Technical Manual

Thermo Scientific SMARTchoice Lentiviral shRNA

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I. Thermo Scientific SMARTchoice Lentiviral shRNA

A. Maximizing successful gene silencing

The Thermo Scientific SMARTchoice Lentiviral shRNA platform is an innovative set of tools and experimental workflows for the application of rationally designed lentiviral shRNA vectors. The purpose behind the SMARTchoice™ concept is to provide the researcher with the most effective tools for delivering and expressing genetic content in their cells of interest. The SMARTchoice platform extends the advanced design of Thermo Scientific SMARTvector 2.0 Lentiviral shRNA and broadens the range of cells to which RNA interference (RNAi) can be applied. The SMARTchoice workflow incorporates a unique tool that allows the researcher to first evaluate and then choose in a modular fashion the vector configuration that is most effective for shRNA expression in the particular cells of interest. Poor shRNA performance can be traced to poor expression, which often is directly attributable to low transcriptional activity of the promoter regulating shRNA expression. The ability to assess promoter activity in the specific cells of interest prior to conducting a gene silencing experiment is a novel, cost-effective strategy for vector-based RNAi. SMARTchoice provides a level of assurance for successful knockdown not currently accessible with other commercially available systems.

This technical manual describes the experimental workflow for successful gene silencing in your cells of interest, including selection of the appropriate promoter.

A critical component of the SMARTchoice technology paradigm is the Thermo Scientific SMARTchoice shRNA Promoter Selection Plate, a 96-well plate containing high-titer, transduction-ready lentiviral particles derived from seven unique lentiviral shRNA constructs each containing a different transcriptional promoter. SMARTvector™ lentiviral particles are normalized with respect to functional viral titer, and dilutions of the particles are arrayed across the plate in a format conducive to testing promoter activity with one simple experiment. A TurboGFP (Evrogen, Moscow, Russia) fluorescent reporter expressed by each of the vectors is utilized to facilitate side-by-side comparisons of promoter activity in a particular cell line.

Once it is determined by the researcher which SMARTchoice promoter is most active in the cells of interest, adapted Thermo Scientific SMARTvector 2.0 shRNA vectors are ordered, constructed with the promoter and reporter of choice (TurboGFP or TurboRFP) and delivered as high-titer lentiviral particles. This semi-customizable approach to vector design provides greater flexibility and more importantly, improved likelihood of experimental success.

B. RNA Interference: Silencing genes to better understand biology

RNAi is an endogenous, post-transcriptional gene silencing pathway that uses small, non-coding RNAs to target specific mRNA transcripts for degradation. Target gene knockdown by RNAi can be mediated by synthetic molecules, such as short interfering RNAs (siRNAs), or induced by short hairpin RNA (shRNA) expression constructs introduced into cells by transfection with plasmids or viral transduction (Figure 1).

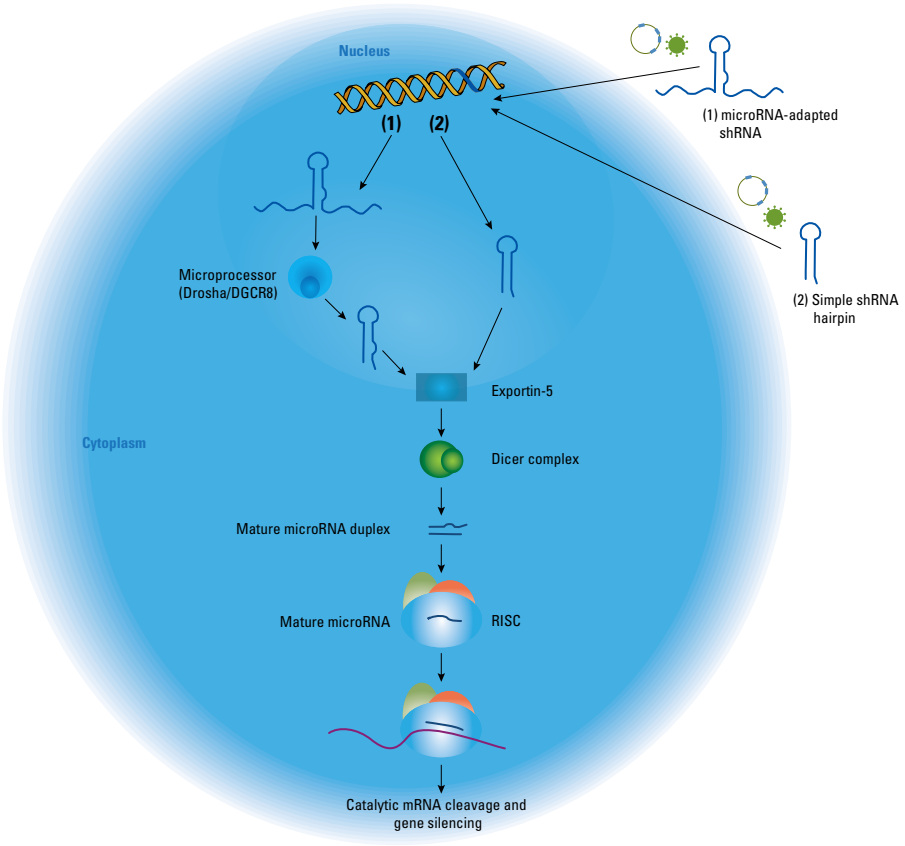


Figure 1. Borrowing the endogenous microRNA pathway to induce RNAi. Vector-based RNAi approaches include the introduction of genetically engineered viral vectors or plasmid-based vectors expressing silencing sequences embedded in an endogenous microRNA scaffold (1) or simple stem-loop shRNA (2). Expressed sequences (1 and 2, shown in blue) enter the endogenous pathway at an early stage and are efficiently processed into potent silencing molecules using the endogenous microRNA mechanism. These approaches lead to target mRNA (shown in purple) cleavage and gene silencing.

Viral vector-based gene silencing occupies a unique niche as a tool for delivering genetic information into a cell. In cases where the cell of interest is refractory to transfection by lipid-mediated methods or when long-term gene silencing is needed to assess experimental outcome, delivery of silencing reagents using viral vectors serves as the method of choice. To that end, there are several viral delivery systems developed using adenoviral, retroviral and lentiviral platforms to deliver shRNA silencing constructs, each of which has its own set of advantages. Pseudotyped lentiviral vectors have proven to be among the most versatile delivery systems by providing 1) delivery of large genomic payloads, 2) broad tropism and 3) long-term transgene expression by integration of the viral payload into the host genome. In addition, lentiviral vectors mediate sustained, long-term expression in both dividing and non-dividing cells, *in vitro* and *in vivo*. The SMARTvector 2.0 vector was developed in order to exploit these well-characterized properties associated with stability and broad tropism, as well as the high titers that are achievable with lentiviral vector-based technologies.

The general process by which a lentivirus transduces a cell is illustrated in Figure 2. (1) Upon binding the cell, the viral genome is delivered into the cytoplasm and (2) is reverse transcribed from RNA to DNA. (3) The DNA intermediate is imported into the host cell nucleus where (4) it is stably integrated into the host genome. The silencing construct is then constitutively expressed and (5) processed into shRNAs that enter the RNAi pathway to affect target gene knockdown. With each cellular division, the integrated virus is replicated and passed on to the daughter cells, thus ensuring continued expression of the targeting sequence throughout the population. Other viral transduction systems, such as retroviruses, integrate into the host genome of dividing cells; however, lentiviruses distinguish themselves by their ability to integrate into the genomes of non-dividing cells. Thus, lentiviral platforms are well-suited for targeting genes in cells derived from primary tissues, stem cells and neuronal and hematopoietic lineages.

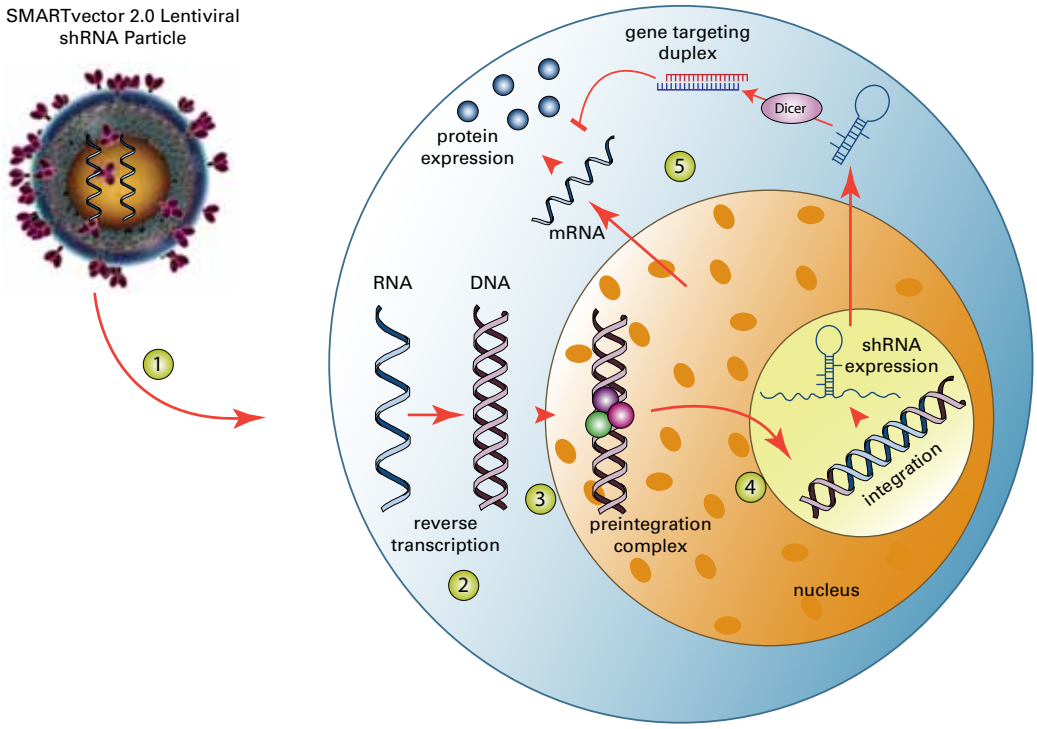


Figure 2. The SMARTvector 2.0 system utilizes lentiviral vectors to stably deliver and express gene silencing triggers capable of entering the RNAi pathway. (See above text for details.)

