TECHNICAL MANUAL

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INSPIRED CELL SOLUTIONS

SMARTvector™ Inducible Lentiviral shRNA

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1 Introduction to SMARTvector Inducible Lentiviral shRNAs

SMARTvector Inducible Lentiviral shRNA vector

SMARTvector™ Inducible Lentiviral shRNA platform is an innovative system ideally suited for RNA interference (RNAi). The purpose is to provide the researcher with the most effective tools for delivering and expressing genetic content in their cells of interest. The platform is based upon the advanced design of SMARTvector Lentiviral shRNA (microRNA scaffold and rational design). SMARTvector Inducible Lentiviral shRNA vectors utilize the Tet-On 3G bipartite induction system. This tightly regulated system consists of an inducible RNA polymerase II promoter, which has been optimized for both minimal basal expression and potent activation upon induction (Loew, 2010). In the presence of doxycycline, the TRE3G promoter is bound and activated by the constitutively expressed Tet-On 3G transactivator protein, which is also encoded within the Inducible shRNA vector. Together, the Tet-On 3G protein and TRE3G promoter permit tight regulation of the shRNA expression, including potent induction, even at the low doxycycline doses that are required *in vivo*. (See http://dharmacon.gelifesciences.com/shrna/smartvector-inducible-lentiviral-shrna/).

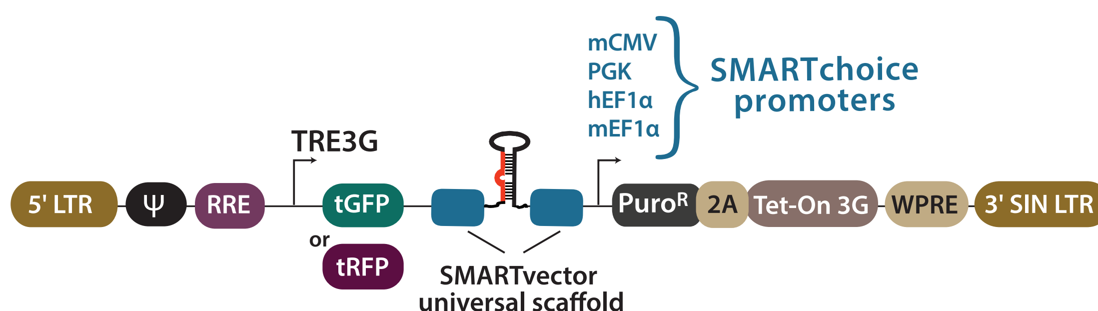

Figure 1. Elements of the SMARTvector Inducible Lentiviral shRNA vector.

Vector Element	Utility
5' LTR	5' Long Terminal Repeat is necessary for lentiviral particle production and integration of the construct into the host cell genome
Ψ	Psi packaging sequence allows lentiviral genome packaging using lentiviral packaging systems
RRE	Rev Response Element enhances titer by increasing packaging efficiency of full-length lentiviral genomes
TRE3G	Inducible promoter with Tetracycline Response Elements which is activated by the Tet-On 3G protein in the presence of doxycycline
tGFP or tRFP	TurboGFP or TurboRFP reporter for visual tracking of transduction and expression upon doxycycline induction
SMARTvector universal scaffold	Optimized proprietary scaffold based on native primary microRNA in which gene-targeting sequence is embedded
Puro^R	Puromycin resistance gene permits antibiotic selection of transduced cells
2A	Self-cleaving peptide that enables the expression of both Puro^R and Tet-On 3G transactivator from a single RNA pol II promoter
Tet-On 3G	Encodes the doxycycline-regulated transactivator protein, which binds to TRE3G only in the presence of doxycycline
WPRE	Woodchuck Hepatitis Post-transcriptional Regulatory Element enhances transgene expression in target cells
3' SIN LTR	3' Self-inactivating Long Terminal Repeat for generation of replication-incompetent lentiviral particles

SMARTvector Inducible Lentiviral shRNA constructs for genes of interest can be ordered at:

http://dharmacon.horizondiscovery.com/rnai/shrna/smartvector-inducible-lentiviral-shrna/ with one of four well-characterized promoters and optional formats with TurboGFP (Evrogen, Moscow, Russia) or TurboRFP (Evrogen, Moscow, Russia) as the fluorescent reporter **(Figure 1)**. Choose to order an individual construct, or Set of 3 constructs, at 100 µL or 200 µL volumes of ≥ 1 × 10^7 TU/mL lentiviral particles.

Recommended experimental controls

Including both inducible positive and negative controls in all experiments assures rigorous, high-confidence data, and is required for accurate data interpretation. In addition, they are cost-effective reagents for experimental optimization, such as: determining optimal conditions for transduction and doxycycline induction prior to transductions with SMARTvector Inducible Lentiviral shRNAs targeting a specific gene.

The following SMARTvector Inducible Lentiviral shRNA controls are available with options for all four promoters and two fluorescent reporters:

- SMARTvector Inducible Non-targeting shRNA Control
- SMARTvector Inducible GAPD Positive shRNA Control (human, mouse and rat)
- SMARTvector Inducible PPIB Positive shRNA Control (human, mouse and rat)

2 Protocol for transduction of lentiviral particles

Workflow checklist

Before you order:

- ○ Determine Materials Required for experiment
- ○ Select Promoter

  If the optimal promoter for your cell type is unknown, empirically test using the SMARTchoice Inducible Non-targeting Control 4-Pack **(See Appendix: Identify the optimal promoter)**.

Order product with the optimal promoter/reporter combination for:

- ○ Your cells and gene(s) of interest
- ○ Positive controls targeting GAPDH or PPIB
- ○ Non-targeting controls

After you order:

- ○ Determine Puromycin Concentration

  Prior to receiving your products, determine the minimum concentration of puromycin required to kill non-transduced cells.

- ○ Determine Optimal Transduction Conditions

  Using the shRNA controls, determine optimal cell density and conditions for transduction **(See Appendix: Determine cell density and transduction conditions)**

- ○ Determine Functional Titer

  Using shRNA controls empirically identify the correct volume needed for each batch of SMARTvector Inducible Lentiviral shRNA particles based upon your cells of interest **(See Appendix: Determining functional titer)**.

- ○ Evaluate Gene Silencing and Phenotype for Gene of Interest

  Using optimized conditions, transduce SMARTvector Inducible Lentiviral shRNAs for gene of interest and monitor induced silencing with assay of choice. **See protocol below:** Gene silencing protocol using SMARTvector Lentiviral shRNAs.

