh medium supplemented with the appropriate concentration of puromycin every 2-3 days depending on the growth of cells.
5. Examine cells daily and identify the minimal concentration of antibiotic that efficiently kills all non-transfected/transduced cells between 3-6 days following addition of puromycin.

**Puromycin selection**

If adding antibiotic for selection, use the appropriate concentration as determined based on the above kill curve.

1. Add medium containing antibiotic 24 or hours post-transfection or post-transduction, respectively, to begin selection

**It is important to wait at least 24 hours after transfection before beginning selection.**

2. Cells can be harvested for transgene expression 24-72 hours after starting selection.
3. If longer selection is required for cells to be confluent, replace selective medium approximately every 2-3 days.
4. Monitor the cells daily and observe the percentage of surviving cells. Cells surviving selection will be expressing the transgene.
5. If generating stable cell lines (optional), select and grow for 10-15 days.
6. Once non-transfected cells are eliminated and/or you have selected for stably transfected cell lines if desired, you can proceed to assay for target gene expression. RT-qPCR, Western blot analysis or other appropriate functional assay can be used; compare treated samples to untreated, reporter alone, non-silencing control, or other controls as appropriate.

## Protocol VI – Packaging lentivirus

The pGIPZ 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 Kit (Cat #TLP5912 or TLP5917). The Trans-Lentiviral shRNA Packaging System allows creation of a replication-incompetent, HIV-1-based lentivirus which can be used to deliver and express your shRNA of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral shRNA Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes (Kappes 2001). For protocols and information on packaging pGIPZ with our Trans-Lentiviral shRNA Packaging System, please see the product manual available on our website.

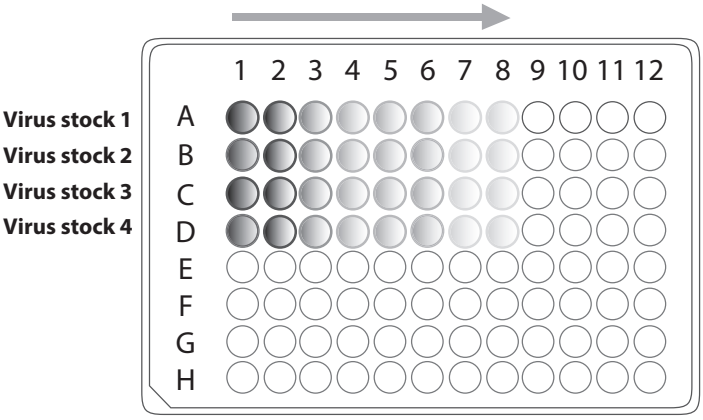



**Figure 6.** Five-fold serial dilutions of virus stock.

## Protocol VII – Titering

**Viral titering**

Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of choice **IF YOU HAVE PRODUCED VIRAL PARTICLES YOURSELF**. This protocol uses the HEK293T cell line that is available as part of our Trans-Lentiviral shRNA Packaging Kit (Cat #TLP5918). You can use a standard HEK293T cell line as an alternative.

**If you have generated a lentiviral stock of the expression control (such as GIPZ non-silencing construct), 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, each 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 media. Utilize the plate as shown in (Figure 6) using one row for each virus stock to be tested. Use the procedure below (starting at step 4) for dilution of the viral stocks. The goal is to produce a series of 5-fold dilutions to reach a final dilution of 390,625-fold.
3. Add 80 µL of serum-free media to each well.
4. Add 20 µL of thawed virus stock to each corresponding well in column 1 (five-fold dilution).

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

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

**Pipette up and down 10-15 times and discard pipette tip.**

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

**Pipette up and down 10-15 times and discard pipette tip.**

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

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

8. Label 24-well plate as shown in (Figure 7) using one row for each virus stock to be tested.
9. Remove culture media from the cells in the 24-well plate.
10. Add 225 µL of serum-free media to each well.
11. Transduce cells by adding 25 µL of diluted virus from the original 96-well plate (Figure 6) to a well on the 24-well destination plate (Figure 7) containing the cells. **For example, transfer 25 µL from well A2 of the 96-well plate into well A1 in the 24-well plate (Table 6).**
12. Incubate transduced cultures at 37 °C for 4 hours.
13. Remove transduction mix from cultures and add 1 mL of DMEM (10% FBS, 1% Pen-Strep).
14. Culture cells for 48 hours.
15. Count the TurboGFP expressing cells or colonies of cells (Figure 8).