C. Innovative lentiviral vector design

The SMARTvector 2.0 Lentiviral shRNA vector design (Figure 3) takes advantage of the ability to eliminate over 70% of the lentiviral genome without compromising essential elements required for efficient transduction and stable integration. With deletion of the nonessential genome, the SMARTvector 2.0 vector backbone utilizes the available genomic "cargo space" by including the following features to make viral-mediated gene silencing efficient, safe and easy:

1. *Optimized microRNA scaffold:* microRNAs are the endogenous substrates of the RNAi pathway. Transcribed in the nucleus as stem-loop structures containing imperfect base-pairing in the stem, these non-coding RNAs are processed by multiple elements of the RNAi pathway to generate short ~19-23 base-pair duplexes capable of associating with the RNA-induced Silencing Complex (RISC) to target genes for silencing. Thermo Fisher Scientific scientists screened a panel of human microRNAs and selected an efficiently processed microRNA scaffold for the SMARTvector 2.0 design. Targeting sequences embedded in this microRNA scaffold are expressed under control of a constitutive promoter and are readily incorporated into the host cell's RNAi machinery.

2. *Rationally designed shRNA targeting sequences:* Studies have shown that potent synthetic siRNAs do not typically provide efficient silencing when expressed from a plasmid. Targeting sequences used in SMARTvector shRNA constructs are designed using a proprietary, microRNA scaffold-specific, rational design algorithm for DNA-based RNAi. The SMARTvector algorithm selects target sequences on the basis of numerous variables such as position-dependent nucleotide preferences, secondary structure and thermodynamic stability profiles, and includes seed-based filters to minimize potential off-target effects.

3. *TurboGFP or TurboRFP reporter genes:* The SMARTchoice platform allows SMARTvector shRNA constructs to include either a TurboGFP or TurboRFP (Evrogen, Moscow, Russia) reporter gene to facilitate assessment and optimization of experimental transduction conditions. This allows for visual tracking of shRNA expression.

4. *Mammalian drug-selection marker:* The puromycin resistance (Puro^R) gene is a feature of all SMARTvector 2.0 constructs and allows for selection and isolation of clonal populations when generating stable cell lines.

5. *RNA polymerase II promoter:* An RNA polymerase II promoter drives expression of the fluorescent reporter, puromycin resistance gene and shRNA.

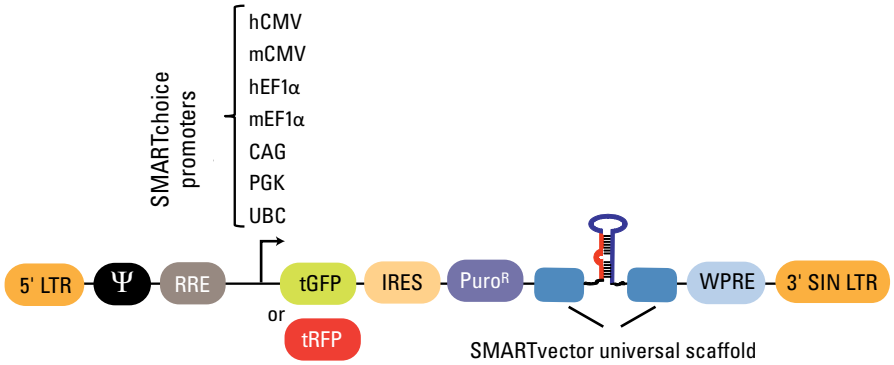


Figure 3. Elements of the SMARTvector 2.0 Lentiviral shRNA vector.

Vector Element	Utility
5' LTR	5' Long Terminal Repeat
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
IRES	Internal Ribosomal Entry Site allows expression of TurboGFP 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
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

D. Importance of promoter activity for lentiviral RNAi

To fully harness the utility of lentiviral vector approaches in RNAi-mediated gene silencing, careful attention must be paid to not only the design of the shRNA, but also the design of the vector used to deliver and express the shRNA. Cells vary greatly in their efficiency of lentiviral vector uptake, integration and subsequent transgene or shRNA expression. Often the design of the vector has a significant impact on the experimental outcome as well as the reliability of the results. This is particularly true with regard to the promoter selected to drive shRNA expression. 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 4 demonstrate how promoter performance varies significantly across different cell lines which, in turn, influences the potency of gene silencing. In these experiments, SMARTvector 2.0 Non-targeting Control constructs utilizing different SMARTchoice promoters were packaged into lentiviral particles and transduced into several human, mouse and rat cell lines (Figure 4). In some instances, promoter activity correlates with the species from which it is derived, as seen in the human OVCAR-8 and HEK293T cell lines where the human CMV and human EF1α promoters were the most transcriptionally active. Similarly, the mouse CMV and mouse EF1α promoters are most active in the mouse-derived NIH/3T3 cell line. However, promoter activity does not always follow a species-specific expression pattern. The mouse EF1α and mouse CMV promoters are the most active in the human Jurkat and human A549 cell lines, respectively. Additionally, both human and mouse CMV promoters were active in rat PC-12 cells. Thus, choosing the most effective promoter in a particular cell line is not always predictable, and therefore should be determined empirically.

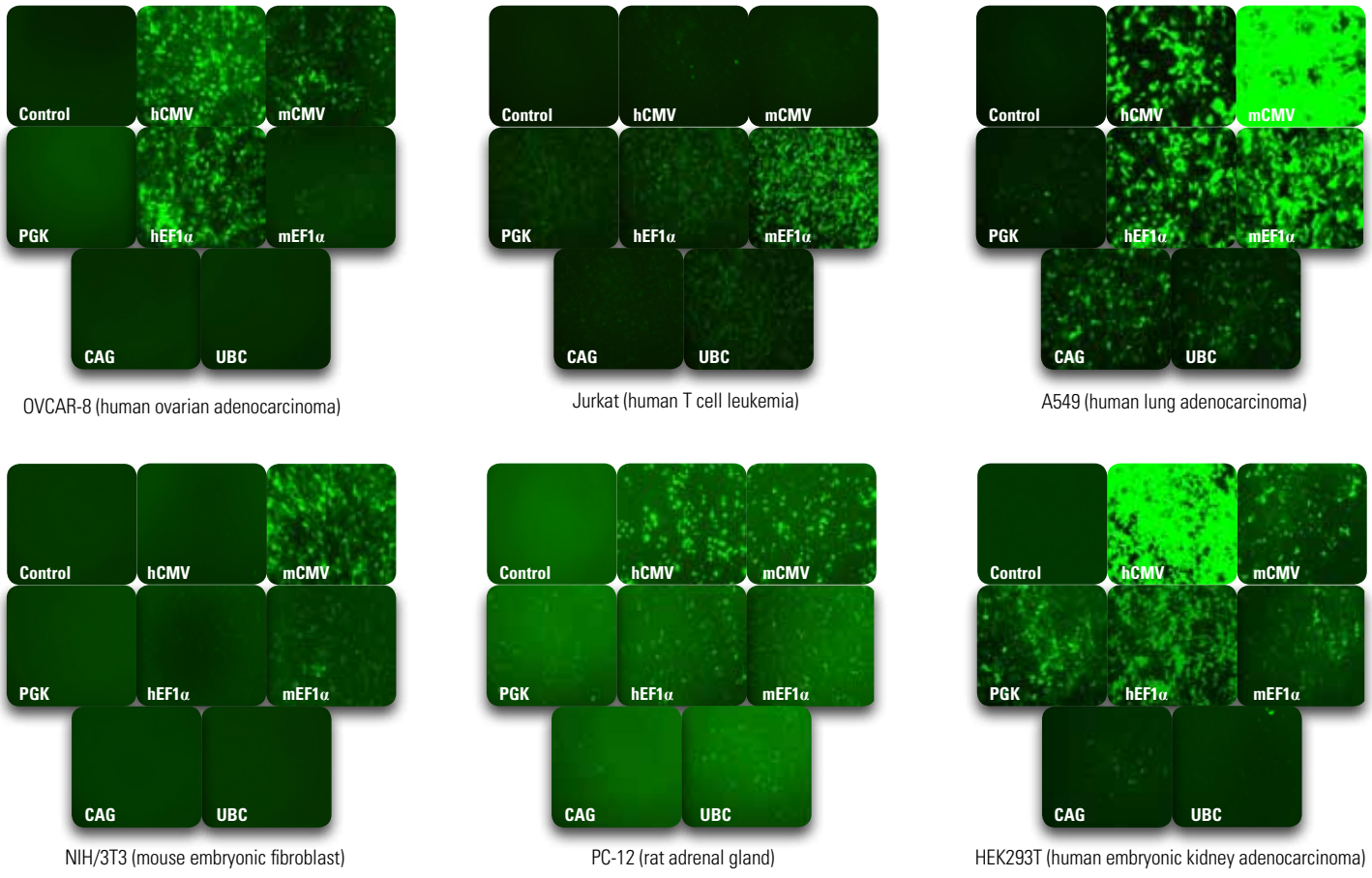
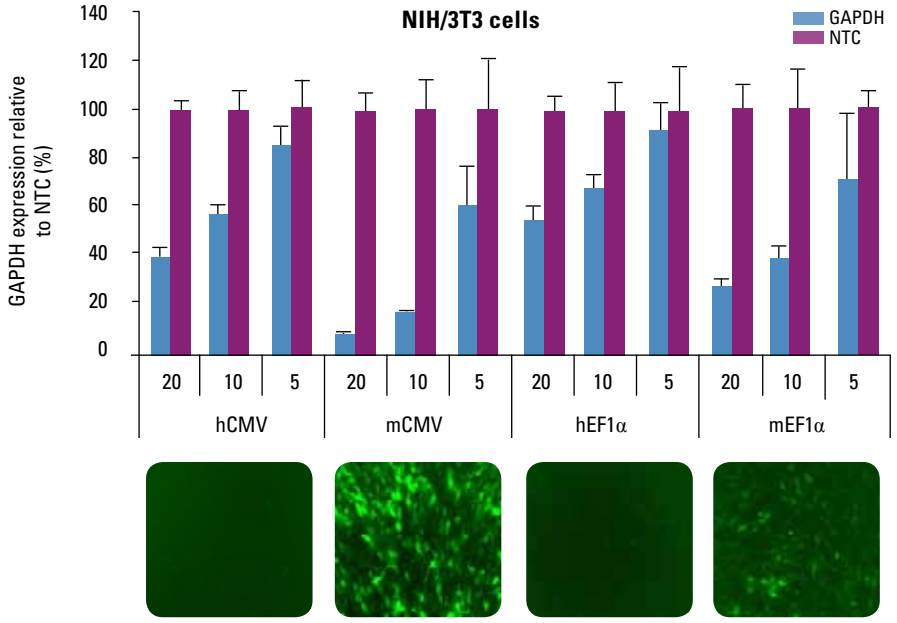


Figure 4. Promoter activity varies across several human and rodent cell lines. Cells were plated at a density of 50,000 cells per well in a 24-well plate and transduced at MOI = 15 with SMARTvector 2.0 Empty Vector Control Particles expressing the TurboGFP reporter. Promoter activity was assessed at 72 hours post-transduction by the fluorescence intensity of TurboGFP.