Materials required

- SMARTvector Inducible Lentiviral shRNA(s) targeting your gene or genes of interest
- Positive and negative controls for optimization with matched promoter/reporter options
    - » SMARTvector Inducible Non-targeting Control shRNA
    - » SMARTvector Inducible Positive Control shRNA

Materials not supplied

- SMARTchoice Inducible Non-targeting Control 4-Pack (Dharmacon, Cat #VSC6847)
- Polybrene (American Bioanalytical, Cat #AB01643)
- 96-well tissue culture plates
- Deep-well 96-well plate (Nunc, Cat #12-565-553)
- Multichannel (8-channel) Pipette
- Cells of interest
- Dulbecco's Modified Eagle Medium (DMEM) High Glucose without L-Glut or Sodium Pyruvate (HyClone, Cat #SH30081.FS)
- Base Medium: antibiotic-free cell culture medium (without supplements serum)
- Growth Medium: antibiotic-free cell culture medium (with serum and/or supplements) recommended for maintenance of the cells
- Transduction Medium: the base cell culture medium containing lentiviral particles (with transduction additives and serum (if necessary).
- Selection Medium: Growth Medium supplemented with the appropriate concentration of puromycin
- Doxycycline hyclate (Thermo Scientific, Cat #ICN19895510)
- Resazurin Cell Viability Reagent

Gene silencing protocol using SMARTvector Inducible Lentiviral shRNAs

The following protocol is designed to rapidly develop cells carrying a single copy of a SMARTvector Inducible Lentiviral shRNA proviral genome, and to induce shRNA expression with an optimized doxycycline dosage. In general, the cells are transduced at low MOI (0.3 TU/cell) to produce a population of cells with one integration event per cell. Following the lentiviral transduction, cells that were not transduced are removed from the population through puromycin selection. The majority of cells that remain after selection will carry a single copy of the inducible shRNA.

Note: While higher MOIs may enhance gene silencing, it is important to consider the possibility of low-level gene knockdown in the absence of doxycycline induction or leakiness. SMARTvector Inducible Lentiviral shRNAs are provided as purified concentrated lentiviral particles to enable a wide range of MOIs that may be required for specific applications. We recommend single-copy lentiviral genome integration for optimal gene silencing with minimal promoter leakiness. Higher MOI may be suitable depending on your application needs.

Determining functional titer in cells of interest

Lentiviral transduction efficiency can vary widely depending on factors such: as target cell type, duration of exposure to lentiviral particles, composition of Transduction Medium and experimental conditions. Therefore, for sensitive applications where a low MOI is desired, the correct volume of lentiviral particles should be empirically identified for each batch of SMARTvector Inducible Lentiviral shRNA particles **(See Appendix: Determining functional titer)**. The

functional titer of SMARTvector Inducible Lentiviral shRNA particles (in HEK293T cells) is reported on the Certificate of Analysis (C of A). We recommend an MOI of 0.3 (adjusted for relative transduction efficiency in your cell type) to ensure single integration of the lentiviral shRNA. The relative transduction efficiency of your cell type will likely be lower than that of HEK293T cells.

Inducible gene silencing by establishing a stable cell population

For cell lines with longer proliferative capacity, stable cell lines may be established by transduction of SMARTvector Inducible Lentiviral shRNA particles and subsequent puromycin selection. These cell lines may be maintained in an uninduced state until expression of the shRNA is desired. Care must be taken so that cells are used within a limited number of passages from completion of selection, depending on the characteristics of the particular cell type.

Day 1:

Transduce cells at a low MOI of 0.3 TU/well in a 96-well, using conditions determined previously.

Day 2:

Change Transduction Medium to Growth Medium.

Day 3:

Trypsinize cells and expand into a larger culture vessel with Selection Medium (containing the predetermined concentration of puromycin sufficient to select for stable integrants).

Day 5:

Replace with fresh Selection Medium.

Note: After cells have been selected in puromycin, it is no longer necessary to maintain antibiotic pressure.

Day 7 or later:

1. Expand the culture until an adequate cell number has been achieved to freeze and archive the cell line.
2. Induce shRNA expression in the experimental cells using the predetermined optimal doxycycline dose.

Day 8 or later:

Check for TurboGFP or TurboRFP expression, or determine gene knockdown.

Note: TurboGFP or TurboRFP expression will become visible at 24 hours and will reach a maximum at 48 to 72 hours post—doxycycline administration. Similarly, gene knockdown, at the mRNA level, is observable at 24 hours and reaches a maximum by 72 hours. Longer periods of exposure to doxycycline may be necessary to achieve some phenotypes.

Inducible gene silencing without establishing stable cell lines

It is not necessary to establish stable cell lines with SMARTvector Inducible Lentiviral shRNAs to perform a knockdown experiment. Depending on the phenotype to be assayed, immortalized, primary, and non-dividing cells may be transduced with SMARTvector Inducible Lentiviral shRNA particles, selected for two to four days in puromycin, induced with doxycycline and assayed within a 7 to 10 day time frame. Using inducible shRNA systems in this context allows temporal separation of phenotypes associated with lentiviral transduction and puromycin selection from phenotypes resulting from knockdown of the target gene.

Day 1:

Plate cells at density optimized for the assay.

Note: **Initial cell density will depend on the desired density at the time of phenotypic analysis, growth rate of cells, and desired experimental timeframe. If targeting an MOI between 0.1 and 0.3, be certain to factor in cell loss resulting from death of untransduced cells during puromycin selection.**

Note: **Make sure to plate additional wells with cells that will be used to monitor the puromycin selection.**

Day 2:

Transduce cells at an MOI of 0.3 in 96-well.

Day 3:

Replace culture Transduction Medium with appropriate Growth Medium.

Day 4:

Replace medium with fresh Selection Medium (containing a puromycin concentration that has been determined to kill untransduced cells in two to three days).

Day 6 or 7:

1. Examine control cells (untransduced) and determine if puromycin selection is complete. If live cells remain, continue puromycin selection for another day.
2. Induce shRNA expression in the cells using the predetermined optimal doxycycline dose.

Note: **Doxycycline induction may be started at this time, or alternatively, the Transduction Medium may be replaced with fresh Selection Medium, and doxycycline induction may be initiated at a later time point. We have not observed any negative effects of continuing puromycin selection while beginning doxycycline induction.**

Day 8 or 9:

1. Check for TurboGFP or TurboRFP expression.
2. Determine knockdown or phenotype.

3 Appendix

Additional protocols

Identify the optimal promoter

SMARTvector Inducible Lentiviral shRNA vector configuration options include four RNA pol II promoter options: mouse CMV, PGK, human EF1α and mouse EF1α; all of which control the expression of puromycin resistance and the Tet-On 3G transactivator. However, the activity of these promoters may not be known for a particular cell of interest prior to RNAi experimentation. By utilizing the SMARTchoice Inducible Non-targeting Control 4-Pack (Cat #VSC6847), the researcher can evaluate which of these promoters are most active in the cells of intended study. After transduction, TurboGFP expression is induced with 1 µg/mL doxycycline, and the activity of each vector configuration is observed as relative intensity of TurboGFP fluorescence. The optimal vector configuration is the one producing the highest level of fluorescence intensity. Once the most active promoter option is qualitatively determined for the specific cells of interest, SMARTvector Inducible Lentiviral shRNAs targeting genes of interest and experimental controls incorporating the optimal promoter and fluorescent reporter (TurboGFP or TurboRFP) of choice can be ordered.

