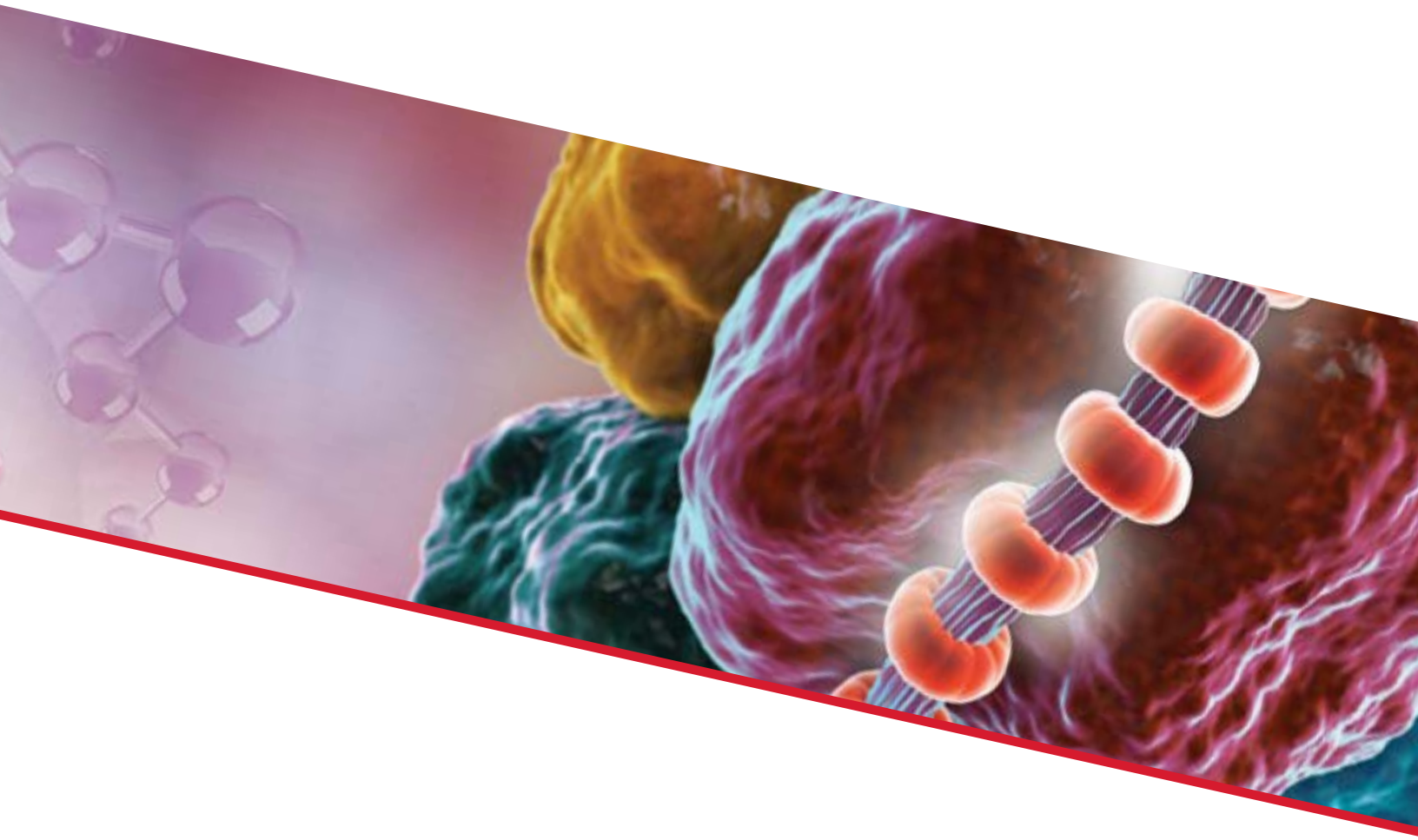
GE Healthcare

Technical Manual

Dharmacon™ shMIMIC Lentiviral microRNA



Contents

1 Introduction to shMIMIC Lentiviral microRNA

The Dharmacon™ SMARTchoice lentiviral vector platform is an innovative system ideally suited for RNA interference (RNAi)-mediated studies. The purpose is to provide the researcher with the most effective tools for delivering and expressing genetic content in their cells of interest. The SMARTvector™ shRNA and shMIMIC microRNA products, which are built upon the SMARTchoice platform, utilize advanced options which include:

- a microRNA scaffold for improved processing
- seven RNA polymerase II promoter options to enable experiments in a broad range of cell types with
- two fluorescent reporter options [TurboGFP (tGFP), TurboRFP (tRFP), Evrogen, Moscow, Russia] and a no-reporter option
- an option of ultra-high titer of 5×10^9 TU/mL

The SMARTchoice workflow incorporates a unique tool, the SMARTchoice Promoter Selection Plate (Cat #SP-001000-01). This is a 96-well plate containing high-titer, transduction-ready lentiviral particles derived from seven unique lentiviral shRNA constructs, each containing a different transcriptional promoter. The SMARTchoice Promoter Selection Plate allows the researcher to evaluate and then choose the vector configuration that is most effective for shRNA expression in the particular cells of interest. This semi-customizable approach to vector design provides greater flexibility, and more importantly, improved likelihood of experimental success relative to static systems (see SMARTchoice web page for more information at http://dharmacon.gelifesciences.com/smartchoice/).

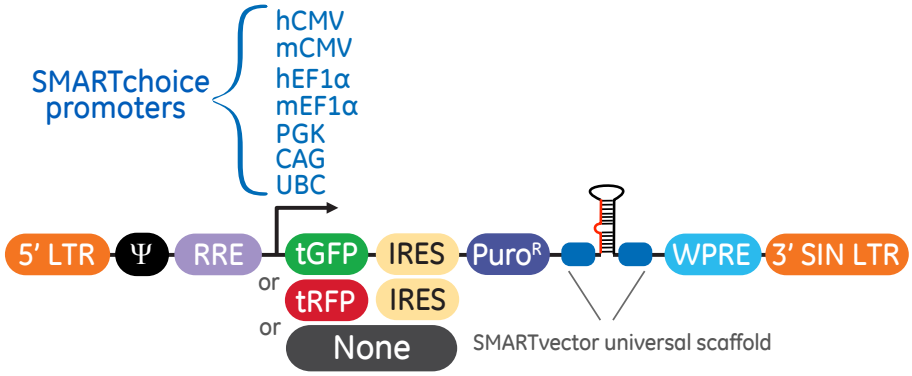


Figure 1. Elements of the SMARTvector shRNA and shMIMIC microRNA lentiviral vector.