As demonstrated in Figure 5, promoter activity, and consequently the level of shRNA expression, has a significant impact on the level of gene silencing. Using various promoters to express an shRNA targeting endogenous GAPDH mRNA transcripts, the promoter with the highest level of activity as assessed by TurboGFP fluorescence subsequently produced the greatest level of gene silencing.

Another key advantage of identifying the optimal promoter using the SMARTchoice shRNA Promoter Selection Plate is the ability to test a range of low to high multiplicities of infection (MOIs) which is useful to identify the most effective MOIs with minimal disruption to cellular viability.

A.



B.

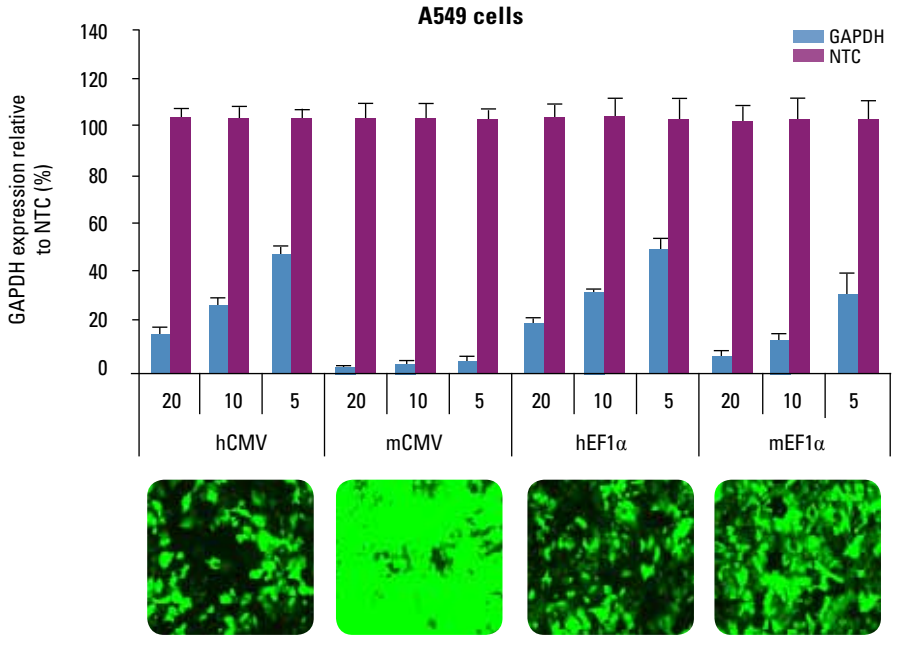


Figure 5. Higher levels of promoter activity correlate with increased gene silencing. A. For assessing promoter activity, cells were plated at a density of 50,000 cells per well in 24-well format and transduced at MOI = 15 with SMARTvector 2.0 Empty Vector Control Particles expressing the TurboGFP reporter. Promoter activity was assessed at 72 hours post-transduction by the fluorescence intensity of TurboGFP. B. For evaluation of gene silencing, cells were plated at 5,000 (NIH/3T3) and 7,000 (A549) cells per well in 96-well format and transduced at MOI = 20, 10 or 5 for NIH/3T3 cells and MOI = 10, 5 or 2.5 for A549 cells. Gene silencing was assessed at 72 hours post-transduction using Thermo Scientific Solaris qPCR Gene Expression Master Mix and Assays amplifying GAPDH (target gene) and RPS18 (reference gene) according to manufacturer's protocols.

Section II. Thermo Scientific SMARTchoice shRNA Promoter Selection Plate

A. Product description

The SMARTchoice shRNA Promoter Selection Plate is designed to allow data-driven promoter selection prior to ordering SMARTvector 2.0 Lentiviral shRNAs targeting specific genes of interest. This unique tool permits the simultaneous evaluation of several 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 and arrayed in a 96-well plate (Figure 6).

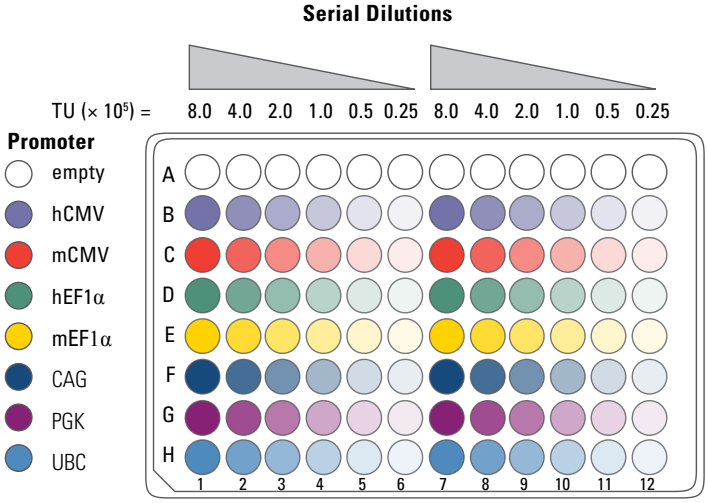


Figure 6. SMARTchoice shRNA Promoter Selection Plate enables straightforward, qualitative assessment of promoters that actively drive expression. Viral particles are arrayed in two-fold dilutions with the highest amount of particles at 8.0×10^5 transducing units (TU). Layout by rows: A. empty wells (DMEM only); B. hCMV – human cytomegalovirus intermediate early promoter; C. mCMV – mouse cytomegalovirus intermediate 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 2.0 lentiviral particles co-express TurboGFP, a puromycin resistance selectable marker and Non-targeting Control (NTC) shRNA in the context of the SMARTvector microRNA-adapted scaffold. The seven promoters represented on the shRNA Promoter Selection Plate are as follows:

hCMV – human cytomegalovirus intermediate early promoter
mCMV – mouse cytomegalovirus intermediate 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 viral 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 non-transducing control. Columns 1 and 7 have the highest number of viral particles [corresponding to 8×10^5 functional 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 viral 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 using a p24 ELISA assay. A description of the titering method can be found in the Appendix along with an explanation of how p24 titers are converted to a functional titer.

B. Recommended workflow

Critical factors contributing to successful gene silencing using lentiviral vector-based RNAi include efficient delivery of the vector to the cell combined with robust and stable expression of the shRNA itself. The SMARTchoice shRNA Promoter Selection Plate allows the researcher to evaluate the strength of various constitutive promoters and consequently the expression level of the shRNA in the cell line or cell type of interest. It is strongly recommended that the optimal promoter is determined prior to ordering tailored SMARTvector 2.0 Lentiviral shRNA Particles containing the promoter and reporter of choice.

The workflow for selecting and ordering SMARTvector shRNA viral particles with the optimal vector configuration is shown in Figure 7. (1) The SMARTchoice shRNA Promoter Selection Plate (#VSC6048) is ordered from Thermo Fisher Scientific. (2) Arrayed lentiviral particles are used to transduce the cells of interest under the appropriate experimental conditions. (3) Promoter strength is assessed by TurboGFP expression driven from each of the unique promoters, with the optimal promoter producing the highest level of fluorescence intensity. (4) Once the most active promoter option is qualitatively determined for the specific cells of interest, order the gene-specific SMARTvector 2.0 Lentiviral shRNA constructs and controls incorporating the optimal promoter and fluorescent reporter (TurboGFP or TurboRFP) of choice. (5) Thermo Fisher Scientific produces high-titer lentiviral particles (6) that are then delivered to the researcher as a transduction-ready reagent for conducting gene silencing experiments.