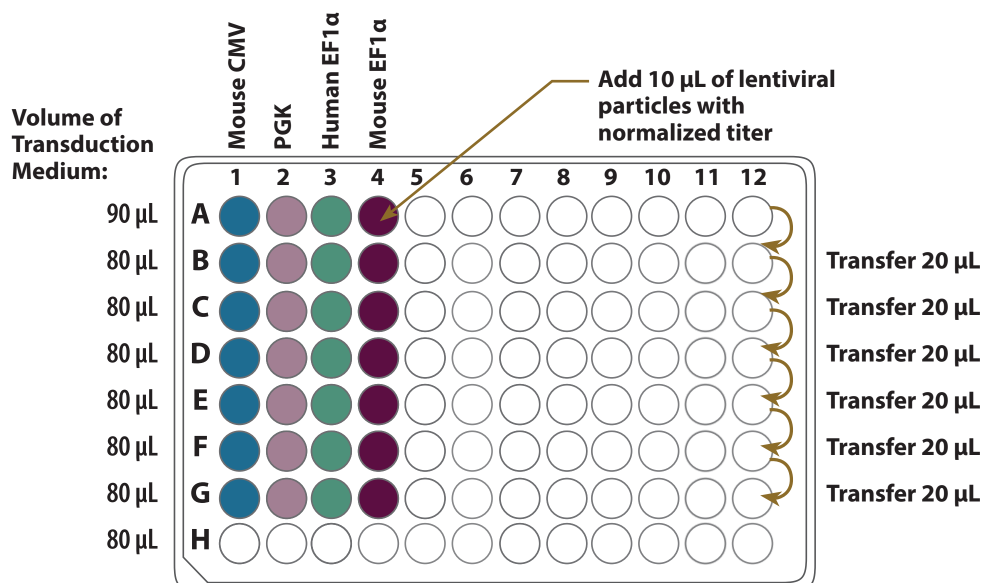

Figure 2. Lentiviral particle dilution plate layout for assessing vector activity in cells of interest using the SMARTvector Inducible Non-targeting Control shRNA by creating a 5-fold serial dilution. There should be no lentiviral particles in Row H wells, only medium.

Day 1:

Plate cells in a 96-well culture plate at optimal cell density in appropriate Growth Medium as determined in previous experiments **(See Appendix: Determine cell density and transduction conditions).** If working with suspension cell lines, plate cells the day of transduction.

Day 2:

1. Prepare 10 mL of pre-warmed Transduction Medium (using serum concentrations as determined previously).
2. Prepare the lentiviral particles dilution plate in which each column represents one of the four SMARTvector Inducible Non-targeting Control vector configurations (mCMV, PGK, hEF1α and mEF1α); see **Figure 2** for an illustration of the recommended plate layout. To the first well at the top of each of four columns (Row A), add 90 µL of Transduction Medium. Then add 80 µL of Transduction Medium to each well in Rows B-H, columns 1-4. Place this plate of Transduction Medium in the incubator until Step 4.

3. Remove your SMARTchoice Inducible Non-targeting Control 4-Pack from the -80 °C freezer and thaw the lentiviral particles while keeping the tubes on ice. Normalize the titers of all four promoter options to the one with the lowest titer using DMEM.
4. To your dilution plate containing Transduction Medium, add 10 µL of each thawed, titer-normalized SMARTvector Inducible Non-targeting Control lentiviral particles to separate wells at the top of each column (Row A). Mix the particles by gently pipetting up and down 3-5 times.
5. With a multichannel pipette, transfer 20 µL of Transduction Medium with lentiviral particles from Row A to Row B. Mix at each transfer step by gently pipetting up and down 3-5 times.
6. Repeat the transfer of 20 µL of Transduction Medium with lentiviral particles from each row to the row below it, through Row G **(see Figure 2);** thus creating 5-fold serial dilutions for each SMARTvector Inducible Non-targeting Control vector configuration. Wells in Row H should contain no lentiviral particles.
7. Remove cell culture plates from the incubator and replace Growth Medium with 25 µL of Transduction Medium containing lentiviral particles from the corresponding well of the lentiviral particles dilution plate.
8. Return cell culture plate with Transduction Medium and lentiviral particles to the incubator.
9. After 6-20 hours (as determined previously **(See Appendix: Determine cell density and transduction conditions**), add 100 µL of medium directly to each well without removing Transduction Medium. Make the necessary adjustments to serum concentration so that the final serum concentration matches Growth Medium after adding to wells.

Day 3:

1. After 24 hours, replace medium with Growth Medium supplemented with 1 µg/mL of freshly dissolved doxycycline.
2. Return plate to incubator and culture for an additional 48 hours.

Day 5:

Examine each well of the 96-well culture plates, and for each vector, identify the row in which approximately 10-30% of the cells are positive for TurboGFP. The majority of cells in this row carry a single integration of the lentiviral genome. Assess TurboGFP fluorescence intensity by FACS or microscopy using the appropriate filters.

The optimal vector for your cell type is the one producing the highest level of fluorescence intensity, when comparing the promoter options at the same number of lentiviral particles.

Note: TurboGFP and TurboRFP expression from a single lentiviral integration can be difficult to see without a high-quality fluorescence microscope. Additionally, phenol red absorbs at both the excitation and emission wavelengths of both of these fluorophores. To enhance the visibility of fluorescing cells, replace the culture medium with a medium that is low in phenol red.

Determine cell density and transduction conditions

Determine cell density and transduction conditions for adherent cells

Successful transduction of cells depends on cell type, cell density, passage number, MOI during transduction, purity of the lentiviral preparation and the presence and/or absence of reagents that facilitate transduction.

Note: Polybrene can be toxic to certain cells. Although Polybrene is not generally necessary to achieve the MOIs recommended for SMARTvector Inducible Lentiviral shRNAs, it may aid transduction for some difficult-to-transduce cell types.

The following optimization protocol allows you to determine lentiviral transduction conditions that are compatible with the cell type of interest in one experiment in a 96-well plate format as depicted in **Figure 3.** This recommended protocol will test cell density, Polybrene concentration, the presence or absence of serum, and the duration of transduction (6 hours or overnight).