**Count each multi-cell colony as 1 transduced cell, as the cells will be dividing over the 48 hour culture period. Figure 8 illustrates this principle of cell counting.**

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

    # of TurboGFP positive colonies counted × dilution factor × 40 = # TU/mL:
    Example: 55 TurboGFP positive colonies counted in well A3
    55 (TurboGFP positive colonies) × 625 (dilution factor) × 40 = 1.38 × $10^6$ TU/mL

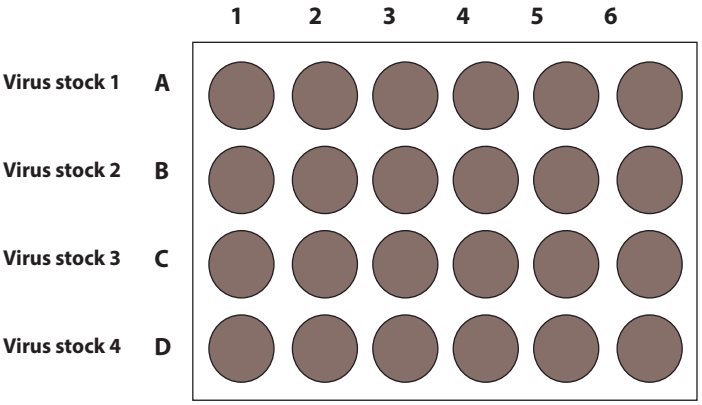



**Figure 7.** Twenty four well tissue culture plate, seeded with HEK293T cells, used to titer the virus.

**Table 6.** Example of set up for dilutions.

| Well (Row A, B, C, or D) | | Volume diluted virus used | Dilution factor |
|---|---|---|---|
| Originating (96-well plate) | Destination (24-well plate) | | |
| A1 | | 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.

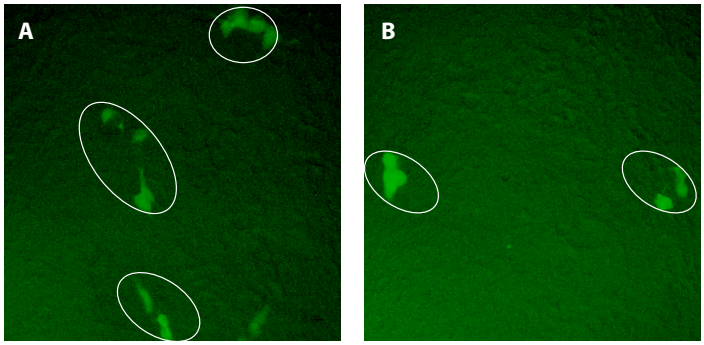



**Figure 8.** Examples of individual colonies.

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 gene silencing

**Multiplicity of infection (MOI)**
To obtain optimal silencing of your shRNA, 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 VIII – 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 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 7). It is strongly recommended that you optimize transduction conditions to suit your target cell line to provide 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 have to optimize the protocol according to your cells of interest.

1. On day 0, plate 5 × $10^4$ cells per well in a 24-well plate. Incubate overnight. **You will be using full growth medium (with serum) at this stage.**
2. The next day (day 1), remove the medium and add an appropriate amount of the virus to acheive the MOI you wish to use. Set up all desired experiments and controls in a similar fashion. **Bring the total volume of liquid up so that it just covers the cells efficiently with serum-free media (see Table 7 for guidelines). If you are using concentrated virus you are likely to use a small volume of virus if you are using unconcentrated virus, you will find you need more virus volume.**

**Table 7.** 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 |

3. Approximately 4-6 hours post-transduction, add an additional 1 mL of full 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 4-6 hours and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.**

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

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

a. If adding puromycin, use the appropriate concentration as determined based on the kill curve (see Protocol V). Incubate cells with the selection medium.
b. Approximately every 2-3 days replace with freshly prepared selective medium.
c. Monitor the cells daily and observe the percentage of surviving cells. Once the non-transduced control cells are dead, the surviving cells in the transduced wells will be expressing the shRNA Optimum effectiveness of the puromycin selection should be reached in 4-6 days with puromycin dependent upon the concentration of puromycin chosen from the kill curve.