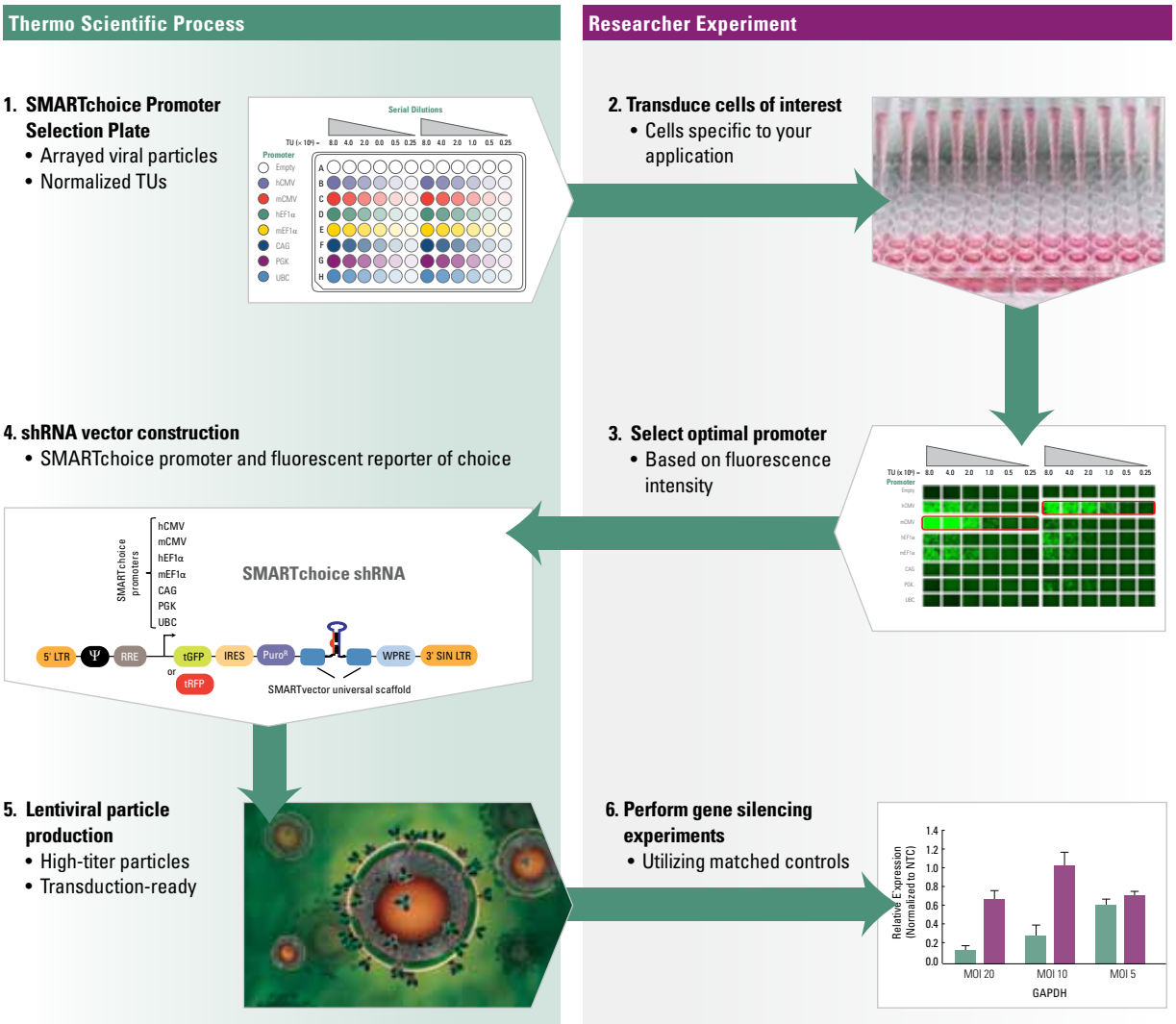


Figure 7. Recommended workflow for determining the optimal vector configuration and ordering customized SMARTvector 2.0 Lentiviral shRNA Particles.

Section III. Protocols for optimization of important cellular conditions prior to transductions

A. Product considerations

i. Safety precautions

The below 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 SMARTchoice shRNA Promoter Selection Plate 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® 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. A biohazardous waste bag should also be placed within the biological safety cabinet.

ii. Materials not supplied

- Polybrene (American Bioanalytical #AB01643)
  Note: The use of Polybrene may improve transduction efficiency of lentiviral particles in some cell types.
- 96-well tissue culture plates (Thermo Scientific #12-565-66)
- Deep-well 96-well plate (Nunc #12-565-553)
- Finnpipette Multichannel (8-channel) Pipette (Thermo Scientific #14-386-349, 14-386-350 or #14-386-352)
- Cells of interest:
  Note: 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.
- Dulbecco's Modified Eagle Medium (DMEM) High Glucose without L-Glut or Sodium Pyruvate (Thermo Scientific #SH3008 IFS)
- Preferred Culture Media: the cell culture media (including serum or supplements) recommended for maintenance and passaging of the cells of interest.
- Base Media: the base media (without serum or supplements) that is used to prepare the Preferred Culture Media.
- Cell viability assay
- Transduction Media: Base Media with or without serum and containing the appropriate concentration of Polybrene as determined to be optimal transduction conditions for cells of interest.
- Foil seals (Thermo Scientific #AB-0745)
- Lysol or bleach

B. Determining cell density and transduction conditions

Prior to performing any transductions with lentiviral particles, it is important to determine what conditions can be tolerated by your particular cells of interest. For example, transducing cells in serum-free media with added Polybrene has been shown to enhance lentiviral transduction efficiency. However, both low serum levels and Polybrene can be toxic to certain cells. Furthermore, determining the best range of cell density at the time of transduction is also important to consider for successful transduction. Finally, each of the viral particles in the Promoter Selection Plate is suspended in DMEM, which may not be the Base Media of the Preferred Culture Media for the cells of interest. The following optimization protocol allows you to determine the culture conditions amenable for transduction by lentiviral particles. The protocol is designed to test multiple conditions in one experiment in a 96-well plate format as depicted below (Figure 8). This recommended protocol will test cell density, Polybrene concentration, the presence or absence of serum and the duration of transduction (6 hours or overnight).

Please note that lentiviral particles are NOT utilized during these steps. The goal of these steps is to determine the most suitable transduction conditions for the cells of interest. Protocols are provided for testing conditions in both adherent and suspension cells prior to any transduction events.

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

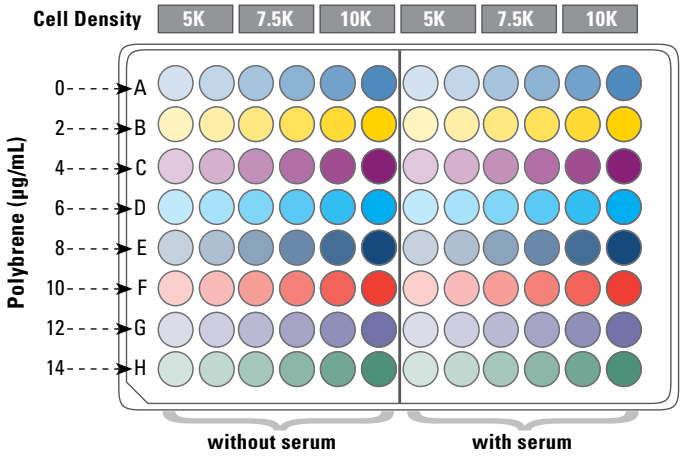


Figure 8. Example of 96-well plate layout for the optimization of transduction conditions. This plate layout tests three cell densities [5,000 (5K), 7,500 (7.5K) and 10,000 (10K) cells per well), seven concentrations of Polybrene (0 - 14 µg/mL) and media with or without serum.

Day 1:

1. Seed cells into two 96-well culture plates at the appropriate cell density following the template depicted in Figure 8. Seed cells in a total volume of 100 µL of the Preferred Culture Media and place 96-well culture plates into incubator for overnight culture under the appropriate conditions (temperature and CO_2 concentration).

   Generally, transductions with lentiviral particles should be performed when cells are approximately 40% confluent. This provides cells the space to replicate in culture and express the TurboGFP marker that is delivered on the vector. 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 8 (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. Preparation of Transduction Media

   Prepare two sets of Transduction Media: one without serum and one with serum

   Transduction Media without serum

   - Prepare 10 mL of Transduction Media containing no serum by pipetting a 1:1 mixture of DMEM to Base Media. *Note that Thermo Fisher Scientific provides lentiviral particles in DMEM. Thus, testing a recipe for suitable transduction media 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 Media formulations containing eight different Polybrene concentrations ranging from 0-14 µg/mL, increasing concentration in 2 µg/mL increments (Figure 9).

   Transduction Media with serum

   - Prepare a similar series of wells with Transduction Media consisting of a 1:1 mixture of DMEM to Base Media and containing serum at the concentration that is appropriate for the cells of interest. For example, if the Preferred Culture Media contains 10% serum, then the final concentration of the Transduction Media should also be 10%.

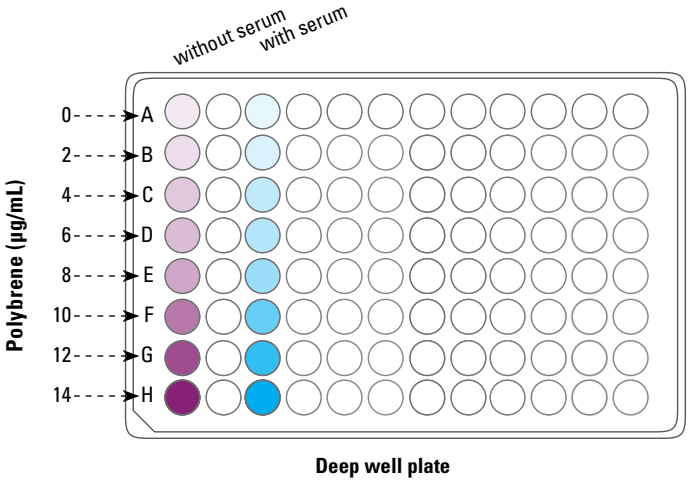


Figure 9. Example of plate layout for preparation of Transduction Media. Transduction Media with (blue wells) or without (purple wells) serum containing a range of Polybrene concentrations in a deep-well, 96-well plate. White wells are empty.

3. Incubation of cells with Transduction Media.
   Two sets of incubations will be performed using each of the Transduction Media 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 Media (with or without serum) containing the range of Polybrene concentrations (0-14 µg/mL) to cells seeded in 96-well culture plate. Transfer Transduction Media in format depicted in Figure 8.
   d. Return culture plates to incubator and culture under the appropriate conditions.
   e. After 6 hours, add 100 µL of Preferred Culture Media directly to each well of plate 1 and return to incubator for overnight culturing.

   At this time, do not remove the Transduction Media 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 media to the 50 µL of Transduction Media in the well will result in a final serum concentration of 10%.

Day 3:

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

Day 4:

1. 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:

1. Examine cultures for cell morphology or presence of phenotypic changes. Cell viability should be determined using any commercially available kit, such as alamarBlue® (TREK Diagnostic Systems) or CellTiter-Blue™ (Promega Corporation).

Select the cell density that had ~ 40% confluency on Day 2. If the cells of interest tolerate serum-free conditions then do not include serum during transduction. Also, choose 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 combined with the highest concentration of Polybrene tolerated by the cells of interest have resulted in the highest transduction efficiencies.

Furthermore, if there is no evidence of significant cellular toxicity with an overnight incubation (16-20 hours), subsequent experiments to identify the preferred promoter should use this incubation time.

Notes:

i. If Polybrene is toxic to the cells, DEAE-Dextran (1-10 µg/mL) may be substituted.

ii. 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 8.

iii. Since the Promoter Selection Plate provides duplicate dilution series, two different incubation conditions could be tested with the Promoter Selection Plate (for example: no serum with and without Polybrene).

iv. The Promoter Selection Plate should be utilized with the optimized conditions identified; further optimization should be performed after the selection of the most active promoter during MOI optimization experiments (step 6 of Figure 6).