Vector Element	Utility
5' LTR	5' Long Terminal Repeat is necessary for lentiviral particle production and integration into host cell genome
Ψ	Psi packaging sequence allows viral genome packaging using lentiviral packaging systems
RRE	Rev Response Element enhances titer by increasing packaging efficiency of full-length viral genomes
tGFP or tRFP	TurboGFP or TurboRFP reporter for visual tracking of transduction and expression
None	No reporter option for use in applications where fluorescence is not required
IRES	Internal Ribosomal Entry Site allows expression of fluorescent reporter and puromycin resistance gene in a single transcript
Puro^R	Puromycin resistance permits antibiotic-selective pressure and propagation of stable integrants
SMARTvector universal scaffold	microRNA-adapted shRNA for gene knockdown or expression of a mature microRNA
WPRE	Woodchuck hepatitis Post-transcriptional Regulatory Element enhances transgene expression in the target cells
3' SIN LTR	3' Self-inactivating Long Terminal Repeat for increased lentivirus safety

Recommended Experimental Controls

Including both positive and negative controls in all experiments assures rigorous, high-confidence data, and is required for accurate data interpretation. Additionally, negative and positive shRNA control lentiviral particles are cost-effective reagents for experimental optimization prior to transductions with shMIMIC Lentiviral microRNA targeting a specific gene, such as determining optimal conditions for transduction.

The following SMARTvector Negative and Positive RNAi Controls are available with all seven promoters and reporter options, and are recommended for use with shMIMIC Lentiviral microRNA:

- SMARTvector Non-targeting Control shRNA
- SMARTvector GAPD Positive Control shRNA (human, mouse and rat)
- SMARTvector PPIB Positive Control shRNA, hCMV/GFP only (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 Promoter Selection Plate (See Appendix: Identify the optimal promoter).

Order product with the optimal promoter/reporter combination for:

- ○ Cells and gene(s) of interest
- ○ Positive controls targeting GAPD 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. A separate antibiotic kill curve protocol is available online.
- ○ 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 shMIMIC Lentiviral microRNA particles based upon your cells of interest (See Appendix: Determine functional titer).
- ○ Evaluate cellular response to microRNA expression
  Using optimized conditions, transduce shMIMIC Lentiviral microRNAs for gene of interest and monitor phenotypic results with assay of choice. See protocol below: Protocol using shMIMIC Lentiviral microRNAs.

Materials required

- shMIMIC Lentiviral microRNA for your microRNA of interest
- Positive and negative controls for optimization with matched promoter/reporter options
  - » SMARTvector Non-targeting Control shRNA
  - » SMARTvector Positive Control shRNA (GAPD or PPIB)

Materials not supplied

- SMARTchoice Promoter Selection Plate (Dharmacon, Cat # SP-001000-01)
- Polybrene (American Bioanalytical, Cat #AB01643)
- 96-well tissue culture plates
- Deep-well 96-well plate (Nunc, Cat #12-565-553)
- Multichannel pipette (8-channel)
- 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 or 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
- Resazurin Cell Viability Reagent

Protocol using shMIMIC Lentiviral microRNA

Gene-specific shMIMIC Lentiviral microRNAs are available as individual constructs supplied as concentrated lentiviral particles. A well-constructed experiment should include a construct expressing the microRNA of interest plus negative and positive controls and untransduced controls, and be performed as biological triplicates. Prior to transduction, cell line-specific optimization must be determined for cell density and transduction medium composition.

Determine functional titer in cells of interest

Lentiviral transduction efficiency can vary depending on factors such as target cell type, duration of exposure to lentiviral particles, composition of transduction medium and experimental conditions. The titer of shMIMIC Lentiviral microRNA particles reported on the Certificate of Analysis (C of A) was measured in HEK293T cells, so the relative transduction efficiency of your cell type will likely be different. Therefore, it is recommended that the correct volume of lentiviral particles should be empirically identified for each batch of shMIMIC Lentiviral particles in the cell type of interest, and testing a range of functionally adjusted multiplicities of infection (MOI) is recommended to ensure sufficient gene knockdown (See Appendix: Determine functional titer).

General protocol

Follow the protocol described below for transducing cells with shMIMIC Lentiviral microRNA and SMARTvector controls. Figure 2 depicts a suggested 96-well plate layout for a well-designed gene silencing experiment using shMIMIC Lentiviral microRNA particles expressing the microRNA(s) of interest, along with the appropriate controls.