**The higher the MOI you have chosen, the more copies of the shRNA and puromycin resistance gene you will have per cell. When selecting with 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 require for your application without going below the minimum antibiotic concentration you have established in your kill curve.**

Once your transduction efficiency is at an acceptable level (with or without puromycin selection performed post-transduction), you can proceed to assay cells for reduction in gene expression or fluorescent reporter activity by reverse transcription quantitative/real-time PCR (RT-qPCR), Western blot analysis or other appropriate functional assay. Compare target gene to untreated, reporter alone (empty vector), non-silencing shRNA, or other negative controls.

**Optimal length of incubation from the start of transfection to analysis is dependent on cell type, gene of interest, and the stability of the mRNA and/or protein being analyzed. RT-qPCR generally gives the best indication of mRNA expression and gene silencing. The use of Western blot analysis to determine knockdown is dependent on quantity and quality of the protein, its half-life, and the sensitivity of the antibody and detection systems used.**

## Protocol IX – Determining relative transduction efficiency

Follow the procedure below to determine the relative transduction efficiency of purchased GIPZ lentiviral particles (Cat #VGH5518, VGM5520, VGH5526). This protocol should only be used with purchased GIPZ shRNA individual clones in viral particle format.

Prior to transducing with purchased GIPZ shRNA individual clones in viral particle format, we recommend determining the relative transduction efficiency of your cell type. Lentiviral titers provided with purchased GIPZ lentiviral particles have been calculated by transducing HEK293T cells. Transduction efficiencies vary significantly by cell type.

The relative transduction efficiency of your cells may be estimated by determining the functional titer of a control virus such as GIPZ Non-silencing control viral particles (Cat #RHS4348) in your cells of interest.

**Follow the procedure below to determine the functional titer of the GIPZ Non-silencing control shRNA viral stock in the mammalian cell line of your choice. The following conditions have been optimized for transduction of HEK293T cells. When determining the relative transduction efficiency of your cell type, use the transduction conditions that have been optimized for your cells of interest.**

1. The day before transduction (day 0), seed a 24-well tissue culture plate with your cells at 5 × $10^4$ cells per well in their respective medium.

**The following day, each well should be no more than 40-50% confluent.**

2. Make dilutions of the Non-silencing control shRNA viral stock in a round bottom 96-well plate using serum-free medium. Utilize the plate as shown in Figure 9 with one row for each replicate (we recommend performing at least two replicates). Use the procedure below for dilution of the viral stock. The goal is to produce a series of five-fold dilutions to reach a final dilution of 390, 625-fold.