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 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 media. Tap bottom of centrifuge tube gently to dislodge cell pellets. Resuspend cells in one centrifuge tube with Base Media containing no serum. The other cell pellet is resuspended in Base Media 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.

   Use your experience with the cells of interest as a guide for the seeding concentration of cells per well. Typically, for suspension cell lines the ideal number of cells per well is in the 10,000-40,000 range and 50,000-200,000 for primary immune cells.

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

   Transduction Media for Suspension Cells
   Prepare 10 mL of Transduction Media without serum by pipetting a 1:1 mixture of DMEM to Base Media. 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 Media 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 4). Polybrene concentration is increased to account for the additional 25 µL of Base Media, which does not already contain Polybrene. For example, well 2 contains 3 µg/mL Polybrene; adding 50 µL of Transduction Media with a Polybrene concentration of 3 µg/mL to 25 µL of Base Media containing no Polybrene will result in a final concentration of 2 µg/mL. Use Table 1 to prepare the eight Transduction Media formulations:

   Two sets of eight wells of Transduction Media should be prepared as before (Figure 9), one set without serum and one with serum.

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

	Polybrene concentration of Transduction Media	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. Incubation of cells with Transduction Media.

   Two sets of incubations will be performed using each Transduction Media formulation (with and without serum): one plate for 6 hour incubation (plate 1) and other plate for 16-20 hour overnight incubation (plate 2).

   Using a multichannel pipette, transfer 50 µL of Transduction Media 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 Media directly to each well in plate 1 (Figure 8) 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 media to the 75 µL of Transduction Media in the well will result in a final serum concentration of 10%.

Day 2:

1. After overnight incubation with Transduction Media (16-20 hours), add 75 µL of Preferred Culture Media directly to each well in plate 2 (Figure 8). Adjust serum concentration as described above to wells containing no serum.

Day 3:

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

Day 5:

1. Cell viability should be determined using any commercially available kit, such as alamarBlue® or CellTiter-Blue.

Select the cell density that had ~ 40% confluency on Day 2. If the cells of interest tolerate serum-free conditions then do not include serum during transduction. Also, choose 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 combined with the highest concentration of Polybrene tolerated by the cells of interest have resulted in the highest transduction efficiencies.

Furthermore, if there is no evidence of significant cellular toxicity with overnight incubation (16-20 hours), subsequent experiments to identify the preferred promoter should use this incubation time.

Notes:

i. If Polybrene is toxic to the cells, DEAE-Dextran (1-10 µg/mL) may be substituted.

ii. 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 8.

iii. Since the Promoter Selection Plate provides duplicate dilution series, two different incubation conditions could be tested with the promoter selection plate (for example: no serum with and without Polybrene).

Section IV. Using the SMARTchoice shRNA Promoter Selection Plate to identify active promoters

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 in the above experiments. The cell culture media to be used in the following protocols with be referred to as the Transduction Media. The specific formulation for the Transduction Media will reflect the optimal Poybrene and serum concentrations previously determined for the cells being transduced. Protocols for transduction of both adherent and suspension cells are included. Please note that 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.

A. SMARTchoice Promoter Selection Plate protocol for adherent cells

Day 1:

1. Seed appropriate number of cells in an appropriate cell culture plate as determined above in a total volume of 100 µL per well in Preferred Culture Media. 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 viral particles on ice. It may take > 30 minutes to thaw viral particles in all wells. After viral particles have thawed, spray the sealed plate with 70% ethanol and carefully wipe excess with a paper towel or Kimwipes®.
   b. Quick-spin viral particles to bottom of the wells by centrifuging (rcf = 300) for 20-40 seconds in a table top centrifuge. Remove plate from centrifuge. Clean Promoter Selection Plate and 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 viral 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 Base Media with Polybrene and serum (at optimal concentrations determined previously in the optimization of general conditions, Section III) to viral 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 Media at a 2X concentration determined during optimization; this will result in 1X Polybrene and serum concentrations to generate 1X Transduction Media with virus.
   b. To each well of the SMARTchoice Promoter Selection Plate, pipette 30 µL of Base Media with 2X Polybrene and serum (as described in 1.2.1) creating ~55 µL total volume per well of 1X Transduction Media with virus. 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 SMARTchoice shRNA lentiviral particles.
   a. Take culture plate from incubator and place in biological safety cabinet. Using an 8-channel pipette, carefully aspirate Preferred Culture Medium from each column of culture plate being careful not to dislodge cells from bottom of well. Dispense Preferred Culture 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 Media with virus 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 Media with virus one column at a time. Tips should be changed following each transfer and deactivated in liquid waste reservoir before disposal. After all columns (dilutions) of Transduction Media with virus are transferred to corresponding wells, place culture plate in incubator and culture under appropriate conditions.
   c. Allow transduction to proceed for 6 hours or overnight as determined previously in the optimization of general conditions, Section III. After the transduction period, add 100 µL of Preferred Culture Medium directly to each well. Adjust serum concentration accordingly.

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

Days 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 between wells (see Figure 10 for an example). Figure 10 depicts the fluorescent images of human A549 and HEK293T cells transduced with viral particles from the SMARTchoice shRNA Promoter Selection Plate.

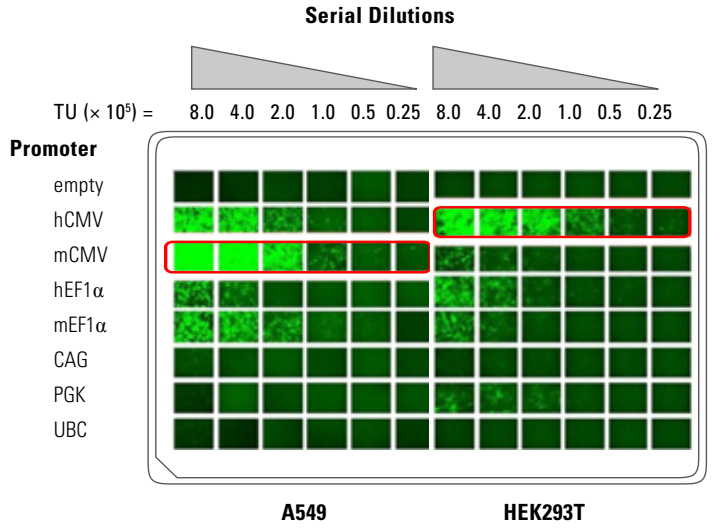


Figure 10. Fluorescense microscopy images of TurboGFP-positive A549 and HEK293T cells for promoter evaluation. Cells were transduced with viral particles from the SMARTchoice shRNA Promoter Selection Plate. Fluorescence intensity demonstrates that the mouse CMV (mCMV) promoter is the optimal promoter in A549 cells, whereas the human CMV (hCMV) promoter is the optimal promoter in HEK293T cells.

For human A549 cells, the mouse CMV promoter consistently ranks highest in fluorescence intensity and therefore would be the optimal promoter choice for delivery and expression these cells. Alternatively, the human CMV promoter consistently ranks highest in HEK293T cells, providing further justification for testing multiple promoters in the specific cells of interest. 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 viral concentrations.

B. SMARTchoice Promoter Selection Plate protocol for suspension cells

Day 1:

1. Using ideal cell concentration determined during optimization experiment, 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 Media, then plate 25 µL cells in Base Media 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 Media; 25 µL would then correspond to 10,000 cells per well.

2. Prepare SMARTchoice shRNA Promoter Selection Plate for transduction in the same manner as described above for adherent cells. Note: Use Polybrene and serum concentration such that the final concentration upon adding 50 µL of Transduction Media with virus 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 Media with virus from each well of Promoter Selection Plate to corresponding wells of culture plate.

   Note: 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 viral particles. This must be done in a Class II/Type A2 biological safety cabinet. 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 and deactivate tips in the liquid waste reservoir before disposing.

4. Allow transduction to proceed for 6 hours or overnight as determined in optimization experiment. After transduction period, add 75 µL of Preferred Culture Medium directly to each well. Adjust serum concentration accordingly.

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

Days 3-5:

Visually inspect cells daily using fluorescence microscopy as described above in Section I, Part A, Days 4-6.

Section V. Ordering SMARTvector 2.0 Lentiviral shRNA Particles with optimal promoter and reporter choices

A. Placing an order

The intent of the SMARTchoice shRNA Promoter Selection Plate is to determine empirically the promoter that is optimal for shRNA expression in the cells of interest. Following assessment of promoter activity, SMARTvector 2.0 Lentiviral shRNA constructs for genes of interest can be ordered at www.thermoscientific.com/SMARTchoice with one of seven well-characterized promoters and either TurboGFP or TurboRFP as the fluorescent reporter (Figure 3). Choose to order an individual SMARTvector construct or Set of 3 constructs at the 100 µL or 200 µL volumes of 1×10^8 TU/mL lentiviral particles. Don't forget to select matching positive and negative controls to complete your experiment. It is imperative to select control particles with the same promoter and reporter options selected for the shRNA targeting the gene of interest.

B. Recommended experimental controls

Negative and positive controls should always be included with any RNAi experiment. Therefore, it is recommended that SMARTvector 2.0 Lentiviral shRNA Negative and Positive Control Particles be ordered in addition to SMARTvector lentiviral particles targeting the gene of interest. Matched controls are available with options for all seven promoters and two fluorescent reporters for Non-targeting, human GAPD, mouse GAPD and rat GAPD control contructs.

SMARTvector 2.0 Non-targeting shRNA Control Particles
SMARTchoice negative controls for RNAi experiments, provided as packaged lentiviral particles ≥ 1×10^8 TU/mL, are designed so that no known gene in human, mouse or rat will be targeted. Negative controls can also be used to assess transduction efficiency and the effects of transduction on the cells of interest. SMARTvector 2.0 Non-targeting Control Particles are available with all seven SMARTchoice promoters and both fluorescent reporters so that RNAi experiments can be performed utilizing the appropriate controls regardless of the promoter chosen for gene-specific constructs.

SMARTvector 2.0 GAPD Control Particles
SMARTchoice GAPD positive control, provided as packaged lentiviral particles at ≥ 1×10^8 TU/mL, is validated to silence glyceraldehyde-3-phosphate dehydrogenase (GAPD, also known as GAPDH). Individual positive control particles targeting GAPD in human, mouse and rat models are available. SMARTvector 2.0 GAPD Control Particles allow assessment of gene silencing in the specific cells of interest. Positive control particles are available with each of the seven SMARTchoice promoters and both fluorescent reporters, as well as individual shRNAs targeting human, mouse and rat GAPD.

