TECHNICAL MANUAL

horizon™
INSPIRED CELL SOLUTIONS

Dharmacon™ shMIMIC Inducible Lentiviral microRNA

Contents

1 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™ SMARTvector™ Promoter Selection Plate
- Dharmacon™ SMARTvector™ Positive and Negative RNAi Controls
- Dharmacon™ SMARTvector™ Inducible shRNAs
- Dharmacon™ SMARTvector™ shMIMIC™ microRNAs
- Dharmacon™ shMIMIC™ Inducible microRNAs
- 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 rerms 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).

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 replication-incompetent 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.

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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), (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 (GentechnikSicherheitsverordnung) 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).

2 Introduction to shMIMIC inducible microRNA lentiviral particles

Dissecting endogenous RNA interference mechanisms: microRNA roles in fundamental biology

microRNAs are endogenous small (primarily 21- to 23-nucleotides) noncoding RNAs that are highly conserved across both mammalian and non-mammalian species. These regulatory RNAs differentially modulate the spatial and temporal expression of messenger RNAs (mRNAs) across the genome and have been estimated to regulate roughly 50% of all mRNAs in vertebrates (Friedman *et al.*, 2009; Bartel 2009).

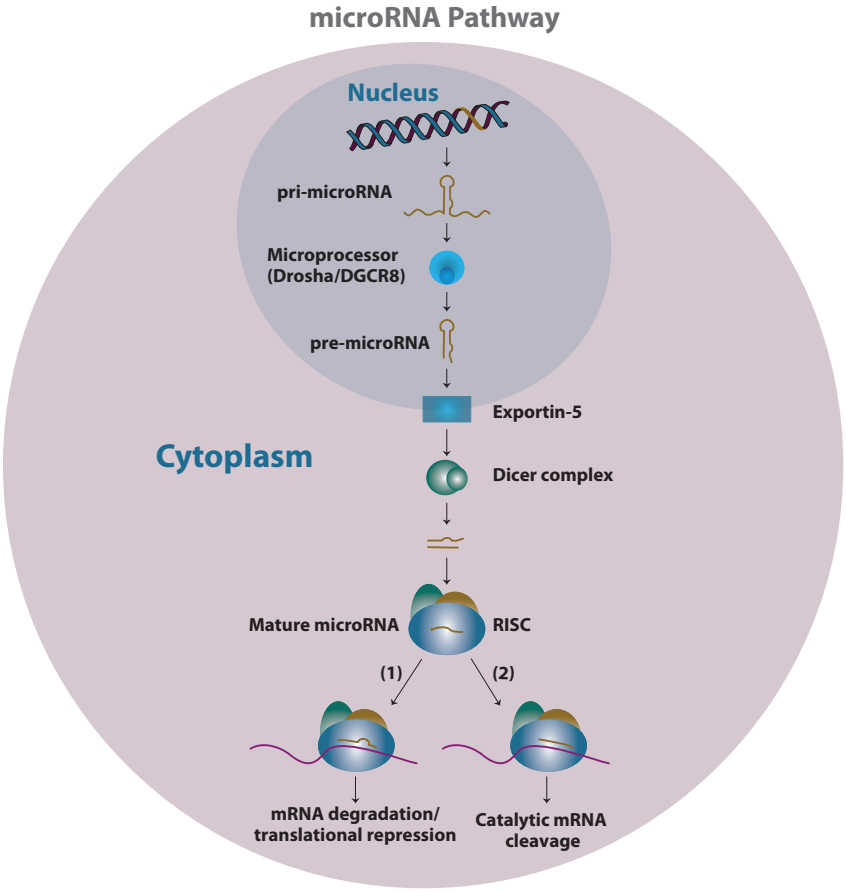

Figure 1. The endogenous mammalian microRNA pathway starting with transcription of the microRNA gene in the nucleus and ending with two options for gene down-regulation: **(1)** seed-mediated mRNA degradation or translational repression and **(2)** catalytic mRNA cleavage mediated by full sequence complementarity.

Since the discovery of the first microRNA gene, lin-4 (Lee *et al.*, 1993; Wightman et al., 1993), thousands of microRNA genes have been identified, representing up to 10% of the genome. An increasing number of investigations reveal a breadth and depth of gene modulation that underscores the importance of these small RNAs.

microRNA biogenesis in mammals begins with the transcription of a microRNA gene that produces a capped and polyadenylated primary microRNA (pri-microRNA) containing local hairpin structures with imperfect base-pairing through the stems **(Figure 1)**. Processing by the Drosha-DGCR8 complex results in a ~ 75 nucleotide stem-loop molecule (the precursor, or pre-microRNA), which is then shuttled out of the nucleus into the cytoplasm (Kim 2005; Kim, Han *et al.*, 2009). Subsequent digestion of the pre-microRNA by the endonuclease Dicer yields a transient, double-stranded microRNA. To modulate gene expression, the double-stranded microRNA unwinds, and the resulting single-stranded mature microRNA associates with a protein complex to form the RNA Induced Silencing Complex (RISC). In this context, the microRNA imperfectly base-pairs primarily with sequences in the 3' untranslated regions (3' UTRs) of target mRNAs and modulates gene expression through transcript destabilization or translational attenuation.

While the list of newly discovered microRNAs continues to grow, the function and gene targets of many microRNAs remain unknown. To address this, researchers utilize synthetic microRNA mimics and inhibitors (such as the Dharmacon miRIDIAN Mimics and Hairpin Inhibitors) to unravel the contribution that individual microRNAs make to both normal and diseased cellular physiology. While synthetic mimics and inhibitors can accurately simulate microRNA gain- and loss-of-function, respectively, and thereby facilitate a greater understanding of microRNA function, in some cases the cell type or the assay being employed is incompatible with use of synthetic molecules. For example, the cells being used in a particular study can be refractory to the principal method of introducing synthetic microRNA mimics into cells, such as, lipid-mediated transfection. In other instances, the assay requires a period of time that extends beyond the effective functional window provided by synthetic molecules. For these reasons, alternative methods for delivering and maintaining expression of microRNA mimics are necessary.

Viral delivery systems provide a particularly effective approach for delivering genetic information into cells. While several viral vector systems have been developed, those based on pseudotyped lentiviral vectors have proven to be among the most versatile, by providing 1) delivery of large genomic payloads, 2) broad tropism, and 3) stable expression by integration of the lentiviral payload into the host genome. Lentiviral vectors are unique in their ability to efficiently deliver genetic payload into both dividing and non-dividing cells. Thus, lentiviral platforms are well-suited for experiments in stem cells and cells derived from primary tissues, neuronal and hematopoietic lineages. Our scientists have combined expertise in microRNA biology and lentiviral vector development to create a novel, proprietary microRNA expression tool. The shMIMIC Inducible Lentiviral microRNA is an ideal microRNA research tool for robust over-expression of mature microRNAs.

Innovative shMIMIC inducible microRNA vector design

The shMIMIC Lentiviral microRNA design is based on a series of systematic experiments carried out during the development of the SMARTvector RNAi platform. The Inducible shMIMIC Lentiviral microRNA vectors combined the advantages of the SMARTchoice platform with those of the state-of-the-art Tet-On® 3G induction system to develop a tightly regulated, single-vector, inducible mature microRNA expression system. **Figure 2** illustrates the general process by which lentiviral-based shMIMC microRNAs enter the cell and integrate the genomic payload, including the microRNA expression cassette, into the host cell genome. With each cellular division, the integrated sequences are replicated and passed on to daughter cells, thus allowing controlled expression of the mature microRNA sequence throughout the population.