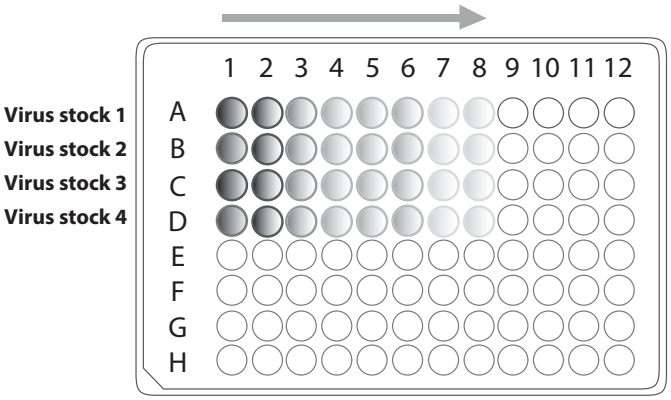



**Figure 9.** Five-fold serial dilutions of virus stock.

3. Add 40 uL of serum-free media to each well in column.
4. Add 80 uL of serum-free media to each well of columns 2-8.

**If desired, include 8 µg/mL polybrene in the dilution medium.**

5. Add 10 µL of thawed control shRNA virus stock to each well in column 1 (five-fold dilution).

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

6. With new pipette tips, transfer 20 µL from each well of column 1 to the corresponding well in column 2.
   **Pipette up and down 10-15 times and discard pipette tip.**
7. With new pipette tips, transfer 20 µL from each well of column 2 to the corresponding well in column 3.
   **Pipette up and down 10-15 times and discard pipette tip.**
8. Repeat transfers of 20 µL 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. Incubate the dilutions of the virus stock for 5 minutes at room temperature.**
9. Label a 24-well plate as shown in Figure 10 using one row for each replicate.
10. Remove culture medium from the cells in the 24-well plate.
11. Add 225 µL of serum-free medium to each well.
12. Transduce cells by adding 25 µL of diluted control shRNA lentivirus from the original 96-well plate (Figure 9) to a well on the 24-well destination plate (Figure 10) containing the cells. **For example, transfer 25 µL from well A2 of the 96-well plate into well A1in the 24-well plate (Table 8).**
13. Incubate transduced cultures at 37 °C for 4-6 hours.

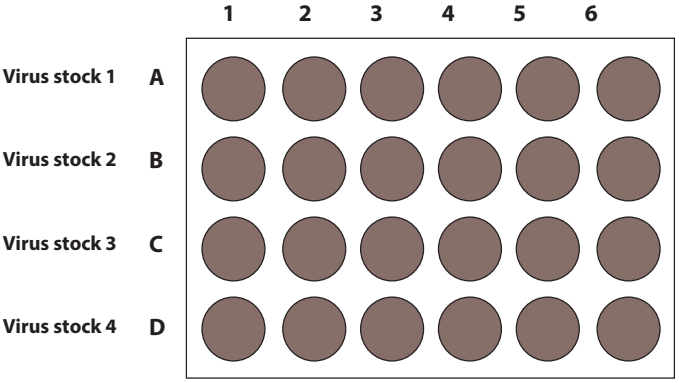



**Figure 10.** Twenty-four well tissue culture plate, seeded with HEK293T cells, used totiter the virus.

14. Add 1 mL of your medium (normal serum concentration).
15. Culture cells for 72 hours.
16. Count the TurboGFP expressing cells or colonies of cells (Figure 11). **Count each multi-cell colony as 1 transduced cell, as the cells will be dividing over the 72 hour culture period. Figure 11 illustrates this principle of cell counting. Count the number of TurboGFP expressing colonies in wells corresponding to at least two viral dilutions.**
17. Transducing units per ml (TU/mL) can be determined using the following formula: # of TurboGFP positive colonies counted × dilution factor × 40 = # TU/mL

**25 µLof diluted virus was added to the cells. This is 1/40th of a mL.**

Example: 55 TurboGFP positive colonies counted in well A3 55 (TurboGFP positive colonies) × 625 (dilution factor) × 40 = $1.38 \times 10^6$ TU/mL.

18. The functional titer calculated for your cell line under your experimental conditions can be used to determine the relative transduction efficiency of your cell type by using the following formula: Functional titer of Non-silencing control shRNA virus stock in your cell line ÷ Titer of Non-silencing control shRNA virus stock as calculated in HEK293T = Relative transduction efficiency.