Section VI. Gene silencing protocols using SMARTchoice Lentiviral shRNA with optimal promoter and reporter selections

A. Recommendations for experimental layout

Gene-specific Thermo Scientific SMARTvector 2.0 Lentiviral shRNA designs are available as individual constructs or sets of 3 supplied as concentrated lentiviral particles. A well-constructed of gene silencing experiment should include at least 3 shRNA designs targeting the gene of interest plus matched negative and positive shRNA controls, performed as biological triplicates. In addition, mock-transduced replicates should always be included. Figure 11 depicts a suggested 96-well plate layout for a well-designed gene silencing experiment using SMARTvector shRNA lentiviral particles. Optimal cell density, serum concentration, Polybrene concentration and transduction time must determined for the cells of interest prior to any transduction. If the SMARTchoice Promoter Selection Plate was used and the protocols followed, these optimized conditions will have already been determined (see Section III).

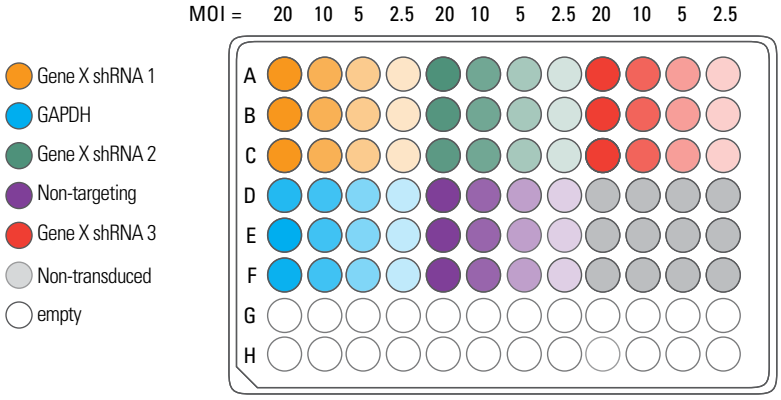


Figure 11. Example of plate layout for typical gene silencing experiment using SMARTvector 2.0 Lentiviral shRNA Particles. Suggested 96-well plate format using three different shRNA constructs (shRNA 1, shRNA 2 and shRNA 3) at four MOI (20, 10, 5 and 2.5 TU/cell) targeting Gene X. Included are positive (GAPDH) and negative (non-targeting) controls and non-transduced cells. White wells are empty.

B. General transduction and gene silencing protocols

Follow the protocol described below for transducing cells with SMARTvector 2.0 Lentiviral shRNA Particles. Utilize the optimized conditions identified in the above experiments to transduce your cells with each of the shRNA vectors targeting the gene(s) of interest along with the appropriate controls. If the SMARTchoice shRNA Promoter Selection Plate was not used and the standard human CMV with TurboGFP configuration is appropriate for your cells, please download the SMARTvector Technical Manual and refer to the protocols published for SMARTvector 2.0 Lentiviral shRNA Particles (www.thermoscientific.com/SMARTvector) to determine optimal conditions for transduction.

C. Transduction of cells with SMARTchoice Lentiviral shRNA

Set up transductions using the optimal cell density, Polybrene concentration, serum concentration and duration as previously determined. Information as to the range of MOIs that should be used to transduce the cells can be obtained from the results of the SMARTchoice Promoter Selection Plate transductions. For example, analysis of the promoter selection results for A549 cells shown in Figure 10 suggests a range of MOIs of 5, 10, 20, and 40 (corresponding to 0.5, 1.0, 2.0 and 4.0 TU/cell, respectively). Taking these optimized transduction conditions into consideration, the following procedures can be followed:

Day 1:

1. Plate cells in biological triplicate in a 96-well culture plate at optimal cell density in appropriate growth medium as determined in previous experiments (see Section III. Protocols for optimization of important cellular conditions in previous experiments (see Section III. Protocols for optimization of important cellular conditions prior to transductions). If working with suspension cell lines, plate cells the day of transduction (see protocol below for recommendations).

   Note: 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.

Day 2:

1. Remove your SMARTchoice Lentiviral shRNA provided as lentiviral particles from -80° C freezer and thaw on ice.

2. Prepare 10 mL of pre-warmed Transduction Media (Polybrene and serum concentrations as determined previously in experiments using the SMARTchoice Promoter Selection Plate).

3. Determine volume of viral particles required per well for highest MOI in a range that will be used to transduce the cells of interest.

   Calculating volume of viral particles 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 × 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 20 (highest MOI)
   - Cell density of 10,000 cells per well at time of transduction
   - Viral Titer is 1×10^8 TU/mL (= 1×10^5 TU/mL)

   Then, total TUs per well is equal to:
   Total TU = (20 TU/cell) × (10,000 cells/well)] / 1×10^5 TU/µL = 2 µL of viral stock/well.
   Therefore, the volume of viral particles with a titer of 1×10^8 TU/mL required for an MOI of 20 is 2 µL per well.

4. Prepare dilutions of lentiviral particles (in pre-warmed Transduction Media) such that 50 µL contains the appropriate number of viral 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 SMARTvector 2.0 Lentiviral shRNA particles including non-targeting and GAPD controls.
   a. Multiply the calculated volume for highest MOI (units in µL) by 7. The volume of viral particles is multiplied by 7 to allow 3 volumes to be used to transduce cells in triplicate, 3 volumes to be used to make next two-fold dilution in series, and 1 volume to represent liquid overage required to accommodate small carryovers during pipetting.
   b. Transfer calculated volume of viral particles into sterile 1.5 mL microfuge tube. Label tube with appropriate MOI (for example "MOI = 20").
   c. Bring volume up to 400 µL with pre-warmed Transduction Media and mix gently by pipetting.
   d. With new pipette tip, transfer 200 µL from the MOI = 20 tube into another sterile 1.5 mL microfuge tube labeled MOI = 10.

e. Bring volume up to 400 µL with pre-warmed Transduction Media and mix gently by pipetting.
f. Repeat steps 4d and 4e for MOI = 5 and MOI = 2.5.

Alternatively, all of these viral dilution steps (4a-f) could be performed in a deep-well plate to enable easier transduction in the 96-well tissue culture plates using an 8-channel pipette.

5. Allow Transduction Media with virus to incubate for 10-20 minutes at room temperature before transducing cells if Polybrene is present in Transduction Media; if Polybrene is not used, proceed directly to transduction of cells (step 6).

6. Remove culture plate(s) from incubator and replace media with 50 µL of Transduction Media with virus. In the "non-transduced" wells, add an equivalent volume of Transduction Media.

7. Return plates to incubator.

8. After 6-20 hours (overnight), add 100 µL of Preferred Culture Media directly to each well without removing media. Make adjustments to serum concentration so that final serum concentration matches Preferred Culture Media after adding to wells.

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

9. Return culture plate to incubator. Incubate for 72-96 hours.

Days 4-6:

1. Determine TurboGFP expression, toxicity and target gene expression. Transduced cells will begin expressing readily detectable levels of TurboGFP or TurboRFP within 48-72 hours post-transduction. Assess TurboGFP or TurboRFP expression by FACS or microscopy using the appropriate filters *(see Table 2 below)*.

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

Test for toxicity using alamarBlue, CellTiter-Blue or a similar assay for viability.

Within 72-96 hours post-transduction, determine knockdown of GAPD at mRNA level with RT-qPCR (Thermo Scientific Solaris reagents) or other appropriate detection method.

Section VII. Appendix

A. Key terms and definitions

Term	Definition
Biosafety Level 2	Biosafety Level 2 defines a set of standards, practices, safety equipment and facilities associated with working with potentially infectious agents. Details of precautions associated with this safety level can be found online at http://oba.od.nih.gov/oba/rac/Guidelines/APPENDIX_G.htm
Drug Selection	SMARTvector 2.0 constructs contain the puromycin resistance gene that allows selection of transduced cells. Following transduction, cells can be selected and clonal populations can be derived.
Long Terminal Repeat (LTR)	The long terminal repeat (LTR) is the control center for retroviral gene expression. All of the requisite signals for gene expression are found in the LTRs: Enhancer, promoter, transcription initiation (capping), transcription terminator and polyadenylation signal. Expression directed by the viral LTR signals is carried out entirely by host cell enzymes (RNA pol II, poly A synthetase, guanyl transferase).
SMARTchoice Promoter	Refers to the internal pol II transcriptional promoter that is used to control expression of the shRNA as well as the fluorescent reporter and puromycin resistance gene. It is the activity of the internal promoter that is being evaluated with the SMARTchoice shRNA Promoter Selection Plate.
microRNA Scaffold	The SMARTvector 2.0 microRNA scaffold is a native, non-coding RNA into which sequences targeting the gene of interest are cloned. This scaffold is readily processed by the endogenous RNAi machinery, providing enhanced performance over other expression cassettes.
Multiplicity of Infection (MOI)	Multiplicity of infection is the ratio of lentiviral transducing particles to cells. An MOI of 10 indicates that there are ten transducing units (TU) for every cell in the well. It is important to note that different cell types require different MOIs for successful transduction and knockdown of the target gene.
Polybrene	Hexadimethrine bromide, also referred to as Polybrene®, has been shown to enhance transduction of mammalian cells 2-10 fold by binding to the cell surface and neutralizing surface charge. The material can be purchased from a number of commercial sources (such as American Bioanalytical, #AB01643).
Puromycin Resistance Gene ($Puro^R$)	The puromycin resistance gene is a mammalian selectable marker gene that blocks the ability of the antibiotic puromycin to inhibit protein synthesis through ribosomal binding.
Self Inactivating (SIN)	Self-inactivating retroviral vectors are constructed by deleting the transcriptional enhancers and/or the enhancers and promoter in the U3 region of the 3' long terminal repeat (LTR). After one round of vector replication, these changes are copied into both the 5' and the 3' LTRs producing an inactive provirus that is replication incompetent.
SMARTvector 2.0 Lentiviral shRNA Particles	Lentiviral particles are intact viral particles capable of transducing cells and expressing shRNA sequences that target specific genes for knockdown using the RNAi pathway. These vectors have been genetically modified to prevent autonomous replication.
Transduction Efficiency	This term refers to the fraction of cells that successfully integrate one or more viral genomes during the transduction process.
Transducing Unit	Unit of measure that refers to the number of viral particles that can transduce a population of cells and integrate into the host genome. Titers are expressed as transducing units per milliliter (TU/mL).
Viral Titer	This is the number of transducing units present in a given volume (1 mL). Titer can be assessed by a number of techniques including antibody- and PCR-based methodologies, flow cytometry and observation of transgene expression following transduction of cells with serial dilutions of viral particles.
Vesicular Stomatitis Virus Glycoprotein (VSVg)	VSVg is an envelope protein derived from vesicular stomatitis virus (VSV). VSVg has previously been shown to provide broad tropism and for that reason is incorporated into the SMARTvector 2.0 lentiviral constructs to enable transduction of a broad range of cell types.