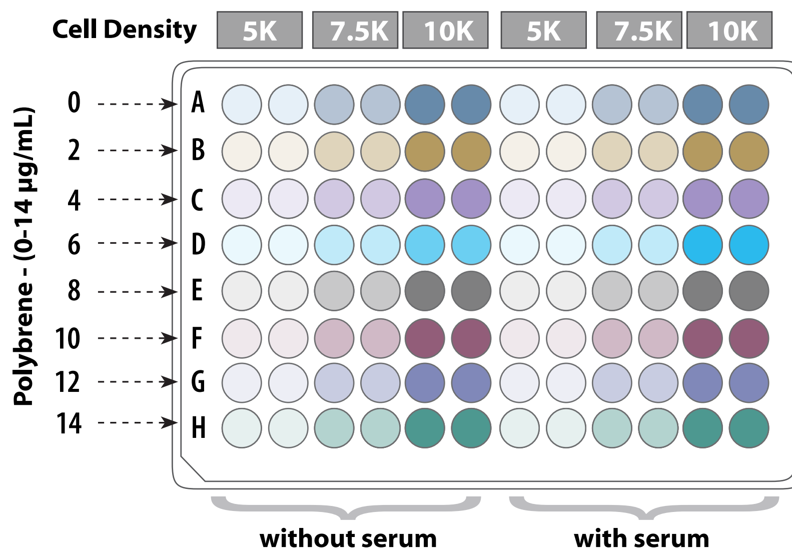

Figure 3. Example of a 96-well plate layout for the optimization of transduction conditions. This plate layout tests three cell densities [5,000 (5K), 7,500 (7.5K) and 10,000 (10K) cells per well], eight concentrations of Polybrene (0 - 14 µg/mL) and media with or without serum.

Day 1:

Seed cells into two 96-well culture plates at the appropriate cell density following the template depicted in **Figure 3.** Use a total volume of 100 µL of the Growth Medium per well and place 96-well culture plates into incubator for overnight culture under the appropriate conditions (temperature and CO_2 concentration).

Day 2:

1. Visually inspect each well under a microscope. Note the confluency for each of the three different cell concentrations seeded on Day 1.

Note: If all three cell concentrations seeded resulted in > 90% confluency, then it is necessary to re-seed the cells using a lower range of cell densities. Generally, transductions with lentiviral particles should be performed when cells are 40% to 80% confluent, depending on the characteristics of the cell line providing the cells with enough surface area to replicate in culture. Because cells can differ significantly with respect to size and morphology, the number of cells seeded should reflect this difference. The number of cells shown in Figure 3 (5,000, 7,500, and 10,000) is specified only to depict a range of cell concentrations that should be seeded across the 96-well plate. The actual number of the cells seeded for the specific cells of interest will vary depending on the size and morphology and will depend on previous experience.

2. Prepare two sets of Transduction Medium: one without serum and one with serum.
   a. Prepare Transduction Medium without serum:
      i. Prepare 10 mL of Transduction Medium containing no serum by pipetting a 1:1 mixture of DMEM to Base Medium.

Note: Lentiviral particles are provided in DMEM. Thus, testing a recipe for suitable Transduction Medium must include DMEM. Although, if the preferred Growth Medium for your cell line contains DMEM this is not necessary, as the cells will tolerate DMEM.

      ii. Transfer 700 µL aliquots of this mixture into eight wells of a sterile deep-well 96-well plate.
      iii. Use these wells to prepare a series of Transduction Medium formulations containing eight different Polybrene concentrations ranging from 0-14 µg/mL, increasing concentration in 2 µg/mL increments (**Figure 4**).

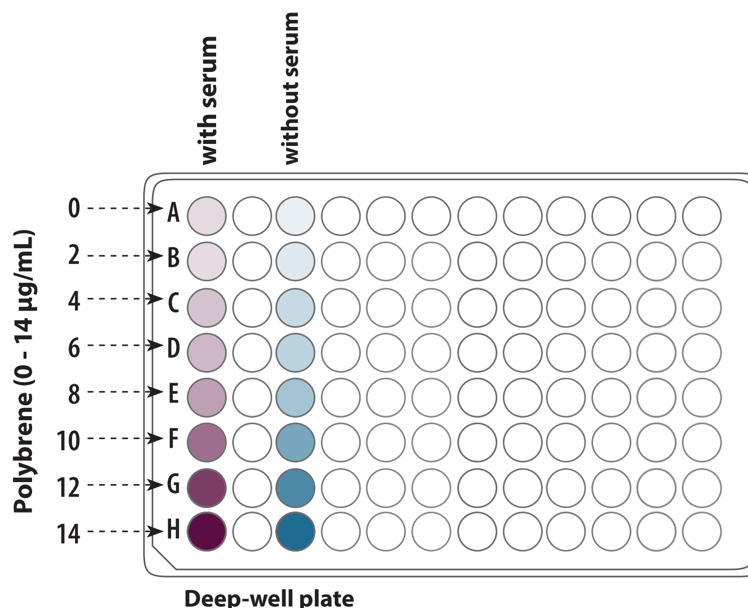

Figure 4. Example of a plate layout for preparation of Transduction Medium. Depicted here is Transduction Medium with (blue wells) or without (purple wells) serum containing a range of Polybrene concentrations in a deep-well, 96-well plate. White wells are empty.

b. Prepare Transduction Medium with serum:

i. Prepare a similar series of wells with Transduction Medium consisting of a 1:1 mixture of DMEM to Base Medium and containing serum at the concentration that is appropriate for the cells of interest.

Note: Again, if the Growth Medium for your cell line contains DMEM simply use 100% of the Base Medium.

3. Perform transduction
   a. Remove culture plates from incubator and place in biological safety cabinet.
   b. Using a multichannel pipette, carefully aspirate Growth Medium from each well of 96-well culture plates being careful not to dislodge cells from bottom of well and dispense into a liquid waste reservoir.
   c. Using a multichannel pipette, transfer 50 µL of Transduction Medium (with or without serum) containing the range of Polybrene concentrations (0-14 µg/mL) to cells seeded in 96-well culture plate. Transfer Transduction Medium in format depicted in **Figure 4**.
   d. Return culture plates to incubator and maintain under the appropriate conditions.
   e. After 6 hours, remove one of the two replicate plates from the incubator and place in biological safety cabinet.
   f. Using a multichannel pipette add 100 µL of Preferred Culture Medium directly to each well of the replicate plate and return to incubator for overnight culturing.

Note: To adjust for the absence of serum (transductions without serum only) make up the difference by including additional serum to the Growth Medium. For example, if your cells require 10% serum, then increase the serum concentration of the Growth Medium to 15%. Adding 100 µL of this culture medium to the 50 µL of Transduction Medium in the well will result in a final serum concentration of 10%.

Day 3:

After overnight incubation with Transduction Medium (16-20 hours) for the second replicate, add 100 µL of Growth Medium directly to each well of plate. Adjust serum concentration as described above to the wells without serum.

Day 4:

After 24 hours, examine all wells of 96-well plates using a microscope and record the confluency and any morphological or phenotypic alterations that may be present.

Day 5:

1. Examine cultures again for cell morphology or presence of phenotypic changes and record observations followed by assaying the cells for viability using any commercially available kit, such as Resazurin or a similar assay for viability.
2. Select the optimal cell density and conditions that result in optimal phenotypic changes for your cell line.