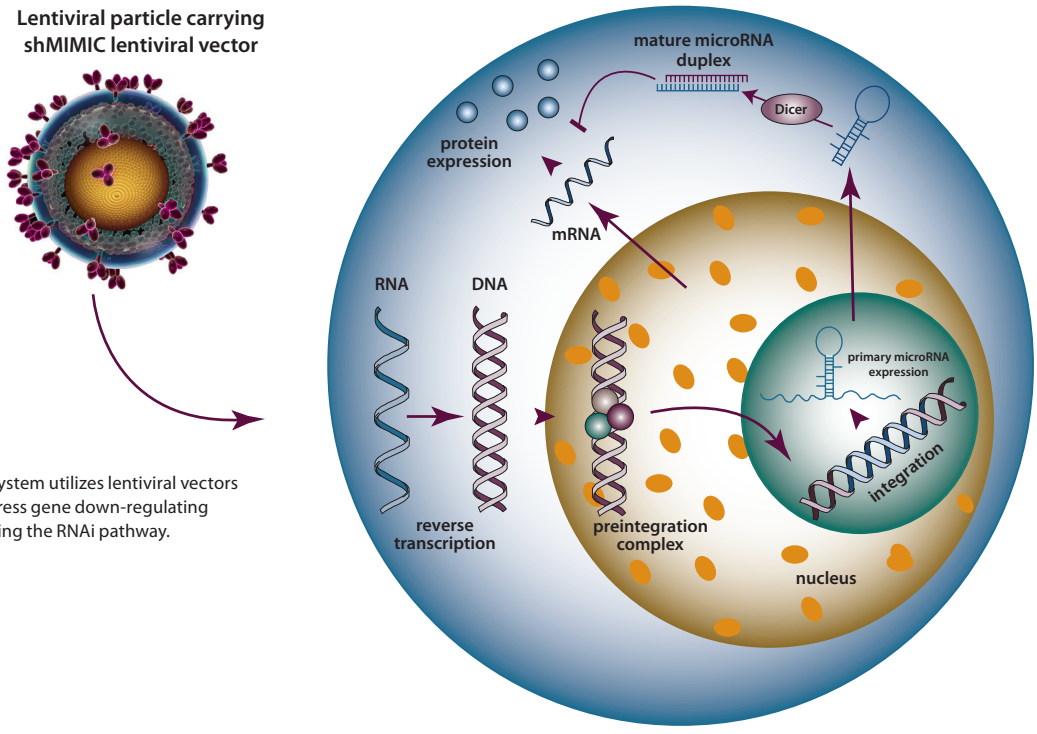

Figure 2. The shMIMIC system utilizes lentiviral vectors to stably deliver and express gene down-regulating triggers capable of entering the RNAi pathway.

shMIMIC Inducible microRNA vectors **(Figure 3)** possess the following enhanced features for robust, regulated and reproducible mature microRNA expression:

1. **Optimized universal microRNA scaffold:** microRNAs are the endogenous substrates of the RNAi pathway. Transcribed in the nucleus as stem-loop structures containing imperfect basepairing in the stem, these noncoding RNAs are processed by multiple elements of the RNAi pathway to generate short ~ 16-28 base-pair duplexes capable of associating with the RNA Induced Silencing Complex (RISC) to target genes for down-regulation. Our scientists screened a panel of human microRNAs and selected an efficiently processed primary microRNA for the universal scaffold.
2. **Rational design for over-expression of each mature human, mouse and rat microRNA in the miRBase database (www.mirbase.org):** Each mature microRNA is systematically embedded within the universal scaffold and cloned into the lentiviral vector backbone. The use of a universal primary context ensures that each mature microRNA is correctly and consistently processed in the cell, thus minimizing sequence variability that can confound interpretation of microRNA-induced phenotypes.
3. **Tightly regulated, inducible shMIMIC microRNA expression:** shMIMIC Inducible microRNAs combine all of the advantages of the SMARTvector platform with those of the recently developed Tet-On® 3G tetracycline-inducible expression system. Our unique, single-vector delivery system permits the rapid development of cells with tightly controlled mature microRNA expression. The minimal leakiness and potent activation of the Tet-On® 3G promoter, an RNA polymerase II promoter, ensures near wild-type expression of the mature microRNA in the absence of doxycycline and robust over-expression upon exposure to doxycycline. This regulated mature microRNA over-expression offers additional experimental control, providing numerous experimental advantages, including regulated over-expression of mature microRNAs that result in cell death.
4. **TurboGFP™ or TurboRFP™ reporter genes:** The shMIMIC Inducible microRNA platform includes either a TurboGFP or TurboRFP (Evrogen, Moscow, Russia) reporter gene to facilitate assessment and optimization of experimental transduction conditions. Because the mRNA encoding the fluorescent reporter is directly linked to the shMIMIC microRNA, expression of TurboGFP or TurboRFP allows for visual tracking of mature microRNA expression. Expression of TurboGFP or TurboRFP occurs only in the presence of doxycycline and fluorescence indicates that the expression of the mature microRNA has been induced.
5. **Mammalian drug-selection marker:** The puromycin resistance ($Puro^R$) gene is a feature of all SMARTchoice lentiviral constructs and enables rapid selection of stable cell lines. In the shMIMIC Inducible microRNA vector, $Puro^R$ is expressed under the control of one of four constitutive promoter options, thus permitting selection of transduced cells independently of mature microRNA expression.
6. **SMARTchoice RNA Pol II promoter options:** The activity of RNA polymerase II promoters can vary in different cellular contexts. The shMIMIC Inducible platform provides a choice of four robust, well-characterized RNA polymerase II promoters (mCMV, PGK, mEF1α and mEF1α) to control the expression of $Puro^R$ and the doxycycline-dependent transactivator, Tet-On® 3G. This ensures that a functional inducible shMIMIC microRNA vector can be utilized in virtually any mammalian cell type of interest.

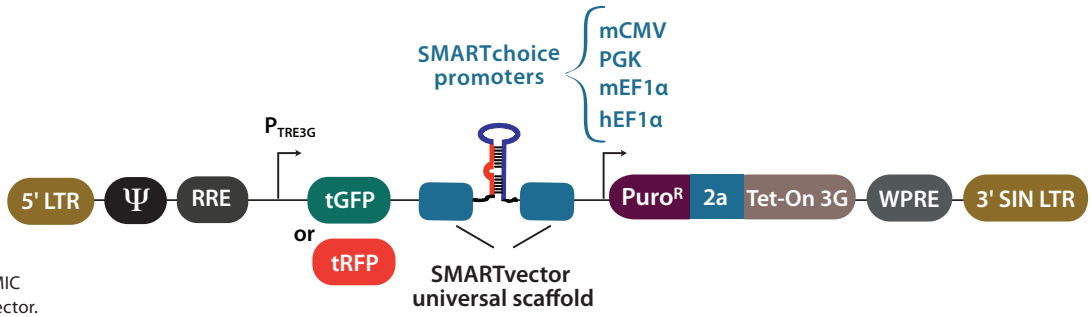

Figure 3. Elements of the shMIMIC Inducible Lentiviral microRNA vector.

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
P_{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 patented scaffold based on native primary microRNA in which mature microRNA 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 P_{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

An optimized inducible mature microRNA expression in a single vector

The shMIMIC Inducible microRNA 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 **(Figure 4)**, which is also encoded within the shMIMIC Inducible Lentiviral microRNA vector. This protein was the product of an *in vitro* evolution and selection process that resulted in significantly higher sensitivity to doxycycline than the original Tet-On® 3G protein (also known as rtTA). Together, Tet-On® 3G protein and TRE3G promoter permit tight regulation of the mature microRNA, including potent induction, even at the low doxycycline doses that are required *in vivo*.

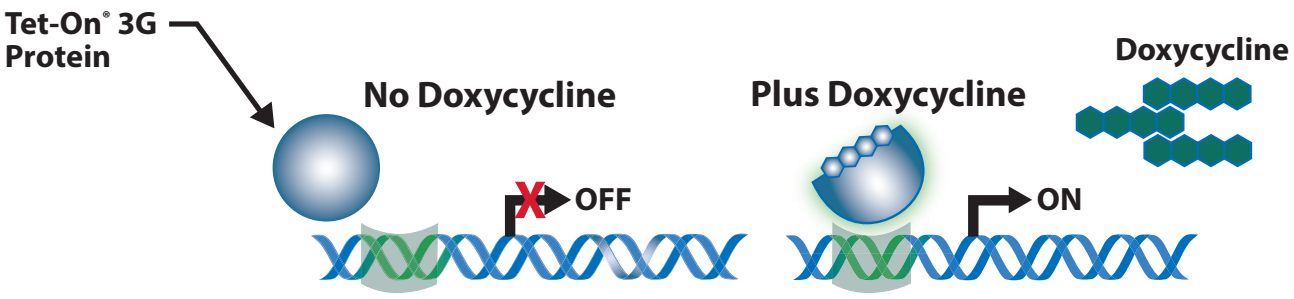

Figure 4. Tet-On® 3G transactivator protein only binds the TRE3G promoter sequence when doxycycline is present, which in turn activates transcription.