**Table 8.** 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.

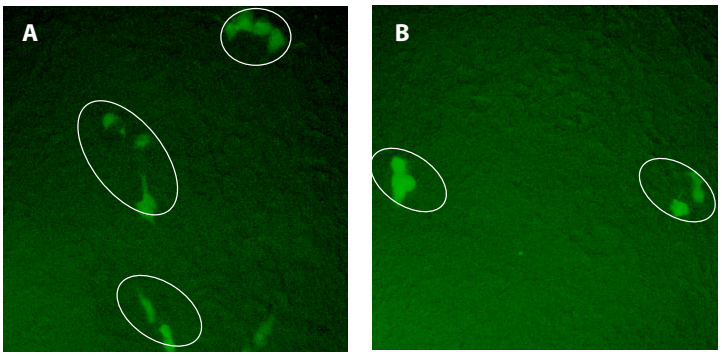



**Figure 11.** Examples of individual colonies.

For example, if the titer of the Non-silencing control shRNA virus stock in HEK293T (as provided on the product specification sheet) is $6.9 \times 10^6$ TU/mL and the functional titer of the control shRNA virus stock in your cell line is $1.38 \times 10^6$ TU/mL, the relative transduction efficiency of your cell type is 0.2. To extrapolate the average functional titer of the provided GIPZ viral particles, multiply the average titer of each plate as provided on the product specification sheet by the relative transduction efficiency of your cell type. In our example, if the titer of the GIPZ viral particles in HEK293T cells is $2 \times 10^6$ TU/mL and the relative transduction efficiency of your cell type is 0.2, the extrapolated average functional titer of that plate your cell type is $4 \times 10^5$ TU/mL.

Once the relative transduction efficiency of the GIPZ virus has been established in your cell line, use the optimized transduction conditions determined in Protocol VIII to transduce your cell line with the purchased GIPZ shRNA individual clones in viral particle format. If the titer of the non-silencing control shRNA virus is not satisfactory in your cell line you might consider choosing a different cell line more permissive to transduction by lentivirus before proceeding.

## Protocol X – PCR

**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 (see Bustin *et al*., 2009).

1. Experimental samples should be run at least in 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. Reverse Transcriptase reactions for cDNA synthesis should always include a No Template Control (NTC) and No Reverse Transcriptase (no RT) control to check for reagents contamination and the presence of contaminating DNA, respectively. Use a robust reverse transcriptase enzyme for cDNA synthesis such as the Thermo Scientific™ Maxima™ cDNA Synthesis Kit for RT-qPCR (Cat #K1641).
3. We have found that normalizing the RNA concentration prior to cDNA synthesis will increase consistency downstream.
4. qPCR should be done at least in 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. Use validated primer sets for SYBR-based assays or primers/probe for probe-based assays.
5. Make sure the mRNA you are using as your internal reference control for qPCR is expressed at a level higher than your target gene's message.
6. Use only high-quality calibrated pipettes, in conjunction with well fitting barrier tips.
7. When pipetting, take the time to visually inspect the fluid in the pipette tip(s) for accuracy and lack of bubbles, especially when using a multi-channel pipette.
8. Be sure to spin your qPCR plate prior to loading in the real-time instrument in order to collect the sample at the bottom of the well and eliminate any bubbles that may have developed.
9. With regard to knockdown experiments using shRNA, it is vitally important that you greatly reduce if not eliminate entirely those cells which are not transduced or transfected from the population. This can be done in several ways: increase the efficiency of your transfection, use a higher mulitplicity of infection (MOI) for your transduction, utilize the puromycin selection marker and select against those cells that do not contain the shRNA or utilize fluorescent sorting to select against those cells that do not contain the shRNA.
10. 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 reference gene expression.
11. 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 transduction and transgene expression. However, it should be noted that small changes in expression levels between an untreated sample and the non-silencing control are to be expected.
12. 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 reference control (such as TBP).
13. Cq values less than 11 for the qPCR internal reference 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 reference control as well as your experimental target to determine an optimum cDNA concentration.
14. It may be necessary to change internal reference controls if conditions in steps 12 and 13 cannot be simultaneously met.

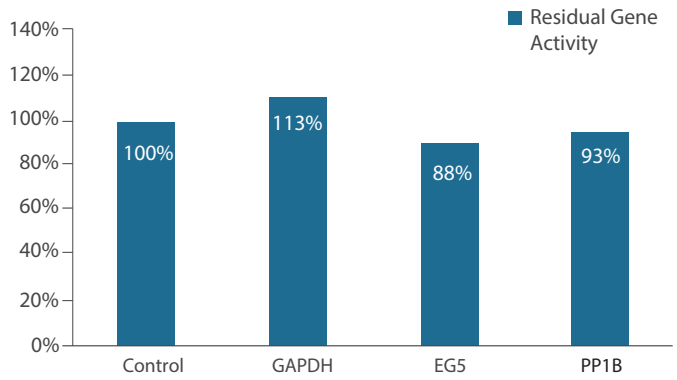



**Figure 12.** Non-silencing lentiviral shRNA control does not knockdown common endogenous genes. The above data represents the baseline amount of GAPDH, EG5 or PP1B mRNA set at 100% in the control. The relative amounts of each of these mRNAs are then represented after treatment with non-silencing shRNA of these genes.