Note: Based on observations from a variety of cell lines and cell types, the lowest concentration of serum generally results in the highest transduction efficiency. Choose the highest concentration of Polybrene with acceptable viability for the cells of interest. If, in subsequent protocols, it is found that transduction efficiency is low, Transduction Medium may be supplemented with this concentration of Polybrene to enhance efficiency. Furthermore, if there is no evidence of significant cellular toxicity with overnight incubation (16-20 hours), then this incubation time may be used for subsequent transductions.

Determine cell density and transduction conditions for suspension cells

Non-adherent or suspension cells should be counted and plated at the time of transduction and not incubated overnight.

Day 1:

1. Determine the number of suspension cells per mL.
2. Transfer enough cells to seed two 96-well culture plates into two sterile centrifuge tubes (include additional 20% volume of cells to ensure adequate volumes for transfer).
3. Centrifuge tubes at low speed to pellet cells.
4. Pour off supernatant containing old culture medium.
5. Tap bottom of centrifuge tube gently to dislodge cell pellets.
6. Resuspend cells in one centrifuge tube with Base Medium containing no serum and cells in the other centrifuge tube in Base Medium containing appropriate concentration of serum for cells of interest. Resuspend in enough volume so that appropriate number of cells are seeded in a total volume of 25 µL per well.

Note: Transduction Medium formulations (with and without serum; 0-14 µg/mL Polybrene concentration) should be prepared essentially as described above. However, because Transduction Medium will be added to cells resuspended in 25 µL of Base Medium (with and without serum), Polybrene and serum concentrations of Transduction Medium will need to be adjusted accordingly.

7. Prepare Transduction Medium without serum for suspension cells:
   a. Prepare 10 mL of Transduction Medium containing no serum by pipetting a 1:1 mixture of DMEM to Base Medium.

Note: Lentiviral particles are provided in DMEM. Thus, testing a recipe for suitable Transduction Medium must include DMEM. If the preferred Base Medium for your cell line contains DMEM this is not necessary, as the cells will tolerate DMEM.

   b. Transfer 700 µL aliquots of this mixture into eight wells of a sterile deep-well 96-well plate.
   c. Use these wells to prepare a series of Transduction Medium formulations containing eight different Polybrene concentrations ranging from 0-14 µg/mL, increasing concentration in 2 µg/mL increments **(Figure 4)**. Polybrene concentration is increased to account for the additional 25 µL of Base Medium, which does not already contain Polybrene. For example, well 2 contains 3 µg/mL Polybrene; adding 50 µL of Transduction Medium with a Polybrene concentration of 3 µg/mL to 25 µL of Base Medium containing no Polybrene will result in a final concentration of 2 µg/mL.
   d. Use **Table 1** to prepare the eight Transduction Medium formulations. Two sets of eight wells of Transduction Medium should be prepared as before **(Figure 4)**, one set without serum and one with serum.

Table 1. Transduction Medium formulations for preparing media with eight different Polybrene concentrations.

	Polybrene concentration of Transduction Medium	Final Polybrene concentration after adding to suspension cells
Well 1	none	0 µg/mL
Well 2	3 µg/mL	2 µg/mL
Well 3	6 µg/mL	4 µg/mL
Well 4	9 µg/mL	6 µg/mL
Well 5	12 µg/mL	8 µg/mL
Well 6	15 µg/mL	10 µg/mL
Well 7	18 µg/mL	12 µg/mL
Well 8	21 µg/mL	14 µg/mL

8. Perform mock transduction
   a. Remove culture plates from incubator and place in biological safety cabinet.
   b. Using a multichannel pipette, transfer 50 µL of Transduction Medium (with or without serum) containing the range of Polybrene concentrations (0-14 µg/mL) to cells seeded in 96-well culture plate. Transfer Transduction Medium in format depicted in **Figure 4**.
   c. Return culture plates to incubator and maintain under the appropriate conditions.
   d. After 6 hours, remove one of the two replicate plates from the incubator and place in biological safety cabinet.
   e. Using a multichannel pipette add 75 µL of Preferred Culture Medium directly to each well of the replicate plate and return to incubator for overnight culturing.

Note: To adjust for the absence of serum (transductions without serum only) make up the difference by including additional serum to the Preferred Culture Medium. For example, if your cells require 10% serum, then increase the serum concentration of the Preferred Culture Medium to 20%. Adding 75 µL of this culture medium to the 75 µL of Transduction Medium in the well will result in a final serum concentration of 10%.

Day 2:

After overnight incubation with Transduction Medium (16-20 hours), add 75 µL of Preferred Culture Medium directly to each well in plate 2. Adjust serum concentration as described above to wells containing no serum.

Day 3:

Using a microscope, examine all wells of 96-well plate and note any morphological or phenotypic alterations present.

Day 4:

1. Examine cultures for cell morphology or presence of phenotypic changes. Cell viability should be determined using any commercially available kit, such as Resazurin or a similar assay for viability.
2. Select the optimal cell density and conditions that result in optimal phenotypic changes for your cell line.

Note: Based on observations from a variety of cell lines and cell types, the lowest concentration of serum generally results in the highest transduction efficiency. Choose the highest concentration of Polybrene with acceptable viability for the cells of interest. If, in subsequent protocols, it is found that transduction efficiency is low, Transduction Medium may be supplemented with this concentration of Polybrene to enhance efficiency. Furthermore, if there is no evidence of significant cellular toxicity with overnight incubation (16-20 hours), then this incubation time may be used for subsequent transductions.

Determine functional titer

Note: The functional titer of SMARTvector Lentiviral shRNA constructs in HEK293T cells is reported on the Certificate of Analysis (C of A). The relative transduction efficiency of your cell type will likely be lower than that of HEK293T cells. The protocol below can be used to determine your cell-line specific functional titer.

Functional titer can be determined either by counting GFP-positive colonies using fluorescence microscopy or by FACS analysis of fluorescent shRNA constructs, or resazurin for non-fluorescent shRNA constructs. The following protocol describes how to estimate functional titer by using non-silencing control lentiviral particles and determining titer by fluorescence microscopy.