Importance of promoter activity for shMIMIC microRNA expression

In order for shMIMIC Inducible microRNAs to function in a broad range of cells, the constitutive promoter must be active enough to produce sufficient quantities of Puro^{R} and Tet-On$^{®}$ 3G transactivator protein to permit both selection of transduced cells and inducible expression of the mature microRNA. Depending on the specific cell type, promoter activity may be nonexistent, weak or silenced by epigenetic mechanisms over time, despite successful transduction and integration. The fluorescent images shown in Figure 5 demonstrate how promoter performance varies significantly across different cell lines which, in turn, can influence the efficacy and potency of over-expressing mature microRNAs.

Due to the proximity of the constitutive and the TRE3G promoters in these vectors, a particular constitutive promoter does not always behave as it would when isolated in a single promoter system. This phenomenon is commonly referred to as promoter interference (Curtin, 2008). It is recommended to test control particles for each vector, using inducible TurboGFP expression as a measure of vector activity in your cell type of interest.

SMARTchoice Inducible vector customization options include four promoter and two reporter options. To determine the optimal shMIMIC Inducible microRNA vector for your cell type of interest, utilize the SMARTchoice Inducible Non-targeting Control 4-Pack to quickly identify the promoter that drives maximal shMIMIC microRNA expression in your cells, simply by examining doxycycline-induced TurboGFP expression **(see Protocol in Chapter 3)**.

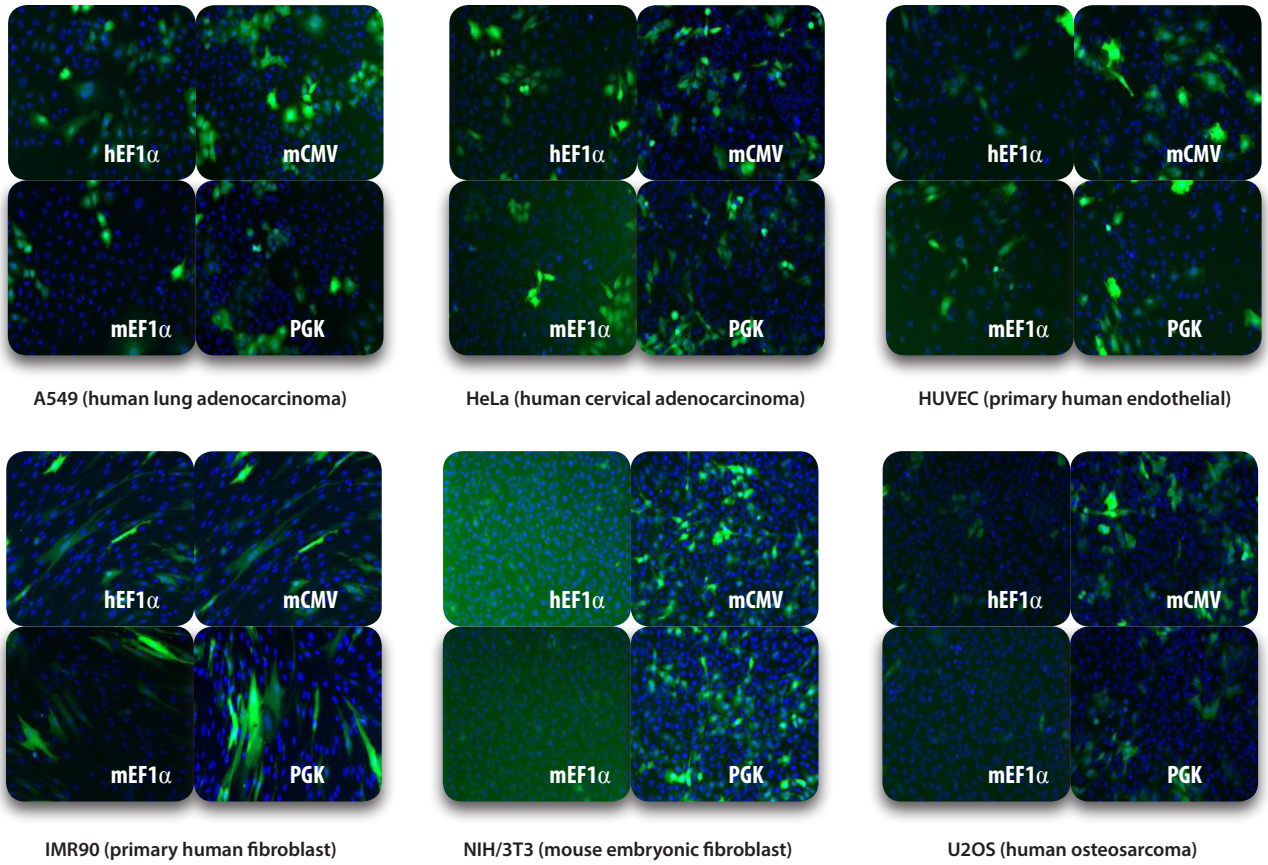

Figure 5. shMIMIC Inducible microRNA promoter activities differ across cell types. Inducible Non-targeting Control lentiviral particles were used to transduce the indicated cell types at MOI = 0.3; 24 hours after transduction, expression of the non-targeting shRNA and TurboGFP was induced with 1 µg/mL doxycycline. After 48 hours of culture in the presence of doxycycline, cells were stained with Hoescht-33342 and nuclei (blue) and TurboGFP (green) were imaged. While ~ 30% of the cells in the field have been transduced, some images may appear to contain fewer than 30% TurboGFP-positive cells due to low TurboGFP expression, indicative of low constitutive promoter activity, in a particular cell type.

Transduction of shMIMIC inducible microRNA lentiviral at recommended low multiplicities of infection (MOI)

The Tet-On® 3G system has been optimized to minimize leaky activity. However, all inducible expression systems display some level of basal or "leaky" activity in the absence of the inducer. The level of basal expression increases as the copy number of shMIMIC microRNA increases with the number of proviral genomes integrated (for example with increasing the multiplicity of infection, MOI). Additionally, toxicity of Tet-On proteins has been reported (Sisson, 2006; Whitsett, 2006; Morimoto, 2009), and the expression level of Tet-On® 3G is directly correlated with MOI. Therefore, in most instances, a single integration of proviral genome per cell provides the optimal balance between minimized leakiness, maximized inducible mature microRNA expression, and minimal non-specific effects resulting from expression of Tet-On® 3G protein.

Nevertheless, because shMIMIC Inducible Lentiviral microRNA are provided as concentrated lentiviral particles, MOIs of greater than one are often achievable. While higher MOIs may enhance phenotypic results by increasing the expression of the mature microRNA , it is important to consider the possibility of low-level mature microRNA expression in the absence of doxycycline induction.

3 Protocols for optimization of transduction conditions and choosing the optimal SMARTchoice inducible vector

Safety precautions

The following protocols describe how to determine optimal conditions tolerated by cells of interest under which transductions can subsequently be performed. The goal is to optimize cell density, Polybrene concentrations, presence or absence of serum and duration of transduction. It is critical to comprehensively read and understand the recommended steps in each protocol prior to initiating the promoter selection assay. Preparation and handling of the shMIMIC Inducible Lentiviral microRNAs and subsequent transduction plates must always be performed within a Class II/Type A2 biological safety cabinet. Any surfaces coming in contact with the lentiviral particles, including pipette tips and plastic ware, should be sanitized by bleach or Lysol™ disinfectant prior to disposal. To facilitate disposal, set up a liquid waste reservoir inside the biological safety cabinet consisting of a 1 L beaker containing 100 mL of bleach or Lysol™ disinfectant. A biohazardous waste bag should also be placed within the biological safety cabinet.