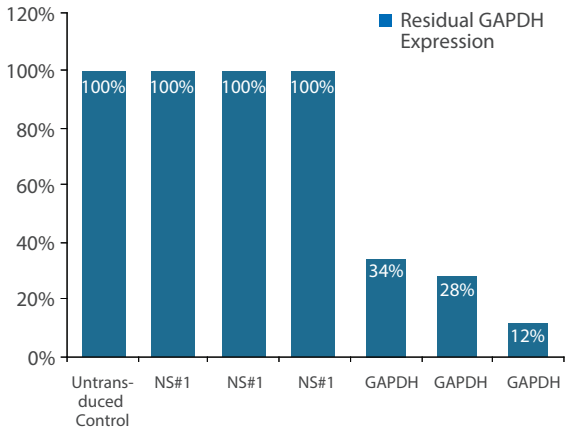



**Figure 13.** HEK293T cells were transduced with lentiviral particles expressing GAPDH or Non-silencing shRNA at variable MOIs ranging from 9-48. The graph depicts the residual levels of GAPDH relative to Non-silencing control.

## Controls and validation

**RNAintro shRNA starter kits**

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

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

**Controls**

The EG5 and GAPDH GIPZ lentiviral shRNA vectors have been validated as positive controls for RNAi experiments performed using the GIPZ shRNA-containing lentiviral vectors. These shRNA have been tested in transduction based experiments and have shown efficient knockdown at both mRNA and protein levels. The EG5 control has been validated to knockdown human EG5 by RT-qPCR (Figure 14 and 15) and in situ hybridization of cells in tissue culture. The GAPDH control has been validated to knockdown human and mouse GAPDH by RT-qPCR (Figure 13). The GIPZ Non-silencing lentiviral shRNA vector has been validated as a negative control for RNAi experiments performed using the GIPZ shRNA-containing lentiviral vectors (Figure 12).

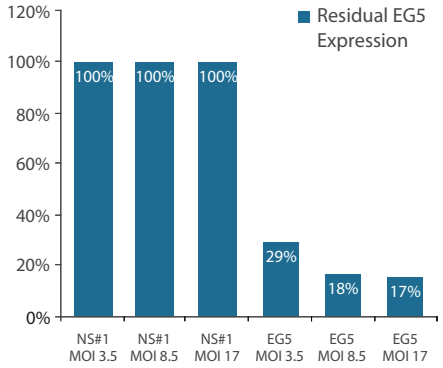



**Figure 14.** HEK293T cells were transduced with lentiviral particles expressing EG5 or non-silencing shRNA at MOIs of 3.5, 8.5 and 17. The graph depicts the residual levels of EG5 relative to its non-silencing control.