B. Viral packaging

During the preparation of lentiviral particles, the transfer plasmid (containing the shRNA gene-targeting construct) and the helper plasmids (containing the elements essential for viral packaging) are co-transfected into the viral packaging cell line, such as HEK293T. The viral vector genomes are produced, encapsulated and released as virion particles from the cells into the surrounding medium. Transduction of cells with this viral supernatant (medium containing the viral 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 viral supernatant can often be toxic. This is due in part because the viral 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 viral preparations, we have developed a proprietary production and manufacturing process leveraging the Thermo Scientific Trans-Lentiviral Packaging System that effectively concentrates viral particles while at the same time greatly reduces packaging cell debris. The benefits of these methodologies are clear: researchers receive preparations of purified, high-titer virus ($> 1 \times 10^8$ transducing units per mL) that afford the option of transducing at a range of MOIs without excessive levels of toxicity.

C. Viral titers

Since the SMARTchoice shRNA promoters have varying transcriptional activities in any particular cell type, it is not feasible to accurately measure functional viral titers based on expression of the TurboGFP or TurboRFP fluorescent reporters following transduction. Instead, a p24 ELISA is used to measure lentiviral particle titer. Because p24 titers overestimate functional titers, a reference control SMARTvector 2.0 Lentiviral shRNA Particle (containing hCMV and TurboGFP) of known functional titer is included in the p24 ELISA. The functional titer of the reference control virus is determined by transducing HEK293T cells with serial dilutions of the viral particles. Transduced cells are then counted under fluorescence microscopy and the titers are calculated as TU/mL. 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 virus with the reference control virus. For the correlation to be more accurate and consistent, all of the SMARTvector 2.0 lentiviral particles, including the reference control, are produced using the same lot of Trans-Lentiviral Packaging Mix and same lot of transfection reagents.

D. Stability and storage

Thermo Scientific SMARTvector 2.0 Lentiviral shRNA Particles are shipped on dry ice as 25 µL aliquots and must be stored at -80° C. Under these conditions, viral particles are stable for at least 12 months. Repeated freeze-thaw cycles should be avoided, as this is expected to negatively affect viral titer. When setting up a gene silencing experiment using SMARTchoice gene silencing reagents, viral particles should be thawed on ice, aliquoted into smaller volumes (if necessary) and immediately returned to -80° C.

E. Quality assurance and control

SMARTvector 2.0 Lentiviral shRNA Particles undergo stringent quality control at multiple steps during the manufacturing process. These include:

1. Restriction enzyme analysis of the vector and sequencing of the entire insert to verify sequence integrity.
2. Sequence confirmation of the shRNA.
3. Viral titer confirmation using p24 ELISA, followed by conversion to a functional titer by correlating p24 values of a SMARTvector 2.0 Lentiviral shRNA Particle of known functional titer.
4. Confirmation of fluorescent reporter expression (TurboGFP or TurboRFP) following transduction in HEK293T cells.
5. Thorough examination of each batch of viral particles to ensure preparations are free from mold and bacterial contamination.
6. A Certificate of Analysis with specified viral titers is included with each shipment.

F. Biosafety features

Historically, the greatest safety risk associated with a lentiviral delivery platform stems from the potential generation of recombinant viruses that are capable of autonomous replication. The SMARTchoice Lentiviral shRNA platform minimizes these hazards to the greatest degree by combining a disabled viral 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 SMARTvector shRNA lentiviral particles 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 viral 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 viral LTR.

Additional safety features are incorporated by the manufacturing process itself. Generation of SMARTvector shRNA lentiviral particles requires a viral packaging step during which the expression construct containing the silencing 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 viral particle capable of autonomous replication. Commercially available 3rd generation lentiviral vector systems separate the viral 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 viral 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 viral components strictly to the packaging cell line. A detailed description of the Trans-Lentiviral Packaging System can be found in Wu, *et al.* 2000.

G. General containment considerations

The major risks associated with the use of lentiviral vectors are (1) the potential for generating a replication competent lentivirus, and (2) the potential for oncogenesis due to the insertional activation/inactivation of key regulatory genes; for example, activation of an oncogene, or inactivation of a tumor suppressor.

To mitigate these risks, either BSL-2 or enhanced BSL-2 containment is required when handling any lentiviral vector particles. For guidance on containment for lentiviral vectors, please 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

The Trans-Lentiviral Packaging System used to create SMARTvector Lentiviral shRNA particles ensures that the end user receives a safe product that can be effectively used in a BSL-2 environment to silence gene expression or over-expression a mature microRNA in a wide range of cell types. It is vital that the protocols supplied with the viral particle product for appropriate handling and storage are fully understood and followed precisely. We strongly advise that users of this product have extensive experience with cell culture techniques.

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

In the U.S., download the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition, Feb 2007 here: http://www.cdc.gov/biosafety/publications/index.htm.

See also: NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines), September 2009, downloadable here: http://oba.od.nih.gov/rdna/nih_guidelines_oba.html.

For additional questions concerning the design or production of SMARTchoice shRNA products, please contact Thermo Fisher Scientific, Molecular Biology Products, Technical Support:
E-mail: techservice.genomics@thermofisher.com
Toll-free phone: (800) 235-9880, option 2

H. Frequently Asked Questions (FAQs)

For answers to questions that are not addressed here, please email technical support at techservice.genomics@thermofisher.com with your question, or call us at (800) 235-9880 (toll-free, in the U.S.) or (303) 604-9499.

What is the difference between SMARTvector 2.0 Lentiviral shRNA Particles and SMARTchoice products?
The SMARTvector 2.0 lentiviral backbone is constructed with the human CMV promoter to drive expression of TurboGFP, the puromycin resistance gene and the shRNA. The SMARTchoice platform now extends the utility of the original lentiviral vector by allowing for semi-customization of the SMARTvector 2.0 backbone. It is now possible to order SMARTvector lentiviral particles with your choice of seven different promoters (human and mouse CMV, human and mouse EF1α, CAG, PKG or UBC) and two fluorescent reporters (TurboGFP or TurboRFP) for increased shRNA expression in more cells of interest.

I have been using SMARTvector 2.0 Lentiviral shRNA, do I need to change to SMARTchoice shRNA?
Not necessarily. SMARTchoice is a platform by which multiple promoter and reporter options exist for SMARTvector 2.0 shRNA constructs. The original SMARTvector 2.0 backbone utilizes human CMV to drive expression of the shRNA; this promoter is very active in many cell lines and cell types, and successful gene silencing is achievable. However, hCMV promoter activity can vary from cell to cell and less-than-optimal promoter activity may impact shRNA functionality. To identify the most active promoter in your cells of interest, we recommend using the SMARTchoice shRNA Promoter Selection Plate, which will allow you to assess seven different promoters in a single experiment.

Can I use the SMARTchoice shRNA 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 shRNA performance.

I think I know the best promoter for my cells of interest. Do I still need to evaluate promoter activity with the SMARTchoice shRNA Promoter Selection Plate?
Promoter activity varies in different cell lines and cell types. We have demonstrated that this may affect the expression of the shRNA and the level of gene silencing that can be achieved. If you have previous data and/or knowledge that a particular promoter is active in your cells, then gene-specific shRNAs can be ordered without testing the SMARTchoice shRNA 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 gene silencing as well as understanding promoter activity. Promoter activity varies in different cell lines and cell types, and we highly recommend using the SMARTchoice shRNA 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 gene silencing compared to the same shRNA 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 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 SMARTvector constructs without a fluorescent reporter?
No. SMARTchoice reporter options include TurboGFP or TurboRFP. Fluorescent reporters facilitate visual tracking of transduction efficiency and shRNA expression.

Will SMARTvector 2.0 shRNA sequences target the same gene in human, mouse and rat cell lines?
Due to the polymorphisms that exist among these organisms, SMARTvector 2.0 Lentiviral shRNA gene silencing constructs designed against one organism generally do not target other organisms. For this reason, the SMARTvector 2.0 algorithm has been employed to pre-design constructs for each gene in the human, mouse and rat genomes.

Can I use the same SMARTchoice promoter to express the shRNA in multiple cell lines?
As a general rule, we recommend using the SMARTchoice shRNA 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 vector-based gene silencing, which can vary widely across cell lines and among species. The SMARTchoice shRNA 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 SMARTvector 2.0 Lentiviral shRNA Particles?
Our SMARTvector viral particles are provided at a functional titer of 1×10^8 (± 20%) transducing units (TU) per milliliter (mL), as determined by p24 ELISA of the viral particles. The p24 titer is then converted to a functional titer by correlating with values of a reference control of known functional titer (SMARTvector 2.0 Lentiviral shRNA Particles containing hCMV and TurboGFP) 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 SMARTvector 2.0 Lentiviral shRNA transducing units (functional viral 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 and silencing of the target gene. 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, media conditions (see Section III) and MOIs (see Section VI), the process of transduction requires nothing more than incubating SMARTchoice Lentiviral shRNA Particles with your target cell population for 6 to 20 hours.

For any additional questions, our expert staff is available to help you optimize a transduction protocol for your specific needs (See Section IX, Contact Information).

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.

Is gene silencing by SMARTchoice shRNA products transient or permanent?
The SMARTvector 2.0 genome integrates into the host genome. As the cells divide, each integrated copy is replicated and transferred to the daughter cell progeny and can permanently express the shRNA.

How many cells can I transduce with the amount of SMARTvector 2.0 lentiviral vector particles provided?
The number of cells that can be transduced with each SMARTvector 2.0 Lentiviral shRNA 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 protocol section above (see Section VI).