Materials not supplied

- Polybrene (American Bioanalytical Cat #AB01643)

The use of Polybrene may improve transduction efficiency of lentiviral particles in some cell types and is optional.

- 96-well tissue culture plates (Nunc™ Cat #12-565-66).
- Deep-well 96-well plate (Nunc™ Cat #12-565-553).
- Multichannel (8-channel) Pipette.
- Cells of interest. **See note in blue box.**
- Dulbecco's Modified Eagle Medium (DMEM) High Glucose without L-Glut or Sodium Pyruvate (Hyclone™ Cat #SH30081.FS).
- Doxycycline hyclate (Fisher Scientific Cat #ICN19895510).
- alamarBlue™ Cell Viability Reagent (Thermo Scientific Cat #PI88952).

- **Preferred culture medium:** the cell culture medium (including serum or supplements) recommended for maintenance and passaging of the cells of interest.
- **Base medium:** the base medium (without serum or supplements) that is used to prepare the Preferred culture medium.
- **Transduction medium:** Base medium with or without serum and containing the appropriate concentration of Polybrene as determined to be optimal transduction conditions for cells of interest.
- Lysol™ disinfectant or bleach.

The passage number of cells can have a significant effect on lentiviral transduction efficiency. For this reason, cells of low passage number (typically < 10) should be used in all experiments, and accurate records that document cell passage number should be maintained. If you are using a recently thawed vial of cells, allow at least one passage after the thaw before performing optimization procedures.

Determining cell density and transduction conditions

Successful transduction of cells depends on a number of factors including the 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. Additionally, your lentiviral particles are suspended in DMEM, which may not be the base medium of the preferred culture medium for the cells of interest.

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

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

i. Optimization of cell density and conditions in preparation for transductions: protocol for adherent cells

Day 1:

1. Seed cells into two 96-well culture plates at the appropriate cell density following the template depicted in Figure 6. Seed cells in a total volume of 100 µL of the preferred culture medium and place 96-well culture plates into incubator for overnight culture under the appropriate conditions (temperature and CO_2 concentration).
2. Generally, transductions with lentiviral particles should be performed when cells are approximately 40% confluent. This provides cells the space 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 6 (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.

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. *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.*
2. Prepare two sets of transduction medium, one without serum and one with serum.

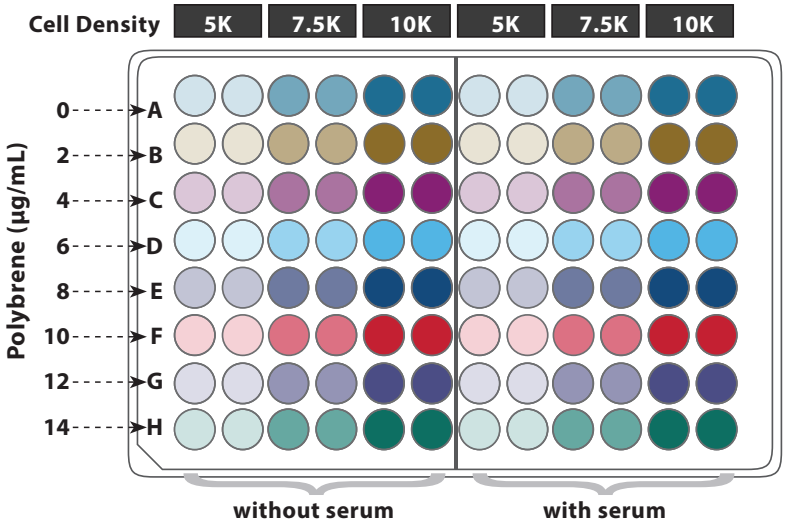

Figure 6. Example of 96-well plate layout for the optimization of transduction conditions. This plate layout tests three cell densities [5,000 (5K), 7,500 (7.5K) and 10,000 (10K) cells per well], eight concentrations of Polybrene (0-14 µg/mL) and medium with or without serum.

Transduction medium without serum:

» Prepare 10 mL of transduction medium containing no serum by pipetting a 1:1 mixture of DMEM to base medium. Note that Dharmacon provides lentiviral particles in DMEM. Thus, testing a recipe for suitable transduction medium must include DMEM.

» Transfer 700 µL aliquots of this mixture into eight wells of a sterile deep-well 96-well plate. 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 7)**.

Transduction medium with serum:

» 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. For example, if the preferred culture medium contains 10% serum, then the final concentration of the transduction medium should also be 10%.

3. Incubate cells with Transduction Medium.

Two sets of incubations will be performed using each of the transduction medium formulations (with and without serum): one plate for six hours (plate 1) and the other plate for an overnight incubation for 16-20 hours (plate 2).

a. Remove culture plates from incubator and place in biological safety cabinet.

b. Using a multichannel pipette, carefully aspirate preferred culture medium from each well of 96-well culture plates being careful not to dislodge cells from bottom of well. Aspirate 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 7.

d. Return culture plates to incubator and culture under the appropriate conditions.

e. After 6 hours, add 100 µL of preferred culture medium directly to each well of plate 1 and return to incubator for overnight culturing. At this time, do not remove the transduction medium from the well prior to adding the preferred culture medium. 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 15%. Adding 100 µL of this culture medium to the 50 µL of transduction.

- **If Polybrene is toxic to the cells, DEAE-Dextran (1-10 µg/mL) may be substituted.**
- **If no-serum conditions affect cellular viability then optimization can be performed using a lower serum concentration (1-3%) and the same plate format as in Figure 7.**

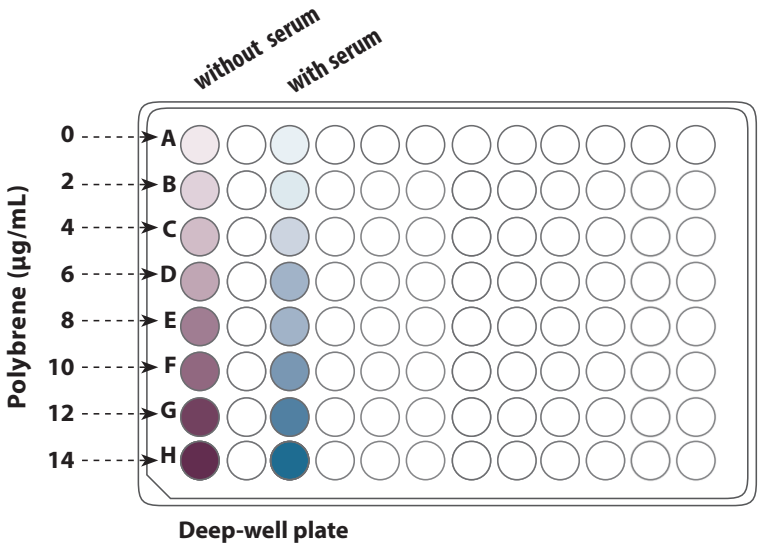

Figure 7. Example of plate layout for preparation of Transduction Medium. Transduction Medium with (blue wells) or without (purple wells) serum containing a range of Polybrene concentrations in a deep-well, 96-well plate. White wells are empty.

Day 3:

After overnight incubation with Transduction Medium (16-20 hours), add 100 µL of Preferred Culture Medium directly to each well of plate 2. Adjust serum concentration as described above to the wells without serum.

Day 4:

Using a microscope, examine all wells of 96-well plates. Record the confluency and any morphological or phenotypic alterations that may be present.

Day 5:

Examine cultures for cell morphology or presence of phenotypic changes. Cell viability should be determined using any commercially available kit, such as alamarBlue Cell Viability Reagent (Thermo Scientific).