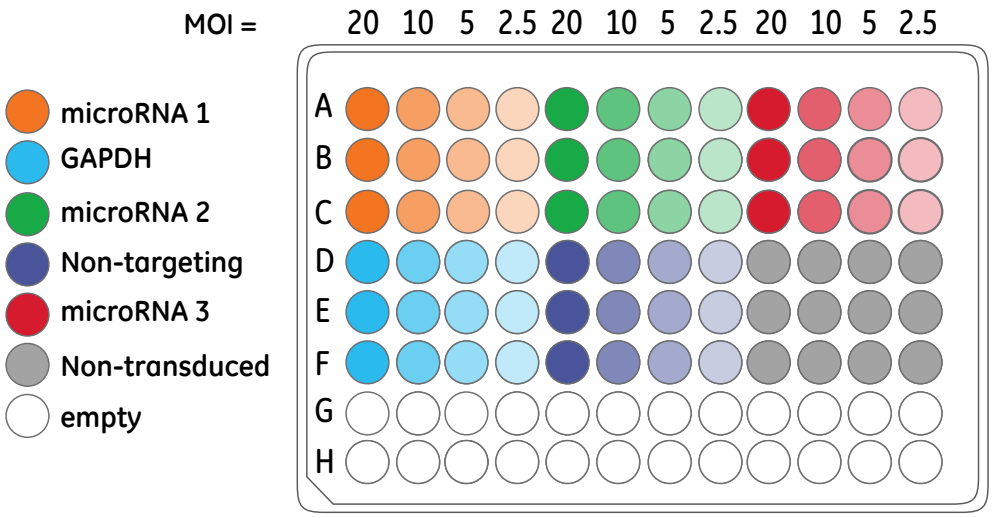


Figure 2. Example of plate layout for typical experiment using shMIMIC Lentiviral microRNA particles. Suggested 96-well plate format using three different shMIMIC constructs (microRNA 1, microRNA 2 and microRNA 3) at four MOI (20, 10, 5 and 2.5 TU/cell). Included are positive (GAPD) and negative (non-targeting) controls and non-transduced cells.

Day 1:

Plate cells in biological triplicate in a 96-well culture plate at previously determined density (See Appendix: Determine cell density and transduction conditions).

Day 2:

1. Remove your microRNA and control lentiviral particles from -80° C freezer and thaw on ice.
2. Prepare 10 mL of pre-warmed Transduction Medium (Polybrene and serum concentrations as determined previously (See Appendix: Determine cell density and transduction conditions).

At all steps of these procedures, it is important to pipette slowly into wells to avoid dislodging adherent cells.

3. Transduce cells at previously determined optimal conditions and MOIs ranging from 5-20 in a 96-well plate . We recommend using a functionally adjusted titer for optimal scientific accuracy (See Appendix: Determine cell density and transduction conditions and Determine functional titer).
   a. Prepare dilutions of lentiviral particles (in pre-warmed Transduction Medium) such that 50 µL contains the appropriate number of lentiviral particles per cell (TU/cell) to obtain desired MOI. Dilutions should be prepared using sterile 1.5 mL microfuge tubes (or deep-well plate). Use the following protocol to make a two-fold serial dilution for each of the shMIMIC Lentiviral microRNA particles and SMARTvector controls.
      i. Multiply the calculated volume for highest MOI (units in µL) by 7. The volume of lentiviral particles is multiplied by 7 to allow 3 volumes to be used to transduce cells in triplicate, 3 volumes to be used to make the next two-fold dilution in series, and 1 volume to represent liquid overage required to accommodate small carryovers during pipetting.
      ii. Transfer calculated volume of lentiviral particles into sterile 1.5 mL microfuge tube. Label tube with appropriate MOI (for example "MOI = 20").
      iii. Bring volume up to 400 µL with pre-warmed Transduction Medium and mix gently by pipetting.
      iv. With new pipette tip, transfer 200 µL from the MOI = 20 tube into another sterile 1.5 mL microfuge tube labeled MOI = 10.
      v. Bring volume up to 400 µL with pre-warmed Transduction Medium and mix gently by pipetting.

vi. Repeat steps iv and v for MOI = 5 and MOI = 2.5.

vii. If Polybrene is present in Transduction Medium, allow Transduction Medium with lentiviral particles to incubate for 10-20 minutes at room temperature before transducing cells. If Polybrene is not used, proceed directly to transduction of cells.

viii. Remove culture plate(s) from incubator and replace medium with 50 µL of Transduction Medium with lentiviral particles. In the "non-transduced" wells, add an equivalent volume of Transduction Medium.

ix. Return plates to incubator.

x. After predetermined transduction time of 6-20 hours (See Appendix: Determine cell density and transduction conditions), add 100 µL of Growth Medium directly to each well without removing medium.

xi. Return culture plate to incubator. Incubate for 24 hours.

Day 4:

Check for TurboGFP or TurboRFP expression, or for no reporter determine gene knockdown as well as test for toxicity using Resazurin or a similar assay for viability.

If you have a reporter, TurboGFP or TurboRFP expression will become visible after 24 hours and will reach a maximum at 48 to 72 hours post-transduction. Similarly, gene knockdown, at the mRNA level, is detectable at 24 hours and reaches a maximum typically by 72 hours.

Establishing a stable cell population

For cell lines with longer proliferative capacity, stable cell lines may be established by transduction of shMIMIC Lentiviral microRNA particles and subsequent puromycin selection. These cell lines may be maintained for long-term studies. Care must be taken that cells be used within a limited number of passages from completion of selection; this will depend on the characteristics of the particular cell type.

Days 1-4:

Follow the "General protocol" above until Day 4.

Day 4:

Change to Selection Medium containing appropriate concentration of puromycin.

Day 5 or 6:

Trypsinize cells and expand into a larger culture vessel with medium containing puromycin in order to select for cells with stably integrated shMIMIC construct.

Day 8 or later:

1. Expand the culture until an adequate cell number has been achieved to freeze and archive the cell line.
2. Check for TurboGFP or TurboRFP expression, or for no reporter determine gene knockdown as well as test for toxicity using Resazurin or a similar assay for viability.

3 Appendix

Additional protocols

Identify the optimal promoter

The SMARTchoice Promoter Selection Plate is designed to allow data-driven promoter selection prior to ordering SMARTvector Lentiviral shRNAs or shMIMIC Lentiviral microRNAs for specific genes of interest. This unique tool permits the simultaneous evaluation of seven different promoters in order to select one which is most effective in your cell line or cell type of interest. The Promoter Selection Plate contains transduction-ready lentiviral particles representing seven different promoters arrayed in a 96-well plate (Figure 3).