1. The day before transduction, seed a 96-well cell culture plate (Destination Plate) with your cells at the density determined during transduction optimization. Grow cells overnight.
2. Make dilution medium using serum and Polybrene conditions determined during transduction optimization. Make dilutions of inducible non-silencing control lentiviral particles in a round-bottom 96-well plate (Dilution Plate). As shown in **Figure 5 and Table 2**, use one row of the plate for each replicate of the dilution series of the lentiviral stock. We recommend performing two replicates. The procedure for dilution of the lentiviral stock is described below and results in a series of five-fold dilutions to reach a final dilution of 390,625-fold.
    a. Add 40 µL of dilution medium to wells A1 and B1. Add 80 µL of dilution medium to each well A2-A8 and B2-B8.
    b. Thaw non-silencing control lentiviral particles on ice and then add 10 µL each to wells A1 and B1. Mix contents of each well by pipetting up and down 10-15 times. Discard pipette tip.
    c. Transfer 20 µL from wells A1 and B1 to the corresponding wells in column 2. Mix contents of each well by pipetting up and down 10-15 times. Discard pipette tip.
    d. Repeat transfer of 20 µL for columns 2 through 8, mixing 10-15 times for each dilution.
    e. Allow lentiviral-Polybrene complexes to form for 3-5 minutes at room temperature.
3. Remove culture medium from the cells in the 96-well plate Destination Plate
4. Transfer 25 µL of each dilution of lentiviral particles from the Dilution Plate to the corresponding wells in the Destination Plate, being careful to not create bubbles.
5. Incubate the cells for 4-24 hours (as determined during transduction optimization).
6. Add 75 µL of Preferred Culture Medium to cells without removing medium. Make the necessary adjustments to serum concentration so that the final serum concentration matches the Preferred Culture Medium after adding to wells.
7. After 24 hours, replace medium with Preferred Culture Medium supplemented with 1 µg/mL of freshly dissolved doxycycline.
8. Culture cells for an additional 48-72 hours (as determined during transduction optimization).
9. Choose one well in the Destination Plate for counting fluorescence-expressing colonies of cells. This should be a well in which individual colonies of cells can be visualized and counted. Count each multi-cell colony as one transduction event, as the cells have been dividing over the culture period **(Figure 6)**. Calculate the average number of fluorescent-positive colonies from the same destination well of each replicate.

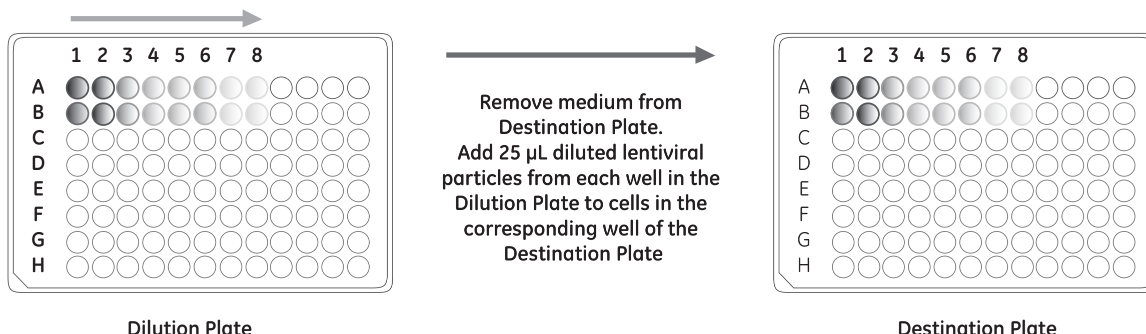

Figure 5. Diagram for dilution series of lentiviral particles (Dilution Plate) and addition to cells (Destination Plate).

Table 2. Example setup for lentiviral particle dilution series.

Well	Dilution Plate		Dilution factor	Volume of diluted lentiviral particles used in transduction (Destination Plate)
	Lentiviral particle serial dilution volume	Volume of dilution medium		
A1	10 µL (*control)	40 µL	5	25 µL
A2	20 µL (from A1)	80 µL	25	25 µL
A3	20 µL (from A2)	80 µL	125	25 µL
A4	20 µL (from A3)	80 µL	625	25 µL
A5	20 µL (from A4)	80 µL	3125	25 µL
A6	20 µL (from A5)	80 µL	15 625	25 µL
A7	20 µL (from A6)	80 µL	78 125	25 µL
A8	20 µL (from A7)	80 µL	390 625	25 µL

*Control (non-silencing shRNA control lentiviral particles). Repeat identical dilution series in wells B1 to B8.

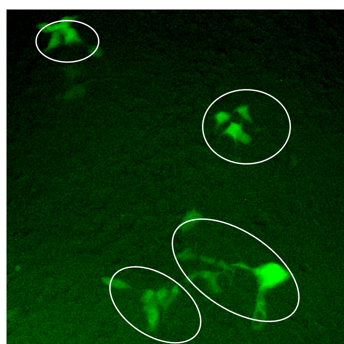

Figure 6. Example of individual colonies in HEK293T cells 72 hour post-transduction. Four colonies are circled. Imaged at 40x magnification.

Functional titer in transducing units per mL (TU/mL) can be determined using the following formula: Number of TurboGFP-positive colonies × Dilution factor (Table 2) ÷ 0.025 mL (Volume of diluted lentiviral particles used) = Functional titer of non-silencing control lentiviral particles stock in your cell line (TU/mL)

Relative transduction efficiency of your cell type can be determined by using the following formula:
Functional titer of non-silencing control in your cell line (TU/mL) ÷ Titer of non-silencing control lentiviral particles stock as calculated by Dharmacon in HEK293T (TU/mL) (reported on the C of A) = Relative transduction efficiency of your cell line

Use the calculated relative transduction efficiency of your cell line to calculate the anticipated functional titer for each lentivirus using the following formula:
Relative transduction efficiency of your cell line × Titer of the lentivirus as calculated by in HEK293T cells (TU/mL) = Anticipated functional titer in your cell line (TU/mL)

Calculating Examples

If you counted 58 fluorescent-positive colonies in well A7 of the destination plate, the titer of the non-silencing control lentiviral particles in your cell line would be calculated as follows:

58 (transduction positive colonies) × 78,125 (dilution factor) ÷ 0.025 mL (volume of diluted lentiviral particles used) = 1.8×10^8 TU/mL functional titer of non-silencing control in your cell line

If the titer for the non-silencing control lentiviral particles on the product insert was listed as 9.0×10^8 TU/mL, the relative transduction efficiency of your cell type would be determined as follows:

1.8×10^8 TU/mL (functional titer in your cell line) ÷ 9.0×10^8 TU/mL (titer as indicated on product insert) = 0.2 relative transduction efficiency

If the relative transduction efficiency of your cell line is 0.2 and the titer of a lentiviral stock, as indicated on the C of A, is 5.0×10^8 TU/mL, the anticipated functional titer of the pool in your cell line would be determined as follows: 0.2 (relative transduction efficiency) × 5.0×10^8 TU/mL (titer as indicated on product insert) = 1.0×10^8 TU/mL anticipated functional titer in your cell line

Optimize doxycycline concentration

The Tet-On 3G induction system employed in the SMARTvector Inducible Lentiviral shRNA constructs generally allows robust shRNA induction at doxycycline doses between 0.1 µg/mL and 1.0 µg/mL, although this can depend on the cell type in use. Furthermore, intermediate expression of the shRNA, and therefore intermediate gene silencing, may be achievable at doses between 10 ng/mL and 100 ng/mL, although this will require optimization to obtain consistent results. In some cell types, doses of doxycycline > 0.5 µg/mL have been observed to affect cell viability. We strongly recommend that for each cell type, a doxycycline-dose curve should be performed to identify the lowest effective concentration of doxycycline resulting in the least effect on cell viability. The following protocol uses the SMARTvector Inducible Negative and Positive RNAi Controls to optimize the doxycycline dose for both maximal knockdown and maximal cell viability. This experiment is recommended whenever SMARTvector Inducible Lentiviral shRNAs are used in a new cell line or type.