Select the initial cell density seeded that resulted in approximately 40% confluency on Day 2. If the cells of interest can tolerate serum-free conditions then exclude serum during transduction. Note the highest concentration of Polybrene with acceptable viability for the cells of interest. Based on observations from a variety of cell lines and cell types, the lowest concentration of serum generally results in the highest transduction efficiency. If, in subsequent protocols, it is found that transduction efficiency is low, Transduction Media may be supplemented with this concentration of Polybrene to enhance efficiency. **See notes above in blue box.**

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.

ii. Optimization of cell density and conditions in preparation for transductions: protocol 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. 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). Centrifuge cells at low speed to pellet cells. Pour off supernatant containing old culture medium. Tap bottom of centrifuge tube gently to dislodge cell pellets. 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 the appropriate number of cells are seeded in a total volume of 25 µL per well.
2. 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.

   Transduction medium for suspension cells:

   » Prepare 10 mL of Transduction Medium without serum by pipetting a 1:1 mixture of DMEM to Base Medium. Transfer 700 µL aliquots of this mixture into eight wells of a sterile deep-well 96-well plate. Use these wells to prepare a series of Transduction Medium formulations containing eight different Polybrene concentrations such that the final concentration will range from 0-14 µg/mL in 2 µg/mL increments (as depicted in Figure 6). 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.

   » Use **Table 1** to prepare the eight Transduction Medium formulations. Two sets of eight wells of Transduction Medium should be prepared as before **(Figure 7)**, 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

3. Incubate cells with Transduction Medium.
   Two sets of incubations will be performed using each Transduction Medium formulation (with and without serum): one plate for 6 hour incubation (plate 1) and the other plate for 16-20 hour overnight incubation (plate 2). Using a multichannel pipette, transfer 50 µL of Transduction Medium to appropriate wells and place 96-well culture plates into incubator under appropriate conditions (temperature and CO_2 concentration).
4. After 6 hours, add 75 µL of Preferred Culture Medium directly to each well in plate 1 and return to incubator for overnight culturing. Adjust serum concentration in wells containing no serum. For example, if cells require 10% serum, increase the serum concentration of 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 4-5:

Examine cultures for cell morphology or presence of phenotypic changes. Cell viability should be determined using any commercially available kit, such as alamarBlue™ Cell Viability Assay Reagent.

Select the appropriate density (for example, ~ 40% confluency for the specific cells of interest) on Day 2. If the cells of interest can tolerate serum-free conditions then exclude serum during transduction. Note the highest concentration of Polybrene with acceptable viability for the cells of interest. Based on observations from a variety of cell lines and cell types, the lowest concentration of serum generally results in the highest transduction efficiency. 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. **See notes below in blue box.**

- **If Polybrene is toxic to the cells, DEAE-Dextran (1-10 µg/mL) may be substituted.**
- **If no-serum conditions affect cellular viability then optimization can be performed using a lower serum concentration (1-3%) and the same plate format as in Figure 7.**

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.

iii. Using the SMARTchoice inducible non-targeting control 4-pack to identify the optimal vector for a given cell type

SMARTchoice Inducible 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 simultaneously evaluate which of these promoters are most active in the cells of intended study, and maximize the opportunity for experimental success. Following the optimization of basic transduction conditions (see Protocol i. or ii.), cells are transduced with the SMARTchoice Inducible Non-targeting Control 4-Pack lentiviral particles under the appropriate conditions. 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, shMIMIC Inducible microRNA Lentiviral Particles or glycerol stocks expressing microRNA of interest and experimental controls incorporating the optimal promoter and fluorescent reporter (TurboGFP or TurboRFP) of choice can be ordered.

Day 1:

Plate cells in a 96-well culture plate at optimal cell density in appropriate growth medium as determined in previous experiments (see Protocol i. and ii.). 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 particle dilution plate in which each column represents one of the four SMARTchoice Inducible Non-targeting Control vector configurations (mCMV, PGK, hEF1α and mEF1α); see Figure 8 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 Non-targeting controls to the one with the lowest titer using DMEM.

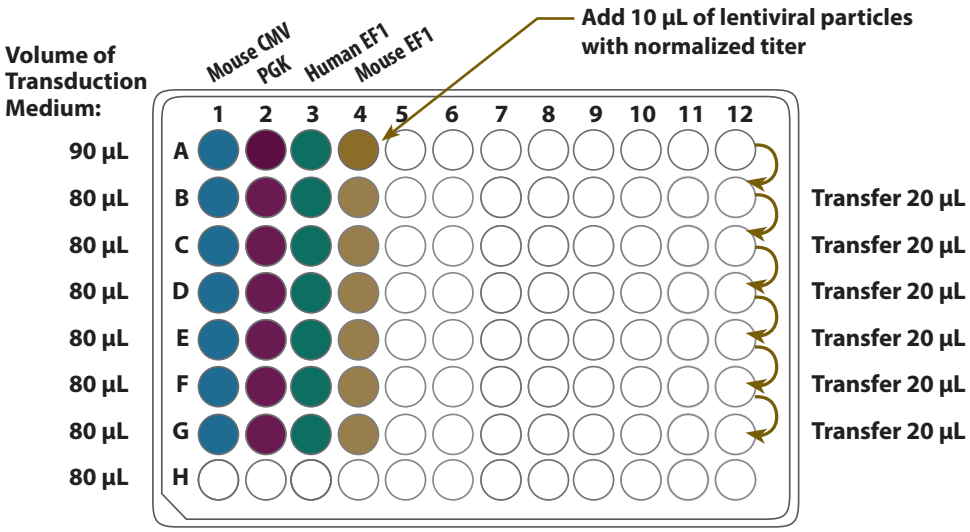

Figure 8. Lentiviral particle dilution plate layout for assessing vector activity in cells of interest using the SMARTvector Inducible Non-targeting Control by creating a 5-fold serial dilution. There should be no lentiviral particles in Row H wells, only medium.

4. To your dilution plate containing transduction medium, add 10 µL of each thawed, titer-normalized 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 down to Row G **(Figure 8)**, thus creating 5-fold serial dilutions for each 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 cell culture medium with 25 µL of transduction medium with lentiviral particles from the corresponding well of the lentiviral particle dilution plate.
8. Return cell culture plate with transduction medium and lentiviral particles to the incubator.
9. After 6-20 hours (as determined in protocols i. or ii.), add 100 µL of preferred culture medium directly to each well without removing medium. Make the necessary adjustments to serum concentration so that the final serum concentration matches preferred culture medium after adding to wells.

Doxycycline is unstable in solution. It is recommended that a fresh working solution be stored for no more than three days at 4 °C. Additionally, when inducing cells with doxycycline, the medium should be replaced with fresh medium containing fresh doxycycline every 24-48 hours.

Day 3:

1. After 24 hours, replace medium with preferred culture 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 viral genome. **See note in blue box below (page 16).** Assess TurboGFP fluorescence intensity by FACS or microscopy using the appropriate filters **(Table 2)**.

The optimal vector for your cell type is the one producing the highest level of fluorescence intensity.

Table 2. Excitation and emission maxima for TurboGFP and TurboRFP fluorescent reporters.

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

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 fluorophores. To enhance the visibility of fluorescing cells, replace the culture medium with a medium that is low in phenol red.

iv. Determining optimal puromycin selection conditions

Day 1:

Using the same cell type and cell densities to be used in subsequent transduction procedures, plate cells and culture overnight.

Day 2:

Replace complete growth medium with growth medium supplemented with a range of puromycin concentrations (0.1-10 µg/mL).

Day 4:

Replace medium with fresh medium supplemented with the appropriate concentration of puromycin. Examine cells daily and identify the minimum concentration of puromycin that efficiently kills all cells between 2-4 days following addition of puromycin.