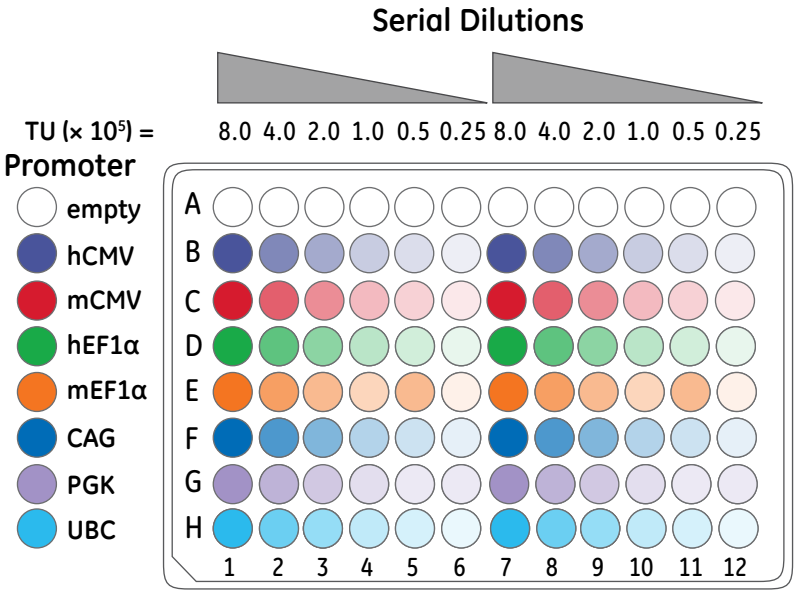


Figure 3. SMARTchoice Promoter Selection Plate enables straightforward, qualitative assessment of promoters that actively drive expression. Lentiviral particles are arrayed in two-fold dilutions with the highest amount of particles at 8.0 × 10^5 transducing units (TU). Layout by rows: **A. empty** wells (DMEM only); **B. hCMV** – human cytomegalovirus immediate early promoter; **C. mCMV** – mouse cytomegalovirus immediate early promoter; **D. hEF1α** – human elongation factor 1 alpha promoter; **E. mEF1α** – mouse elongation factor 1 alpha promoter; **F. CAG** – chicken beta actin hybrid promoter; **G. PGK** – mouse phosphoglycerate kinase promoter; **H. UBC** – human ubiquitin C promoter.

The arrayed SMARTvector Lentiviral shRNA particles co-express the TurboGFP reporter, a puromycin resistance selectable marker and Non-targeting shRNA Control (NTC) in the context of the SMARTvector microRNA-adapted scaffold. The seven promoters represented on the SMARTchoice Promoter Selection Plate are as follows:

hCMV – human cytomegalovirus immediate early promoter

mCMV – mouse cytomegalovirus immediate early promoter

hEF1α – human elongation factor 1 alpha promoter

mEF1α – mouse elongation factor 1 alpha promoter

CAG – chicken beta actin hybrid promoter

PGK – mouse phosphoglycerate kinase promoter

UBC – human ubiquitin C promoter

Columns 1-6 on the plate are duplicated in columns 7-12, allowing optimization experiments to be performed as replicates, or using two different transduction conditions or two different cell lines. Each well contains lentiviral particles suspended in 25 µL of DMEM containing no serum, with the exception of Row A, which contains DMEM only and is intended for use as the untransduced control. Columns 1 and 7 have the highest number of lentiviral particles

[corresponding to 8 × 10^5 transducing units (TU)* in 25 µL] and serial two-fold dilutions are made in columns 2-6 and 8-12, respectively.

> *For example, transduction of 10,000 cells with 25 µL of lentiviral particles from well B1 would represent an MOI = 80, and the stepwise dilutions in wells B2, B3, B4, B5 and B6 would represent MOI equal to 40, 20, 10, 5 and 2.5, respectively.*

*For an accurate assessment of promoter activity, all lentiviral particles are normalized to equivalent functional titers in HEK293T cells using a p24 ELISA assay. A description of the titering method can be found in the FAQ section along with an explanation of how p24 titers are converted to a functional titer.

Transduction of cells of interest using lentiviral particles from the SMARTchoice Promoter Selection Plate should be performed using the cell number, Polybrene concentration, serum level and duration as determined previously. (See Appendix: Determine cell density and transduction conditions). The specific formulation for the Transduction Medium will reflect the optimal Polybrene and serum concentrations previously determined for the cells being transduced. Protocols for transduction of both adherent and suspension cells are included.

The SMARTchoice Promoter Selection Plate is not a cell culture plate. Cells should not be added to, nor should transductions be carried out in the SMARTchoice Promoter Selection Plate.

SMARTchoice Promoter Selection Plate protocol for adherent cells

Day 1:

Seed appropriate number of cells in an appropriate cell culture plate as previously determined in a total volume of 100 µL per well in Growth Medium. Incubate plate overnight under appropriate conditions.

Day 2:

1. Prepare the SMARTchoice Promoter Selection Plate as follows:
    a. Remove the SMARTchoice Promoter Selection Plate from freezer and thaw lentiviral particles on ice. It may take > 30 minutes to thaw lentiviral particles in all wells. After lentiviral particles have thawed, spray the sealed plate with 70% ethanol and carefully wipe excess with a paper towel or Kimwipes®.
    b. Quick-spin lentiviral particles to bottom of the wells by centrifuging (rcf = 300) for 20-40 seconds in a table top centrifuge. Remove plate from centrifuge. Wipe the Promoter Selection Plate and clean the inside of centrifuge using 70% ethanol. Carefully dry plate with paper towel or Kimwipes.
    c. Within a Class II/Type A2 biological safety cabinet, carefully remove foil seal from Promoter Selection Plate being careful not to splash lentiviral particles out of wells. Using forceps to hold edge of foil seal, immerse seal in liquid waste reservoir and dispose of in biohazardous waste bag.
2. Add Transduction Medium [with Polybrene and serum at optimal concentrations determined previously; (See Appendix: Determine cell density and transduction conditions)] to lentiviral particles:
    a. If Polybrene was determined intolerable to cells and will not be used in transductions, skip this step and proceed directly to Step 3. Add Polybrene and serum to Base Medium at a 2x concentration determined during optimization; this will result in 1x Polybrene and serum concentrations to generate 1x Transduction Medium with lentiviral particles.
    b. To each well of the SMARTchoice Promoter Selection Plate, pipette 30 µL of Base Medium with 2x Polybrene and serum (as described above) creating ~ 55 µL total volume per well of 1x Transduction Medium with lentiviral particles. An 8-channel pipette is recommended for all transfers to and from Promoter Selection Plate. Use new tips for each transfer and deactivate any lentiviral particles in pipette tips by aspirating and dispensing bleach or Lysol® into and out of tips prior to disposal.

c. Place a new foil seal over the Promoter Selection Plate and quick-spin plate by centrifuging (rcf = 300) for 20-40 seconds in a table top centrifuge.

d. Incubate plate at room temperature for 10-20 minutes.