Day 1:

Plate cells in biological triplicates in a 96-well culture plate at optimal cell density in appropriate Growth Medium as determined in previous experiments **(See Appendix: Determine cell density and transduction conditions)**. If working with suspension cell lines, plate cells the day of transduction **(See protocol below for recommendations)**.

Day 2:

1. Thaw the inducible positive and negative control lentiviral particles on ice.
2. For both the positive and negative controls, prepare enough Transduction Medium with lentiviral particles to transduce 24 wells of a 96-well plate at an MOI of 0.3.
3. Remove culture plates from incubator and replace Growth Medium with 25 µL of Transduction Medium with lentiviral particles according to the plate layout in **Figure 7**.
4. Return culture plate to incubator.
5. After 6-20 hours (as determined in previous experiments **(If unknown see Appendix: Determine cell density and transduction conditions)**, add 100 µL of Growth Medium directly to each well without removing medium. Make adjustments to serum concentration so that final serum concentration matches Growth Medium after adding to wells.

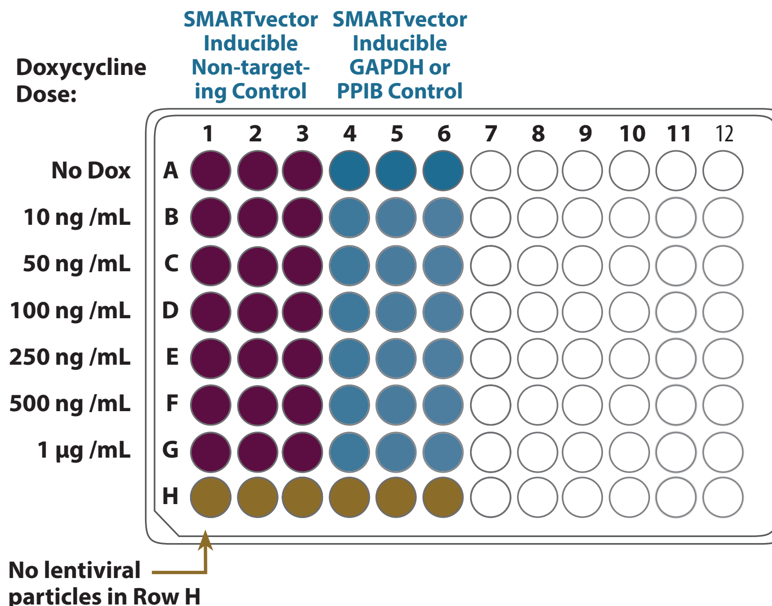

Figure 7. Plate layout for transductions using SMARTvector Inducible positive and negative control shRNAs (at MOI of 0.3) and induction of shRNA expression at multiple doxycycline concentrations.

Day 3:

Replace medium on cells with fresh Growth Medium.

Days 4, 6 and 7:

Replace medium on cells with fresh Selection Medium supplemented with the optimal dose of puromycin for the target cell line.

Day 8:

1. Ensure that puromycin selection is complete by examining Row H and determining that no viable cells remain.
2. Replace culture medium on cells with fresh Growth Medium supplemented with 0, 10 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL, 1 µg/mL of freshly dissolved doxycycline (**Figure 7**).

Day 10:

Replace culture medium on cells with fresh Growth Medium, maintaining doxycycline at the doses from Day 8.

Day 11:

1. Test for toxicity using Resazurin or a similar assay for viability.
2. Determine knockdown of GAPD or PPIB positive control genes at the mRNA level relative to negative control using RT-qPCR techniques.
3. Choose the doxycycline dose that results in maximal GAPD or PPIB gene silencing while having a minimal effect on cell viability. This doxycycline dose will be appropriate for all Inducible shRNAs of the same vector configuration in the tested cell type.

Stability and storage

SMARTvector Inducible Lentiviral shRNA particles are shipped on dry ice as 25 µL aliquots and must be stored at -80 °C. Under these conditions, lentiviral particles are stable for at least 12 months. Repeated freeze-thaw cycles should be avoided, as this is expected to negatively affect titer. Once thawed, unused particles lentiviral particles should be kept on ice, aliquoted into smaller volumes (if necessary) and immediately returned to -80 °C.

Quality assurance and control

SMARTvector Inducible Lentiviral shRNA particles are subject to stringent quality control at multiple steps during the manufacturing process, including:

1. Sanger sequencing of each clone to ensure integrity of shRNA sequence;
2. lentiviral particle titering using flow cytometry or p24 ELISA, followed by conversion to a functional titer based on a matched-vector reference control of known functional titer in HEK293T cells;
3. confirmation of fluorescent reporter expression (TurboGFP or TurboRFP) following transduction and doxycycline induction in HEK293T cells;
4. examination of each batch to ensure preparations are free from mold and bacterial contamination; and
5. generation of Certificate of Analysis (C of A) with specified lentiviral functional titers for each batch, included with each shipment.

Frequently asked questions (FAQs)

Do I have to select my cells with puromycin to achieve gene silencing?

This depends on how the SMARTvector Inducible Lentiviral shRNA constructs are used. We recommend transducing your cells at a target MOI of 0.3. At low MOIs such as these, the majority of cells will not have a proviral genome integrated into their genomic DNA immediately following transduction. Therefore puromycin selection is necessary to remove untransduced cells from the population, resulting in a cell population in which the majority of the remaining cells carry a single lentiviral integration. Puromycin selection may not be necessary if cells are transduced at high MOI.

What happens if I transduce at higher MOI than recommended (> 1)?

At higher MOIs, more copies of the TRE3G and shRNA will be present. Each copy of the TRE3G promoter will contribute some low-level of basal shRNA expression. Therefore at higher MOI, more "leaky" shRNA expression and gene knockdown may be observed. In addition, Tet-On proteins, including Tet-On 3G have been shown to be toxic when highly expressed. Higher MOI transductions will result in higher Tet-On 3G expression, and potentially more striking effects on cell viability. However, for some limited applications, a higher rate of lentiviral integration may be preferred.

How do I choose the best promoter for my cells?

We have found the mCMV- and PGK-based vectors to be functional in most cell types. However, in cells such as mouse embryonic stem cells, we have found the mEF1α and hEF1α vectors to be more effective. To ensure functionality in your cell type of interest, please use the SMARTchoice Inducible Non-targeting Control 4-Pack to assess the cell-specific activity of the four promoter options.

Can I culture my cells in doxycycline continuously to treat this as a constitutive knockdown vector?

Yes.