4 Ordering shMIMIC inducible microRNA lentiviral particles or glycerol stocks with vector-matched positive and negative RNAi controls

Placing an order

shMIMIC Inducible microRNA lentiviral particles and glycerol stocks can be ordered at dharmacon.horizondiscovery.com/rnai/microrna/#all with one of four well-characterized promoters and either TurboGFP or TurboRFP as the fluorescent reporter **(Figure 3)**. Order individual lentiviral constructs at 100 µL or 200 µL volumes of $\geq 1 \times 10^7$ TU/mL lentiviral particles, or glycerol stocks. It is imperative to select matching positive and negative controls with the same promoter and reporter options selected for the mature microRNA of interest.

Recommended experimental controls

Inducible negative and positive RNAi controls are cost-effective reagents for experimental optimization such as determining optimal conditions for transduction and doxycycline induction, prior to transductions with specific shMIMIC Inducible miRNA lentiviral particles. Including both positive and negative controls in all experiments assures rigorous, high-confidence data, and is required for accurate data interpretation.

We strongly recommended that SMARTvector Inducible Negative and Positive RNAi Controls be ordered in addition to lentiviral particles targeting the gene of interest. Vector-matched controls are available with options for all four promoters and two fluorescent reporters.

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

SMARTvector inducible non-targeting control

SMARTvector Inducible negative controls for RNAi experiments, provided as packaged lentiviral particles at ≥ 1×10^7 TU/mL, or glycerol stocks are designed so that no known gene in human, mouse or rat will be targeted, thus providing a baseline to which microRNA-specific effects can be normalized. Negative controls can also be used to assess transduction efficiency and the effects of transduction on the cells of interest. The SMARTvector Inducible Non-targeting Controls are available with all four SMARTchoice Inducible promoter options and both fluorescent reporters so that RNAi experiments can be performed utilizing the appropriate controls regardless of the promoter chosen for gene-specific constructs.

Although little or no shMIMIC microRNA expression occurs in the absence of doxycycline, it is strongly recommended that a vector-matched non-targeting control is used to control for potential cellular phenotypes that may be associated with doxycycline exposure and/or expression of the fluorescent reporter.

SMARTvector inducible positive control shRNAs

SMARTvector Inducible oositive control shRNAs are provided as packaged lentiviral particles at ≥ 1×10^7 TU/mL or glycerol stocks. The inducible shRNA positive controls permit confirmation of transduction efficiency and gene knockdown through consistent, potent silencing of non-essential, abundantly expressed reference genes. The inducible GAPDH positive control shRNA is validated to silence glyceraldehyde-3-phosphate dehydrogenase, while the inducible PPIB positive control shRNA is validated to target peptidylprolyl isomerase B (also known as cyclophilin B). Individual positive control shRNAs targeting GAPDH and PPIB in human, mouse and rat models are available in each SMARTvector Inducible vector promoter and reporter options.

5 Mature microRNA over-expression protocols using shMIMIC inducible lentiviral microRNAs

Optimal usage of shMIMIC Inducible microRNA lentiviral particles

In most instances, we recommend relying on single-copy lentiviral genome integration for optimal mature microRNA expression with minimal leakiness (Chapter 2). However, because shMIMIC Inducible microRNAs are provided as concentrated lentiviral particles, MOIs of greater than one are often achievable if required for a specific application. While higher MOIs may enhance over-expression of the mature microRNA, it is important to consider the possibility of low-level microRNA expression in the absence of doxycycline induction.

The following protocols are designed to rapidly develop cells carrying a single copy of a shMIMIC Inducible microRNA proviral genome, and to induce shMIMIC microRNA expression with an optimized doxycycline dosage. In general, to produce populations of cells with a uniform MOI = 1, the cells are first transduced with enough lentiviral particles to produce an MOI between 0.1 and 0.3. 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 shMIMIC microRNA.

Determining functional titer in the cells of interest

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.

Calculating the volume of lentiviral particles needed for a given MOI

Calculate the total number of transducing units (TU) that would be added to a well for a given MOI with the following equation:

$$TU = (MOI \times CN)/VT$$

where, MOI = the desired MOI in the well (units are TU/cell); CN = number of cells in the well; VT = Viral Titer (units are TU/µL).

For example, if the experiment requires per well:

- MOI of 0.3
- Cell density of 10,000 cells per well at time of transduction; and
- Lentiviral titer as functionally determined for your cell type and conditions is 1×10^7 TU/mL (= 1×10^4 TU/µL)

Then, total TUs per well is equal to:

$$\text{Total TU} = [(0.3 \text{ TU/cell}) \times (10{,}000 \text{ cells/well})] / 1 \times 10^4 \text{ TU/µL} = 0.3 \text{ µL of lentiviral stock/well.}$$

Therefore, the volume of lentiviral particles with a titer of 1×10^7 TU/mL required for an MOI of 0.3 is 0.3 µL per well. It is recommended to include biological triplicates. When making lentiviral dilutions, be certain to scale the dilution series such that volumes are sufficient enough to permit accurate pipetting well within the working range of your pipettor.

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 9** and **Table 3**, 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 (only if using Polybrene).
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. C
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 10**). Calculate the average number of fluorescent-positive colonies from the same destination well of each replicate.

Doxycycline is unstable in solution. It is recommended that a fresh working solution be stored for no more than three days at 4 °C. Additionally, when inducing cells with doxycycline, the medium should be replaced with fresh medium containing fresh doxycycline every 24-48 hours.

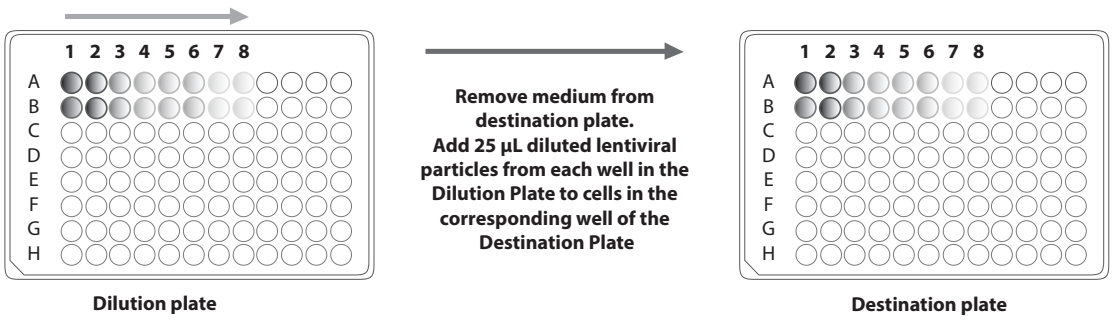

Figure 9. Diagram for dilution series of lentiviral particles (Dilution Plate) and addition to cells (Destination Plate).

Table 3. Example setup for lentiviral particle dilution series.

	Dilution Plate		Dilution factor	Volume of diluted lentiviral particles used in transduction (Destination Plate)
Well	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	15625	25 µL
A7	20 µL (from A6)	80 µL	78125	25 µL
A8	20 µL (from A7)	80 µL	390625	25 µL

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

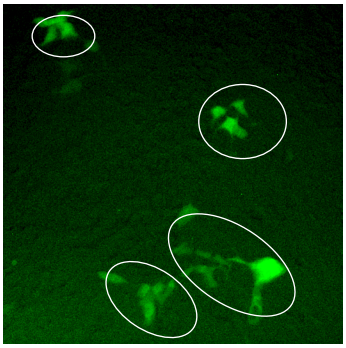

Figure 10. Example of individual colonies in HEK293T cells 72 hour post-transduction. Four colonies are circled. Imaged at 40x magnification.

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

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 fluorophores. To enhance the visibility of fluorescing cells, replace the culture medium with a medium that is low in phenol red.

Calculating the volume of lentiviral particles to use in a transduction

The functional titer of each lot of shMIMIC Inducible microRNA Lentiviral Particles is given in the Certificate of Analysis (C of A). However, lentiviral transduction efficiency varies widely and depends on factors such as target cell type, presence/absence of serum during transduction, duration of exposure to lentiviral particles, presence/absence of transduction enhancers, composition of transduction medium and others. Therefore, for sensitive applications where an MOI of 0.1-0.3 is desired, it is recommended that for each batch of unique cell type and for each variation in transduction conditions, the correct volume of lentiviral particles should be empirically determined using control particles.