3. Perform transduction of cells with SMARTvector Lentiviral shRNA particles

a. Take culture plate from incubator and place in biological safety cabinet. Using an 8-channel pipette, carefully aspirate Growth Medium from each column of culture plate being careful not to dislodge cells from bottom of well. Dispense Growth Medium into liquid waste reservoir. Change tips between columns.

b. Carefully remove foil seal from Promoter Selection Plate. Transduce cells by transferring 50 µL of the Transduction Medium with lentiviral particles from each well of Promoter Selection Plate to corresponding wells of culture plate. It is recommended that an 8-channel pipette be used to transfer Transduction Medium with lentiviral particles one column at a time. Tips should be changed following each transfer. After all columns (dilutions) of Transduction Medium with lentiviral particles are transferred to corresponding wells, place culture plate in incubator with appropriate conditions for the cell line.

c. Allow transduction to proceed for 6 hours or overnight as determined previously in the optimization of general conditions, (See Appendix: Determine cell density and transduction conditions). After the transduction period, add 100 µL of Growth Medium directly to each well. Adjust serum concentration accordingly.

4. Culture transduced cells under appropriate conditions for 48 to 96 hours.

Day 4-6:

Visually inspect cells daily using fluorescence microscopy, flow cytometry or another method suitable for the observation of TurboGFP expression. A qualitative assessment of relative promoter activity can be made by comparing TurboGFP fluorescence intensity among wells. Figure 4 depicts the fluorescent images of human A549 and HEK293T cells transduced with lentiviral particles from the SMARTchoice Promoter Selection Plate.

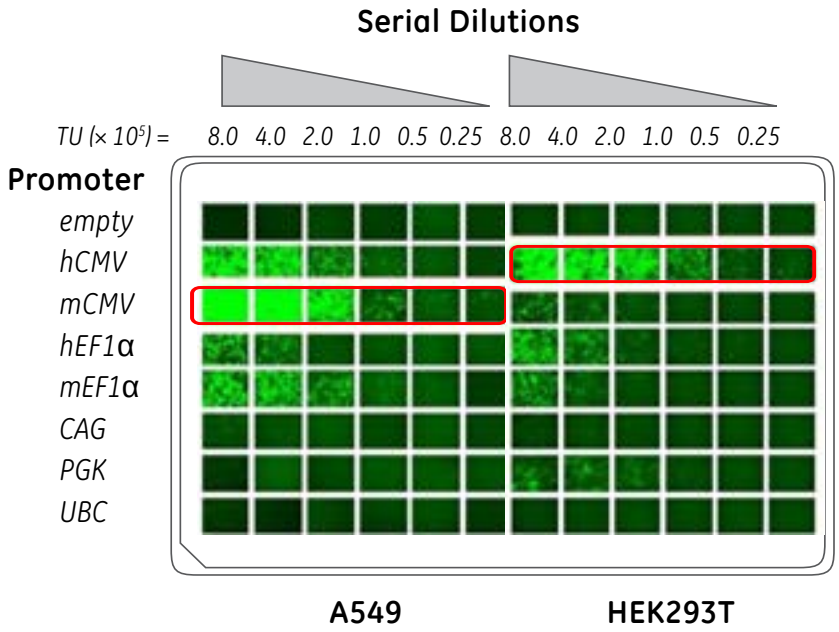


Figure 4. Fluorescense microscopy images of TurboGFP-positive A549 and HEK293T cells for promoter evaluation. Cells were transduced with lentiviral particles from the SMARTchoice Promoter Selection Plate. Fluorescence intensity demonstrates that the mouse CMV (mCMV) promoter is the optimal promoter in A549 cells, whereas the human CMV (hCMV) promoter is the optimal promoter in HEK293T cells.

For human A549 and HEK293T cells, the mCMV and hCMV promoter, respectively, consistently ranks highest in fluorescence intensity and therefore would be the optimal promoter choice for expression in these cell lines. Generally, there will be a clear indication as to which promoter is the most active in the cells of interest. The most active promoter for a particular cell line should be consistent across all six lentiviral particle concentrations.

SMARTchoice Promoter Selection Plate protocol for suspension cells

Day 1:

1. Using ideal cell concentration (See Appendix: Determine cell density and transduction conditions), transfer suspension cells into sterile centrifuge tube. Pellet cells by centrifuging (rcf = 300) for 6 minutes in a table top centrifuge. Following centrifugation, carefully aspirate supernatant. Gently resuspend cell pellet in 3.75 mL of Base Medium, then plate 25 µL cells in Base Medium suspension into each well of 96-well culture plate.

   For example, if the optimal density is 10,000 cells per well, pellet 1.5 × 10^6 cells and resuspend in 3.75 mL of Base Medium; 25 µL would then correspond to 10,000 cells per well.

2. Prepare SMARTchoice Promoter Selection Plate for transduction in the same manner as described above for adherent cells.

Use Polybrene and serum concentration such that the final concentration upon adding 50 µL of Transduction Medium with lentiviral particles to 25 µL of cells (see next step) will give the 1x concentration as determined during the optimization step for your cells of interest.

3. Transduce cells by transferring 50 µL of Transduction Medium with lentiviral particles from each well of Promoter Selection Plate to corresponding wells of culture plate.

As suspension cells settle over time to the bottom of the well, it is recommended that you gently rock or tap the plate a few times (at 30-60 minute intervals) for the first few hours to mix the cells with the lentiviral particles. For more thorough mixing, you may gently pipette up and down 5-10 times using a multichannel pipette. Be sure to change pipette tips from one column to the next.

4. Allow transduction to proceed for 6 hours or overnight as determined in optimization experiment. After transduction period, add 75 µL of Growth Medium directly to each well. Adjust serum concentration accordingly.
5. Culture transduced cells under appropriate conditions for 48 to 96 hours.