How long can I passage my cells in culture before inducing expression with doxycycline?

We have found inducible knockdown in stable cell populations to be consistent over 20 passages, even with just a single initial puromycin selection period. However, long-term silencing of the lentiviral content may occur in some cell types. Therefore, it is generally good practice to freeze early passages of the cell population, and use the cells within a limited passage number.

Do I need to generate a stable cell line expressing Tet-On first?

No, SMARTvector Inducible Lentiviral shRNA particles carry all necessary elements for selection and inducible expression. Cell lines capable of inducible shRNA expression are generated with a single transduction.

Since there is little or no gene knockdown in the absence of doxycycline, why do I need to use a Non-Targeting Control shRNA?

Although doxycycline is generally accepted as "inert," some cellular phenotypes, such as reduced growth rate, may be observed upon exposure to doxycycline. In addition, fluorescent reporters such as TurboGFP and TurboRFP can cause some subtle changes in cellular phenotypes. To rigorously control for both of these effects, it is necessary to use a vector-matched Non-Targeting Control shRNA to identify true shRNA-dependent phenotypes.

Can I use tetracycline to induce expression of the shRNA in SMARTvector Inducible Lentiviral shRNA constructs?

No, Tet-On 3G has been optimized for binding doxycycline, and it responds poorly to tetracycline.

What are the maximum excitation and emission wavelengths for TurboGFP & TurboRFP?

Fluorescent reporter	Excitation wavelength	Emission wavelength
TurboGFP	482 nm	502 nm
TurboRFP	553 nm	574 nm

4 Reference and recommended reading

1. E. Ahler, W.J. Sullivan, *et al*., Doxycycline Alters Metabolism and Proliferation of Human Cell Lines. *PLoS ONE*. **8**(5), e64561 (2013).
2. J.A. Curtin, A.P. Dane, *et al*., Bidirectional promoter interference between two widely used internal heterologous promoters in a late-generation lentiviral construct. *Gene Ther.* **15**(5), 384-390 2008).
3. R. Loew, N. Heinz, *et al*., Improved Tet-responsive promoters with minimized background expression. BMC *Biotechnol.* **10**, 81 (2010).
4. M. Morimoto, R. Kopan. rtTA toxicity limits the usefulness of the SP-C-rtTA transgenic mouse. *Dev. Biol.* **325**(1), 171-178 (2009).
5. T.H. Sisson, J.M. Hansen, *et al*., Expression of the reverse tetracycline-transactivator gene causes emphysema-like changes in mice. Am. J. Respir. *Cell Mol. Biol.* **34**, 552-560 (2006).
6. J.A. Whitsett, A.K. Perl. Conditional control of gene expression in the respiratory epithelium: A cautionary note. Am. J. Respir. *Cell Mol. Biol.* **34**, 519-520 (2006).
7. X. Zhou, M. Vink, *et al*., Optimization of the Tet-On system for regulated gene expression through viral evolution. *Gene Ther.* **13**(19), 1382-1390 (2006).

5 Lentiviral particle product safety level information

This Lentiviral Particle Product Safety Level Information constitutes Product Documentation according to clause 1 of the Product Terms and Conditions. It is applicable to the following lentiviral particle products:

- Dharmacon™ SMARTvector™ shRNAs
- Dharmacon™ shRNAs
- Dharmacon™ Promoter Selection Plate
- Dharmacon™ Positive and Negative RNAi Controls
- Dharmacon™ SMARTvector™ Inducible shRNAs
- Dharmacon™ shMIMIC™ microRNAs
- Dharmacon™ Pooled Lentiviral shRNA Screening Libraries
- Dharmacon™ GIPZ™ shRNAs
- Dharmacon™ Precision™ LentiORFs
- Dharmacon™ Decode™ Pooled RNAi Screening Libraries

The Products are solely for internal research use (as set forth in the Product Terms and Conditions) in laboratories where the containment measures stated below and in applicable laws and regulations are met. Products may not be used for diagnostic, therapeutic or other commercial purposes and may not to be administered to humans for any purpose or to animals for therapeutic purposes. The Products are replication-incompetent, self-inactivating (SIN) and non-pathogenic (do not cause infectious human disease).

Note: Any investigator who purchases Dharmacon lentiviral particle products is responsible for consulting with their institution's health and biosafety personnel for specific guidelines on the handling of lentiviral vector particles. Furthermore, each investigator is fully responsible for obtaining the required permissions for research using and the acceptance of replicationincompetent SIN lentiviral vectors and replication-defective lentiviral particles into their local jurisdiction and institution.

For questions concerning the design or production of the products, please contact our technical support team.

Dharmacon, a Horizon Discovery Group Company
2650 Crescent Drive
Lafayette, CO 80026 USA
Tel: **1-800-235-9880; 303-604-9499**
Fax: 1-800-292-6088; 303-604-9680
Email: ts.dharmacon@horizondiscovery.com

In the US:

For US guidance on containment for lentiviral vectors, please refer to:

1. The Recombinant DNA Advisory Committee (RAC) guidelines for research with lentiviral vectors (https://osp.od.nih.gov/wp-content/uploads/Lenti_Containment_Guidance.pdf)
2. 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);
3. The NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines), Oct 2011 (https://osp.od.nih.gov/wp-content/uploads/2013/06/NIH_Guidelines.pdf).

In the EU:

For the EU directives, please consult the following:

1. Council Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified micro-organisms. (revised version of Directive 90/219/EEC of the European Parliament and of the Council of 23 April 1990 on the contained use of genetically modified micro-organisms, amended by Council Directive 98/81/ EC of 26 October 1998); and
2. Council Directive 2001/18/EC of the European Parliament and of the Council of 12 March on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC.

In Germany:

Required Containment Measures: The containment requirements as stated in the German Genetic Safety Ordinance (Gentechnik-Sicherheitsverordnung) of Safety Level 2* or higher have been assigned to the handling of the above-mentioned lentiviral vector particles. Please note a higher Security Level might be required if the lentiviral vector particles are used for genetic engineering operations with other products which require a higher Security Level. *Safety Level 2: activities of low risk for human health and the environment by the state of scientific knowledge (Stand der Wissenschaft).

For the German regulations, please consult the following:

1. German Genetic Engineering Act (Gentechnikgesetz - GenTG); and
2. Genetic Engineering Safety Ordinance (Gentechnik-Sicherheitsverordnung - GenTSV).

6 Limited Use 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 http://dharmacon.horizondiscovery.com/uploadedFiles/dharmacon-licensing-statements.pdf. 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

t +44 (0) 1223 976 000 (UK) or +1 800 235 9880 (USA); +1 303 604 9499 (USA)

f +44 (0)1223 655 581

w horizondiscovery.com/contact-us **or** dharmacon.horizondiscovery.com/service-and-support

Horizon Discovery, 8100 Cambridge Research Park, Waterbeach, Cambridge, CB25 9TL, United Kingdom

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