Optimizing doxycycline concentration

The Tet-On 3G induction system employed in the SMARTchoice Inducible Lentiviral system generally allows robust shMIMIC microRNA induction at doxycycline doses between 0.1 µg/mL and 1.0 µg/mL, although this depends on the cell type in use. Furthermore, intermediate expression of the shMIMIC microRNA, 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 above 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.

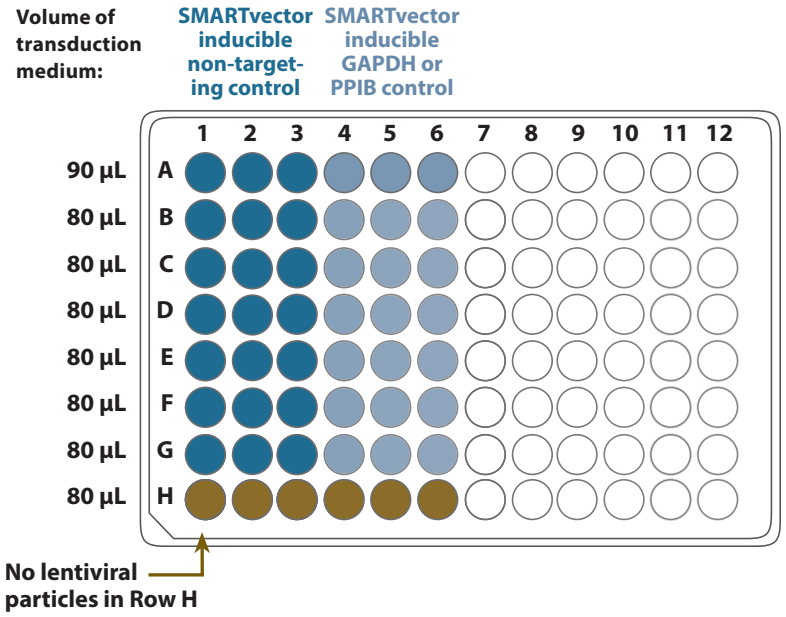

Figure 11. Plate layout for transductions using SMARTvector Inducible Positive and Negative Control shRNAs.

The following protocol uses the SMARTvector Inducible Negative and Positive RNAi Controls to optimize the doxycycline dose for both maximal expression and maximal cell viability. This experiment is recommended whenever shMIMIC Inducible microRNAs are used in a new cell line or type.

Identifying the optimal doxycycline dose for the cells of interest

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 (Chapter 3). If working with suspension cell lines, plate cells the day of transduction (see protocol below for recommendations).

Day 2:

1. Thaw the SMARTvector Inducible positive and negative control shRNA 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 medium with 25 µL of transduction medium with lentiviral particles according to the plate layout in Figure 11.
4. Return culture plate to incubator.
5. After 6-20 hours (as determined in Chapter 3), add 100 µL of preferred culture medium directly to each well without removing medium. Make adjustments to serum concentration so that final serum concentration matches Preferred Culture Medium after adding to wells.

Day 3:

Replace medium on cells with fresh preferred culture medium.

Days 4, 6 and 7:

Replace medium on cells with fresh preferred culture medium supplemented with the optimal dose of puromycin (determined in Chapter 3) 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 preferred culture medium supplemented with 0 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL, 1 µg/mL of freshly dissolved doxycycline (Figure 12).

Day 10:

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

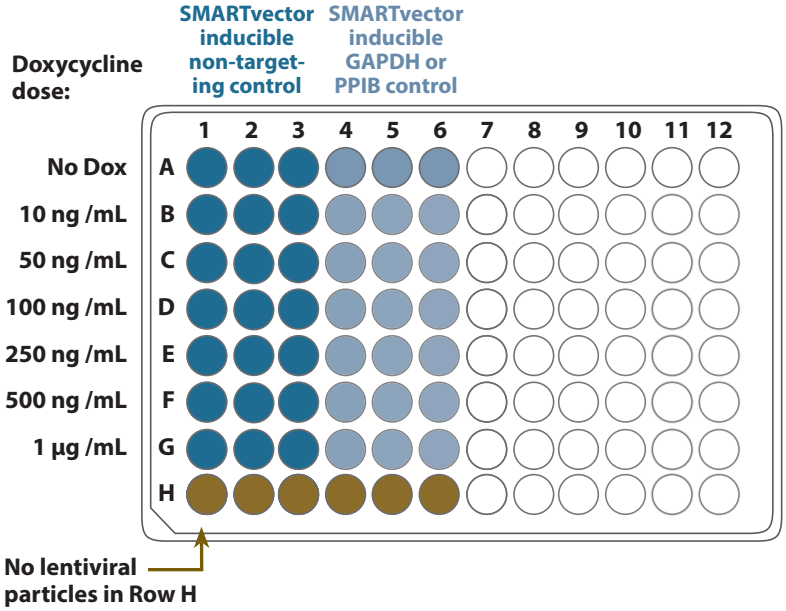

Figure 12. Plate layout for optimizing induction of expression at multiple doxycycline concentrations following transductions using SMARTchoice Inducible Positive and Negative Control shRNAs.

Day 11:

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

Establishing stable cell populations

For transformed cells, immortalized cells, or cell lines with unlimited proliferative capacity, stable cell lines may be established by transduction of shMIMIC inducible microRNAs lentiviral particles and subsequent puromycin selection. These cell lines may be maintained in an uninduced state until expression of the shMIMIC microRNAs is desired. Similarly, some primary cell types capable of 10 or more population doublings may also be used to establish inducible cell systems, but these cells must be used within a limited number of passages from completion of selection, depending on the characteristics of the cell type.

Establishing a stable cell line

Day 1:

Transduce cells at a low MOI of 0.1-0.3 in a 96-well, 24-well or larger format.

Day 2:

Change growth medium.

Day 3:

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

Day 5:

Change growth medium, maintaining the puromycin concentration used on Day 3.

Day 7 or later:

After cells have been selected for two to four days in puromycin, it is no longer necessary to maintain selection. Expand the culture until an adequate cell number has been achieved to freeze and archive the cell line. At this point the cell line is ready for subsequent experiments.

Induce shMIMIC microRNA expression in the stable cell line using the optimal doxycycline dose (in the range of 0.1 µg/mL and 1.0 µg/mL). TurboGFP or TurboRFP expression will become visible in about 24 hours, and will reach a maximum at 48 to 72 hours. Effects of mature microRNA expression on target gene repression at the mRNA and/or protein level can be assessed 72 hours after induction. Phenotypic assay timepoints will need to be optimized depending on assay.

Inducible shMIMC microRNA expression without establishing stable cell lines

It is not necessary to establish stable cell lines with shMIMIC Inducible microRNAs to perform a a mature microRNA gain-of-function experiment. Depending on the phenotype to be assayed, immortalized, primary and non-dividing cells may be transduced with shMIMIC Inducible microRNAs Lentiviral Particles selected for two to four days in puromycin, induced with doxycycline and assayed within a 7 to 10 day timeframe. Using inducible mature microRNA expression systems in this context allows temporal separation of phenotypes associated with lentiviral transduction and puromycin selection from phenotypes resulting from mature microRNA over-expression.

Day 1:

Plate cells at density optimized for the assay. 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 0.1-0.3, be certain to factor in cell loss resulting from death of untransduced cells during puromycin selection. Remember to plate additional wells with cells that will be used as controls to monitor the puromycin selection.

Day 2:

Transduce cells at an MOI of 0.1-0.3 in 96-well or 24-well format.

Day 3:

Replace culture medium.

Day 4:

Replace culture medium with fresh 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 in the untransduced wells, continue puromycin selection for another day.
2. Depending on the phenotype to be examined, doxycycline induction may be started at this time. We have not observed any negative effects of continuing puromycin selection while beginning doxycycline induction. Alternatively, the medium may be replaced with fresh growth medium and doxycycline induction may be initiated at a later time point.
3. Induce shMIMIC microRNA expression in the cells using the optimal doxycycline dose. TurboGFP or TurboRFP expression will become visible at 24 hours and will reach a maximum at 48 to 72 hours. Similarly, microRNA target gene repression at the mRNA and/or protein level is typically observable 72 hours post-induction. Longer periods of exposure to doxycycline may be necessary to achieve some phenotypes.