Day 3-5:

Visually inspect cells daily using fluorescence microscopy as described above in Day 3-4 of protocol above for adherent cells. 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.

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.

Polybrene can be toxic to certain cells. Although Polybrene is not generally necessary to achieve the MOIs recommended for 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 5. This recommended protocol will test cell density, Polybrene concentration, the presence or absence of serum, and the duration of transduction (6 hours or overnight).

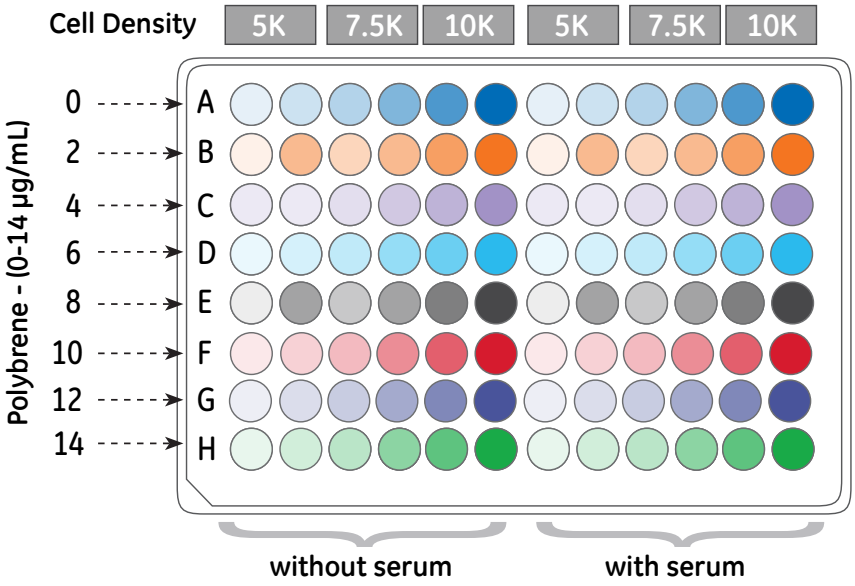


Figure 5. Example of a 96-well plate layout for the optimization of transduction conditions. This plate layout tests three cell densities (5,000 (5K), 7,500 (7.5K) and 10,000 (10K) cells per well), eight concentrations of Polybrene (0-14 µg/mL) and medium with or without serum.

Day 1:

Seed cells into two 96-well culture plates at the appropriate cell density following the template depicted in Figure 5. 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.

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 space for cells 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 5 (5,000, 7,500, a**nd 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

Lentiviral particles are provided in DMEM. Thus, testing a recipe for suitable transduction medium must include DMEM. Although, if the preferred Base Medium for your cell line contains DMEM it is not necessary to test 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. 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.
   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.

ii. 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.

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 5.
   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.
   f. Using a multichannel pipette add 100 µL of Growth Medium directly to each well of the replicate plate and return to incubator for overnight culturing.

To adjust for the absence of serum (transductions without serum only) make up the difference by including additional serum to the Growth Medium. F or 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:

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 the cell line.

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.

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.

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.

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:
   a. Prepare 10 mL of Transduction Medium containing no serum by pipetting a 1:1 mixture of DMEM to Base Medium.

Lentiviral particles are provided in DMEM. Thus, testing a recipe for suitable transduction medium must include DMEM. Although, if the preferred Base Medium for your cell line contains DMEM it is not necessary to test 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 5). Polybrene concentration is increased to account for the additional 25 µL of Base Medium, which does not already contain Polybrene. For example, well (B1) 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 5), one set without serum and one with serum.
8. Perform Transduction
   a. Remove culture plates from incubator and place in biological safety cabinet.

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

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 5.

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 Growth Medium directly to each well of the replicate plate and return to incubator for overnight culturing.

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 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 Growth 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 optimal density and conditions that result in optimal phenotypic changes for the cell line.

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

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, 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 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 10-20 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 6-20 hours (as determined during transduction optimization).
6. Add 75 µL of normal growth medium to cells.
7. Culture cells for 48-72 hours (as determined during transduction optimization).
8. Choose one well in the Destination Plate for counting fluoresence-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.

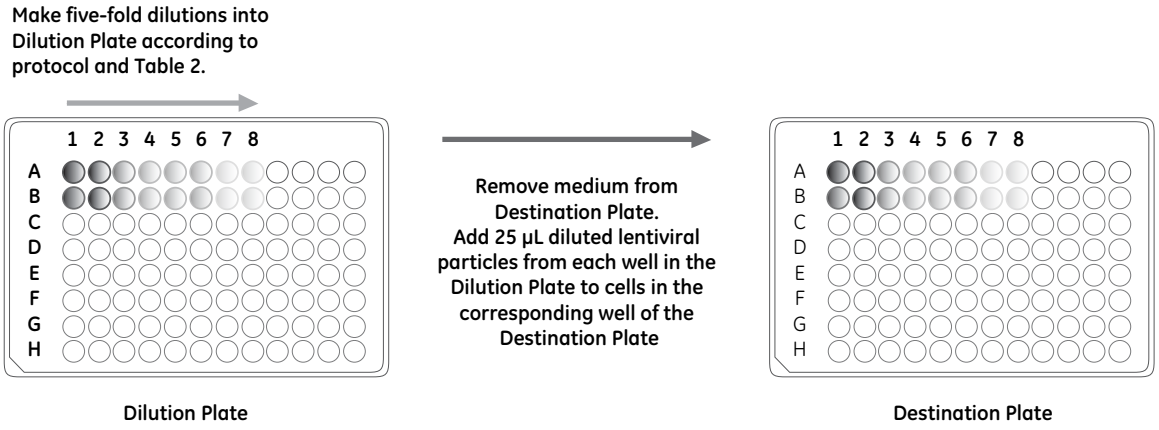


Figure 5. Diagram for dilution series of lentiviral particles (Dilution Plate) and addition to cells (Destination Plate).

Table 2. Example setup for lentiviral particle dilution series.

	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.