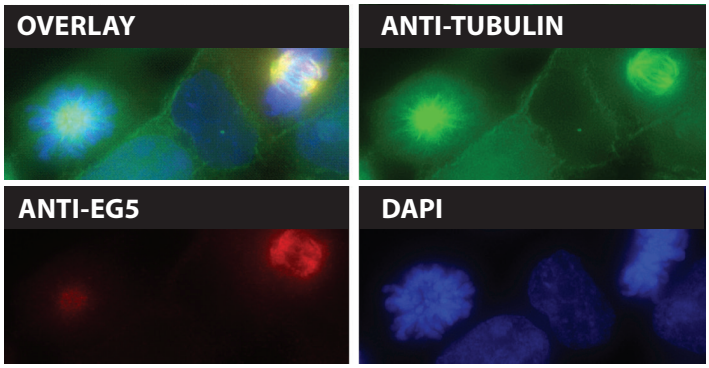



**Figure 15.** The characteristic phenotype observed by the targeting of the EG5 (KIF11) gene results in the formation of half spindles, mitotic arrest and monoastral microtubular arrays (green, see the cell on the left). By contrast, normal cells show bipolar spindles and microtubule networks in mitosis and in interphase (see the cell on the right). The comparative expression of EG5 (red) between the cell on the left and the right shows the extensive knockdown of EG5 in the cell displaying the phenotype (left). The cells were visualized at 100x magnification using aLeica DMIRB fluorescence microscope. HEK293T cells were stained for tubulin (anti-tubulin, green), DNA (DAPI, blue) and EG5 (anti-EG5, red).

**Table 9.** Related Reagents.

| Related Reagents | Dharmacon Cat # |
|---|---|
| GAPDH Verified Positive Control* | RHS4371 |
| EG5 Verified Positive control* | RHS4480 |
| Non-silencing Verified Negative Control* | RHS4346 |
| DharmaFECT kb transfection reagent 1 mL | 2006-01 |
| GIPZ shRNA Empty Vector | RHS4349 |
| Trans-Lentiviral shRNA Packaging System | TLP5912 |
| Trans-Lentiviral shRNA Packaging System with HEK293T Cells | TLP5917 |

*These items also available in the GIPZ lentiviral transfection starter kit (Cat #RHS11851).

## Frequently Asked Questions (FAQs)

**What clones are part of my library 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 (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 GIPZ shRNA constructs in 2x LB broth medium with both 25 µg/mL zeocin and 100 µg/mL carbenicillin for archive replication. For plasmid preparations, grow the constructs in 2x LB broth medium containing only 100 ug/mL carbenicillin.

**What packaging cell line should I use for making lentivirus?**

The GIPZ shRNA vector is tat dependant, so a packaging system that expresses the tat gene. For packaging our lentiviral shRNA constructs, we recommend the Trans-Lentiviral shRNA Packaging Kit (TLP5912, TLP5917). The Trans-Lentiviral Packaging Kit 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 shRNA of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral Packaging Kit uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes (Kappes and Wu et al. 2001). For protocols and information on packaging GIPZ shRNA 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 pGIPZ vector?**

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

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

The titer units are given in transducing units (TU) per mL, so the number 40 is used to convert the 25 µL used in the titration ("volume of diluted virus used", Table 6) to one milliliter.

**What is the sequencing primer for pGIPZ?**

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

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

**Where do you purchase puromycin?**

We purchase puromycin from Fisher Scientific Cellgro (Cat #BP2956-100).

**How many transfections are available in each volume size of DharmaFECT kb transfection reagent?**

The number of transfections that can be performed depends on the size of the culture dish used.

## Troubleshooting

For help with transfection or transduction of your lentiviral constructs, please email technical support at technical@horizondiscovery.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 was the 260/280 ratio of DNA? Over 1.8?
3. What was the transfection efficiency if you used direct transfection? What transfection reagent was used?
4. Were positive and negative knockdown controls used (such as our GAPDH or EG5 validated positive controls and the validated non-silencing negative control)?
5. What were the results of the controlled experiments?
6. How was knockdown measured (for example real-time RT-qPCR or western blot analysis)?
7. What is the abundance and the half-life of the protein? Does the protein have many isoforms?
8. What packaging cell line was used if you are using transduction rather than transfection?
9. What was your viral titer?
10. What was your MOI?
11. Did you maintain the cells in puromycin selection media after transfection or transduction?
12. How much time elapsed from transfection/transduction to puromycin selection?

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

1. Concentration and purity of plasmid DNA and nucleic acids—determine the concentration 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 do not interfere with both DNA/DharmaFECT kb complex formation and cell transfection. This is not the case for other transfection reagents.
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 virus 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 media, the use of polybrene in the media, length of exposure 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 3–5 for factors influencing successful transfection (above).

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**Cited References and additional suggested reading**


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## Lable licenses

The shRNA 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.

### For more information

To find the contact information in your country for your technology of interest, please visit us at **horizondiscovery.com/contact-us**


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