6 Appendix

Lentiviral packaging

During the preparation of lentiviral particles, the transfer plasmid (containing the specific shMIMIC microRNA of interest) and the helper plasmids (containing the elements essential for viral packaging) are co-transfected into the viral packaging cell line, such as HEK293T. The lentiviral vector genomes are produced, encapsulated and released as virion particles from the cells into the surrounding medium. Transduction of cells with this lentiviral supernatant (medium containing the lentiviral particles) can be performed at low or high MOIs. Lower MOIs may be used for more easily transduced cells or when establishing stable cell lines. High MOIs may be needed for difficult-to-transduce cell lines or *in vivo* applications; however, it is important to note that addition of large volumes of low-titer lentiviral supernatant can often be toxic. This is due in part because the lentiviral supernatant also contains cellular debris (metabolites, nucleases and proteases) derived from the packaging and production process that will affect the viability of transduced cells. Recognizing the limitations and requirements for lentiviral preparations, we have developed a proprietary production and manufacturing process leveraging the Dharmacon Trans-Lentiviral Packaging System that effectively concentrates lentiviral particles while at the same time greatly reduces packaging cell debris. Researchers receive preparations of purified, high-titer lentiviral particles ($\geq 1 \times 10^7$ TU/mL).

Lentiviral particle titering

Prior to shipping, SMARTvector Inducible lentiviral particles are titered in one of two ways. The particles are either directly functionally titered by transducing HEK293T cells with serial dilutions of lentiviral particles, inducing TurboGFP expression with doxycycline, and then quantifying TurboGFP-positive cells by flow cytometry. Alternatively, a p24 ELISA is used to measure lentiviral particle titer. Because p24 titers overestimate functional titers, a vector-matched SMARTchoice

Inducible reference control of known functional titer is included in the p24 ELISA. The functional titer of the reference control is determined by flow cytometry as previously described. Including the reference control in the ELISA allows the p24 titer to be converted to a functional titer by correlating the p24 values of the sample with the reference control.

Stability and storage

shMIMIC Inducible microRNA lentiviral 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 lentiviral particles should be kept on ice, aliquoted into smaller volumes (if necessary) and immediately returned to -80 °C.

Quality assurance and control

shMIMIC Inducible microRNA Lentiviral 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 shMIMIC microRNA 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;
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 titers for each batch included with each shipment.

Trans-lentiviral packaging system biosafety features

Historically, the greatest safety risk associated with a lentiviral delivery platform stems from the potential generation of recombinant lentiviruses that are capable of autonomous replication. Dharmacon minimizes these hazards to the greatest degree by combining a disabled lentiviral genome with the proprietary Trans-Lentiviral Packaging System. Starting with the pNL4-3 molecular clone of HIV1 (GenBank Accession Number AF324493), the lentiviral backbone has been modified to eliminate all but the most essential genetic elements necessary for packaging and integration (5' LTR, Psi sequences, polypurine tracts, Rev responsive elements and 3' LTR). Furthermore, all essential replication genes (gag, pol and env) and accessory genes (tat, rev, vif, vpr, vpu and nef) have been removed. Therefore, all lentiviral particles produced by Dharmacon are replication-incompetent, containing less than 30% of the wild-type HIV-1 genome. Additionally, the 3' LTR has been modified to remove the enhancer elements located in the U3 region. These enhancer elements are needed for native transcriptional activity from the lentiviral LTR. The deletion within the 3'-LTR (ΔU3) results in a vector that is self-inactivating (SIN), which greatly reduces the probability of producing recombinant particles and limits cellular toxicity often associated with expression from the lentiviral LTR. Additional safety features are incorporated by the manufacturing process itself. Generation of lentiviral particles requires a packaging step during which the expression construct containing the shRNA sequence is encapsulated into a viral particle structure. Lentiviral vector packaging systems have divided the essential functions amongst multiple plasmids to reduce the risk of generating replication-competent lentivirus (RCL). The split-genome packaging system is designed so that multiple recombination events between the components are required to generate a lentiviral particle capable of autonomous replication. Commercially available 3rd generation lentiviral vector systems separate the envelope, env (such as VSVg) from the gag-pro-pol, which encodes structural and enzymatic functions. The Trans-Lentiviral Packaging System provides an even higher level of safety over 3rd generation packaging systems by further splitting the lentiviral pol [reverse transcriptase (RT) and integrase (IN) functions] from gag-pro. Because the RT and IN enzymes are provided in *trans* to Gag-Pro, additional recombination events are necessary to produce RCLs. 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 lentiviral components strictly to the packaging cell line. A detailed description of the Trans-Lentiviral Packaging System can be found in (Wu, *et al.* 2000).

7 Frequently asked questions

Do I have to select my cells with puromycin?

This depends on the how shMIMIC Inducible microRNAs are used. We recommend transducing your cells at a target MOI between 0.1 and 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 viral 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)?

For some limited applications, a higher rate of lentiviral integration may be preferred. Each copy of the TRE3G promoter will contribute some low-level of basal shMIMIC microRNA expression. Therefore at higher MOI, more "leaky" shMIMIC microRNA expression 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. Monitoring for these effects is suggested if higher MOIs are used.

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 observed shMIMIC Inducible microRNA over-expression 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, shMIMIC Inducible microRNA Lentiviral Particles carry all necessary elements for selection and inducible expression. Cell lines capable of inducible expression are generated with a single transduction.

Since there is little or no shMIMIC microRNA expression 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 mature microRNA-dependent phenotypes.

Can I use tetracycline to induce expression of the mature microRNA in shMIMIC Inducible microRNAs?

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

8 References

1. Bartel DP, MicroRNAs: target recognition and regulatory functions. *Cell.* 136(2), 215-33 (2009).
2. Curtin JA, Dane AP, et al., Bidirectional promoter interference between two widely used internal heterologous promoters in a late-generation lentiviral construct. *Gene Ther.* 15(5), 384-90 2008).
3. Kappes JC, Wu X, Safety considerations in vector development. Somat. *Cell Mol. Genet.* 26(1-6), 147-58 (2001).
4. Kim VN, MicroRNA biogenesis: coordinated cropping and dicing. *Nat. Rev. Mol. Cell Biol.* 6(5),376-85 (2005).
5. Kim VN, Han J, Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell Biol.* 10(2), 126-39 (2009).
6. Krutzfeldt J, Rajewsky N, Silencing of microRNAs in vivo with 'antagomirs'. *Nature.* 438, 685-689 (2005).
7. Lee R, Feinbaum R, The C. elegans heterochronic gene lin-4 encodes small RNAs with complementarity to lin-14. *Cell.* 75(5), 843-54. (1993).
8. Loew R, Heinz N, et al., Improved Tet-responsive promoters with minimized background expression. *BMC Biotechnol.* 10, 81 (2010).
9. Miyoshi H, Blomer U, Development of a Self-Inactivating Lentivirus Vector. *J. Virol.* 72(10), 8150-8157 (1998).
10. Morimoto M, Kopan R. rtTA toxicity limits the usefulness of the SP-C-rtTA transgenic mouse. *Dev. Biol.* 325(1), 171-178 (2009).
11. Sisson TH, Hansen JM, 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).
12. Whitsett JA, Perl AK. Conditional control of gene expression in the respiratory epithelium: A cautionary note. Am. J. Respir. *Cell Mol. Biol.* 34, 519-520 (2006).
13. Wightman B, Ha I, Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4mediates temporal pattern formation in C. elegans. *Cell.* 75(5), 855-62 (1993).
14. Wu X, Wakefield JK, Development of a novel trans-lentiviral vector that affords predictable safety. *Mol. Ther.* 2(1), 47-55 (2000).

9 Limited use licenses

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