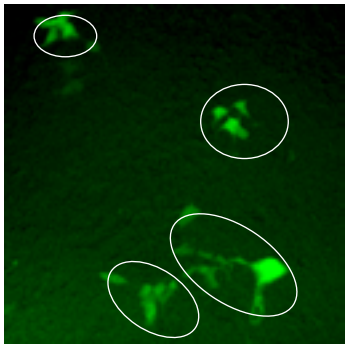


Figure 6. Example of individual colonies in HEK293T cells 72 hour post-transduction. Four colonies are circled. Imaged at 40x magnification.

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

Stability and storage

SMARTvector Lentiviral shRNA particles and shMIMIC Lentiviral microRNA 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

SMARTvector Lentiviral shRNA particles and shMIMIC Lentiviral microRNA 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 construct 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. for fluorescent reporter options: confirmation of fluorescent reporter expression (TurboGFP or TurboRFP) following transduction 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.

Frequently asked questions (FAQs)

Can I use the SMARTchoice Promoter Selection Plate to optimize all of the transduction conditions for my cells of interest?

No. The Promoter Selection Plate is not meant to be used to establish fundamental lentiviral transduction conditions, such as optimal cell density, in your cells of interest. The purpose of the Promoter Selection Plate is to visually assess relative promoter activity. Basic experimental conditions (such as cell density, with or without serum, Polybrene concentration) should be established prior to transduction of viral particles using the SMARTchoice Promoter Selection Plate. The Promoter Selection Plate contains duplicate wells of serially diluted lentiviral particles representing each promoter so that two separate conditions can be tested simultaneously. After identification of the optimal promoter for your cells of interest, additional transduction optimization should be performed to identify appropriate MOIs for optimal performance.

I think I know the best promoter for my cells of interest. Do I still need to evaluate promoter activity with the SMARTchoice Promoter Selection Plate?

Promoter activity varies in different cell lines and cell types. We have demonstrated that this may affect the expression of the construct and the level of expression that can be achieved. If you have previous data and/or knowledge that a particular promoter is active in your cells, then gene-specific lentiviral microRNAs can be ordered without testing the SMARTchoice Promoter Selection Plate. If you are not sure which promoter to choose, then testing your cells with the SMARTchoice Promoter Selection Plate prior to ordering gene-specific shRNAs as lentiviral particles can potentially save you a great deal of time and money.

I have used the human CMV promoter previously for my mouse cell line with low performance. Will a different promoter increase shRNA functionality?

Poor performance may be due to low transduction efficiency or suboptimal promoter activity. Optimization of transduction conditions is essential to successful results as well as understanding promoter activity. Promoter activity varies in different cell lines and cell types, and we highly recommend using the SMARTchoice Promoter Selection Plate to determine the most active promoter in your cells of interest. For example, the mouse CMV promoter in NIH/3T3 cells results in significantly increased promoter activity and greater microRNA expression compared to the same construct expressed from the human CMV promoter in this cell line.

I am using human cell lines in my research; should I always choose a human promoter for best activity?

In some instances promoter activity correlates with the species from which it is derived. However, promoter activity does not always follow a species-specific expression pattern. For example, we have observed mouse promoters to be the most active in some human cell lines, whereas both human and mouse promoters were most active in some rat cells. Choosing the most effective promoter in a particular cell line is not always predictable, and therefore should be determined empirically.

Can I order shMIMIC or SMARTvector constructs with a promoter other than the seven SMARTchoice promoter options?

No. SMARTchoice promoter options include human and mouse CMV, human and mouse EF1α, CAG, PGK and UBC.

Can I order shMIMIC or SMARTvector constructs without a fluorescent reporter?

Yes. SMARTchoice reporter options include TurboGFP, TurboRFP and a no reporter option.

Can I use the same promoter to express the microRNA in multiple cell lines?

As a general rule, we recommend using the SMARTchoice Promoter Selection Plate to identify the promoter with the highest activity in each cell line or cell type. Promoter strength is important to the success of a microRNA expression experiment, which can vary widely across cell lines and among species. The SMARTchoice Promoter Selection Plate is designed to identify the optimal promoter specific for each cell line or cell type.

What is titer?

Titer refers to the number of viral particles or transducing units present per milliliter of solution (TU/mL). The titer value is used in conjunction with the desired multiplicity of infection (MOI) to determine the number of viral particles (or volume of viral particle suspension) required for transduction experiments.

What titers are achieved for shMIMIC Lentiviral microRNA Particles?

Our shMIMIC Lentiviral microRNA particles are provided at either a functional titer of 1×10^8 (± 20%) transducing units (TU) per milliliter (mL) or an ultra-high titer format of 1×10^9 (± 20%), as determined by p24 ELISA of the lentiviral particles. The p24 titer is then converted to a functional titer by correlating with values of a reference control of known functional titer (SMARTvector Lentiviral shRNA particles containing matched promoter) that is included in the p24 ELISA.

What does transducing unit (TU) mean?

Transducing units are the number of functional viral particles in a solution that are capable of transducing a cell and expressing the transgene.

What is MOI?

Multiplicity of infection (MOI) is the ratio of lentiviral transducing units (functional lentiviral particles) to cells. An MOI of 10 indicates that there are 10 transducing units for every cell in the well. It is important to note that different cell types may require different MOIs for successful transduction. For instance, HEK293T cells are highly permissive to lentiviral transduction (MOI of 5-20) while neuronal cells such as SH-SY5Y may require higher MOIs ranging from 10-40.

How do I transduce cells?

Once optimization studies have been performed to identify preferred Polybrene concentrations, cell densities, medium conditions (See Appendix: Determine cell density and transduction conditions) and MOIs (See Appendix: Identify the optimal promoter and MOI), the process of transduction requires nothing more than incubating lentiviral particles with your target cell population for 4 to 20 hours.

How do I determine the transduction efficiency?

The transduction efficiency can be measured by assessing the fraction of cells expressing TurboGFP or TurboRFP after transduction. This can be achieved by simple microscopic observation using a fluorescence microscope or by FACS analysis 48-72 hours after transduction. For constructs that do not express a fluorescent protein reporter, we recommend titering the lentiviral particles produced using a functional lentiviral titration protocol such as limiting dilution with cell viability assay by crystal violet staining or genomic qPCR assay.

How many cells can I transduce with the amount of shMIMIC Lentiviral microRNA particles provided?

The number of cells that can be transduced with each shMIMIC Lentiviral microRNA construct will depend upon the MOI used during the transduction procedure. Procedures and formulas for optimizing transduction for your particular cell type are provided in the Appendix.

How do I know if my target cell type can be transduced with lentiviral particles?

Lentiviral particles are manufactured with a VSVg envelope protein that provides broad tropism. Therefore, most cell types are transduced, albeit, with varying degrees of efficiency. However, the efficiency of is not only a factor of successful transduction, but is also dependent upon the efficiency of transgene expression; this can be assessed by utilizing the SMARTchoice Promoter Selection Plate with the cells of interest. This assay will allow the simultaneous evaluation of seven SMARTchoice promoters.

What quality control is performed on shMIMIC Lentiviral microRNA particles?

shMIMIC Lentiviral microRNA constructs undergo multiple levels of analysis throughout production to ensure the quality of the final product. Restriction enzyme analysis and sequencing are performed on all lentiviral vector constructs to ensure integrity of the lentiviral vector backbone and of the targeting sequence, respectively. Lentiviral packaging cells are carefully monitored for bacterial and/or fungal contaminants. Following packaging, lentiviral particles are titered by p24 ELISA. The p24 titers are adjusted to a functional titer using reference control lentiviral particles titered by flow cytometry to count GFP-positive cells.

Are Dharmacon lentiviral particle products safe to use in the laboratory? What precautions should be taken when handling SMARTvector and shMIMIC lentiviral particles?

Lentiviral delivery systems have been employed in many research laboratories around the world without incident. Handling of lentiviral reagents requires extensive experience with cell culture techniques. It is vital that the protocols and the safety guidelines described in the Appendix for appropriate handling and storage are fully understood and followed precisely. For additional guidance on containment of lentiviral vectors, we strongly recommend the user refer to the Recombinant DNA Advisory Committee (RAC) guidelines for research with lentiviral vectors: http://oba.od.nih.gov/oba/rac/Guidance/LentiVirus_Containment/pdf/Lenti_Containment_Guidance.pdf. Also, details of the BSL-2 equipment, facilities and protocols can be found at http://www.cdc.gov/OD/ohs/biosfty/bmbl4/ bmbl4s3.htm.

What is a SIN vector?

A self-inactivating (SIN) vector is a retroviral vector that contains a non-functional or modified 3' long terminal repeat (LTR) sequence. This sequence is copied to the 5' end of the vector genome during integration, resulting in the inactivation of promoter activity of both LTRs.

Can SMARTvector Lentiviral shRNA particles be further propagated in the lab?

No. SMARTvector products are engineered for maximum biosafety and are therefore replication incompetent.

What is Polybrene?

Polybrene, also known as hexadimethrine bromide, is a small, positively charged molecule that binds to cell surfaces and neutralizes surface charge. Polybrene has been shown to enhance transduction of mammalian cells by 2-10 fold. Alternatives include DEAE-Dextran.

What MOI and cell density should I be using?

The MOI used in your experiments will depend upon the cell type employed, the applicability of reagents that enhance transduction (such as Polybrene), the appropriate medium conditions and the density at which cells are plated (See Appendix: for recommended optimization protocols).

How do I select for stably transduced cells?

The SMARTvector construct contains the puromycin resistance gene (PuroR) that allows for production of stable cell lines. It is recommended to optimize the concentration of puromycin for your cells of interest to result in 100% cell death of untransduced cells after 4 to 6 days.

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 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™ Lentiviral shRNAs
- Dharmacon™ SMARTvector™ Inducible Lentiviral shRNAs
- Dharmacon™ shRNAs
- Dharmacon™ Promoter Selection Plate
- Dharmacon™ Positive and Negative RNAi Controls
- Dharmacon™ Inducible Lentiviral 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).

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.

GE Healthcare Dharmacon, Inc.
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@ge.com (North America) or TS.Dharmacon.eu@ge.com (Europe)

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 (http://oba.od.nih.gov/oba/rac/Guidance/LentiVirus_Containment/pdf/Lenti)
2. Containment_Guidance.pdf);
3. 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);
4. The NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines), Oct 2011 (http://oba.od.nih.gov/rdna/nih_guidelines_oba.html).

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).

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BENITEC TECHNOLOGY LICENSED PATENTS

Name Inventor	Title	Patent No.	Application No.
Graham/Rice	Control of Gene Express	ZA 2000/4507* AU2001100608* SG75542* US 6,573,099* GB2353282* AU 743316* NZ 506648* US 10/346853* PCT/AU/99/00195* BR PI9908967-0* CA 2323726* CB 99804255-2* CZ PB2000-3346* EP 99910039.9* US10/646,070 US 10/759,841* EP 04015041.9* AU2005211538	HK 01105904.3* HU PO101225* IN 2000/00 169/DEL* JP P2000-537990 KR 7010419/2000* MX008631* PL P.343064* SK PV 1372-2000* AU 35647/02* NZ 525941* SG200205122-5* US 09/646807* PP2492/98* AUPP2499/98* US 10/821,710* US 10/821,726* AU 2005209648
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Grahm, et al.	Double-Stranded Nucleic Acid	AU 2003906281 AU 2003906281 US 10/861,191	AU 2004902279 PCT/AU04/000759

*Indicates a "Co-Owned Patents" are jointly owned by Benitec and CSIRO.

OXFORD TECHNOLOGY

PCT Application (filing date)	Priority Application(s) (filing date)	National Application	Issued Patents
	US 07/586,603 (21 Sept 1990)	US 08/361,839	5,817,491
PCT/US91/05699 (9 Aug 1991)	US 07/658,632 (19 Feb 1991)	EP 91915104.3 JP 3-514518 AU 84302/91 CA 2,104,396	0572401 3547129 663470 2,104,396
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V2-0417