How do I know if my target cell type can be transduced with SMARTchoice Lentiviral shRNA Particles?
SMARTchoice viral 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 gene silencing 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 shRNA Promoter Selection Plate assay with the cells of interest. This assay will allow the simultaneous evaluation of seven SMARTchoice promoters.

What quality control is performed on SMARTchoice Lentiviral shRNA Particles?
SMARTvector 2.0 shRNA 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 viral vector constructs to ensure integrity of the lentiviral vector backbone and of the targeting sequence, respectively. Viral packaging cells are carefully monitored for bacterial and/or fungal contaminants. Following packaging, viral particles are titered by p24 ELISA. The p24 titers are adjusted to a functional titer using reference control viral particles titered by flow cytometry to count GFP-positive cells.

Are Thermo Scientific lentiviral particle products safe to use in the laboratory? What precautions should be taken when handling SMARTchoice viral particles?
Lentiviral delivery systems have been employed in many research laboratories around the world without incident. Handling of SMARTvector reagents requires extensive experience with cell culture techniques. It is vital that the protocols supplied with the SMARTchoice Lentiviral shRNA Particles 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 SMARTchoice Lentiviral shRNA Particles be further propagated in the lab?
No. SMARTvector products are engineered for maximum biosafety and are therefore replication incompetent.

How is the product manufactured?
SMARTchoice Lentiviral shRNA products are manufactured using the Thermo Scientific Trans-Lentiviral shRNA Packaging System which has been optimized for achieving high viral titers as well as premium biosafety. The SMARTvector 2.0 shRNA vector construct and the Trans-Lentiviral Packaging plasmids are co-transfected into an HEK293T cell line, and then viral particles are harvested, concentrated and purified to provide a high-titer preparation. All SMARTchoice shRNA particles are manufactured under stringent quality control guidelines (see Section VII., Appendix).

What is Polybrene?
Polybrene, also know 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.

How are SMARTchoice products shipped?
SMARTchoice products are shipped on dry ice for overnight domestic delivery or priority international for delivery outside of the U.S.

How should SMARTchoice products be stored?
SMARTchoice Lentiviral shRNA products must be stored at -80° C. Once thawed, the lentiviral preparation can be aliquoted to convenient volumes and the aliquots stored at -80° C to minimize the number freeze-thaws prior to each experiment.

What is the shelf-life of the SMARTchoice shRNA products?

SMARTchoice Lentiviral shRNA products can be stored at -80° C for 12 months without a significant loss in titer. Each thaw can reduce titer significantly, so freeze-thaws should be avoided as much as possible.

Can SMARTchoice Lentiviral shRNA products be kept at 4° C?
Once thawed, lentiviral particle preparations begin to decrease in titer. We do not recommend storing any lentiviral particles at 4° C.

How are the SMARTvector 2.0 shRNA targeting sequences designed?
We have developed an algorithm specific for the highly processed microRNA scaffold in which the SMARTvector 2.0 targeting sequence of interest is incorporated. The algorithm was derived using the bioinformatics strategies specific for shRNAs; briefly, weighted criteria for features associated with silencing functionality were identified from a teaching set of functional and non-functional shRNA sequences and combined into a selection algorithm.

Are there any differences between titers based upon p24 ELISA and FACS analysis?
A common method for calculating viral titers is to use an ELISA assay, which measures the amount of the viral capsid protein p24 in the supernatant of the packaging cell line after transfection with the transfer and helper plasmids. Because free, unassociated p24 may be present and because not all viral particles containing p24 are competent to transduce cells, estimations of the number of transducing units based on p24 quantification can overestimate the effective or functional titer. For this reason, when titers are assessed for SMARTvector 2.0 Lentiviral shRNA Particles by p24 ELISA, a reference SMARTvector 2.0 Lentiviral shRNA containing hCMV and TurboGFP with known functional titer is included with each assay. The functional titer of the reference lentiviral vector stock is determined by transduction of HEK293T cells with serial dilutions of virus and using flow cytometry to count GFP-positive cells.

What is the minimum titer range for the SMARTchoice Lentiviral shRNA products? Is the customer supplied with supernatant or purified particles?
The SMARTchoice Lentiviral shRNA products are provided at a minimum titer of $1 \times 10^8 \pm 20\%$ TU/mL based on our titering methods. All SMARTchoice products are provided as purified, concentrated viral particles and not as culture supernatant, which can contain toxic cellular debris resulting from the packaging process.

What controls should be included as part of a well-run lentiviral vector-based gene knockdown experiment?
We suggest that both positive and negative controls (for example SMARTvector 2.0 GAPD and Non-targeting Control Particles, respectively) and untreated cells should be included in each gene silencing experiment to ensure the accurate assessment of transduction efficiency, target gene knockdown and cellular viability following transduction.

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 media conditions and the density at which cells are plated. Please see Section III, Optimization protocols for the SMARTchoice shRNA Promoter Selection Plate, for recommended optimization studies that will aid end users in determining these parameters.

How do I select for stably transduced cells?
The SMARTvector 2.0 construct contains the puromycin resistance gene ($Puro^R$) 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 non-transduced cells after 4-6 days.

Can I assay transduced cells without going through the stable cell line selection?
Yes. SMARTchoice Lentiviral shRNA preparations generally provide enough particles to perform transduction experiments without the need for drug selection if the fraction of cells transduced at your chosen MOI is high enough (> 90% cells transduced).

Will SMARTvector 2.0 shRNA sequences target the same gene in human, mouse and rat cell lines?
Due to the polymorphisms that exist among these organisms, SMARTvector 2.0 Lentiviral shRNA gene silencing constructs designed against one organism generally do not target other organisms. For this reason, the SMARTvector 2.0 algorithm has been applied to pre-designed constructs for each gene in the human, mouse and rat genomes.

Can I use the same SMARTchoice promoter to express the shRNA in multiple cell lines?
Promoter strength is important to the success of vector-based gene silencing, which can vary widely across cell lines and among species. The SMARTchoice shRNA Promoter Selection Plate is designed to identify the optimal promoter specific for each cell line or cell type.

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OXFORD TECHNOLOGY

PCT application (filing date)	Priority Application(s) (filing date)	National Applications	Issued Patents
	US 07/586,603 (21 Sept 1990)	US 08/341,839	5,817,491
PCT/US91/01699 (9 Aug 1991) WO 92/14829	US 07/658,632 (19 Feb 1991)	EP 91915104.3 JP 3-514518 AU 84302/91 CA 2,104,396	0572401 3547229 663470 2,104,396
	US 07/1 70,515 (21 March 1988) US 07/1 70,515 (21 March 1988)	US Continuation 08/336,789 US Continuation 08/462,693 US Continuation 10/201,179	5,591,624 5,716,832 7,070,994
PCT/US91/06832 (20 Aug 1991) WO 92/05266	US 07/586,603 (21 Sept 1990)	AU 88436/91 AU Divisional 47984/96	665176 690427
PCT/GB97/02857 (17 Oct 1997) WO 98/1 7815	GB 9621680.9 (17 Oct 1996) GB 9624437.9 (25 Nov 1996)	EP 97909436.4 EP Divisional 98202432.1 US 09/224,014 US Divisional 09/91 5,169 US CIP 10/661,761 US Continuation 11/046,041 JP 10-519086 AU 47122/97 CN 97198767.X NZ 334866	0904392 6,312,682 6,689,956 7,198,784 725143 ZL97198767.X 334866
PCT/GB97/02858 (17 Oct 1997) WO 98/17816	GB 9621680.9 (17 Oct 1996)	GB 9921117.1 EP 97909437.2 US 09/284,011 JP 10-519087 AU 47123/97 CN 97198883.8 NZ 334522	2331522 6,235,522 737801 ZL971 98883.8 334522
PCT/GB97/02969 28 Oct 1997) WO 98/1 8934	GB 9622580.8 (29 Oct 1996)	US CIP 10/328,616 US Continuation 11/155,043 US Continuation 11/726,679 JP 10-520197	6,926,123 7,056,699

BENITEC TECHNOLOGY

LICENSED PATENTS

NAMED INVENTOR	TITLE		PATENT NO. APPLICATION NO
Graham / Rice	Control of Gene Expressa	ZA 2000/4507* AU2001100608* SG75542* US 6,573,099* GB2353282* AU 743316* NZ 506648* US 10/346853* PCT/AU99/00195* BR PI9908967-0* CA 2323726* CB 9804255-2* CZ PB2000-3346* EP 99910039.9* US 10/646,070 US 10/759,841* EP 04015041.9* AU 2005211538	HK 01105904.3* HU PO101225* IN 2000/00 169/CHE* JP P2000-537990* KR 7010419/2000* MX 008631* PL P.343064* SK PV1372-2000* AU 35647/02* NZ 525941* SG200205122-5* US 09/646807* PP2492/98* AUPP2499/98* US 10/646,807* US 10/821,710* US 10/821,726* AU 2005209648
Graham / Rice / M / R	Genetic Silencing	WO 01/70949 GB 237722 AU PQ6363	SG 91678 ZA 2002/7428 AU 2001240375 AU PR2700
Graham, et al.	Double-Stranded Nucleic Acid	US 60/475,827 US 60/550,504 US 60/479,616 US 60/553,920 US 10/861,191	AU 2003906281 AU 2004902279 AU 2003906281 PCT/AU04/000759
Kolvykhalov and Schroeder	Improved Method for Detecting and Characterization of Short Nucleic Acids	US 60/647,317	
Roelvink, et al.	RNAi Expression Constructs	US 60/649,641	US 60/653,580
Roelvink, et al.	RNAi Expression Constructs with Ribozyme	US 60/678,389	

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

Section IX. Contact Information

For technical information or troubleshooting contact Thermo Scientific Molecular Biology Technical Support:
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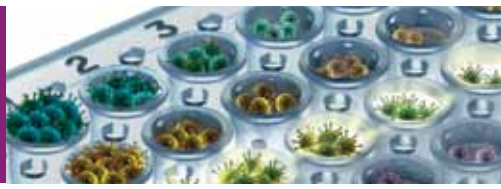
In Europe (EU, Middle East, Africa) Techservice.emea.genomics@thermofisher.com.

Thermo Scientific SMARTchoice Lentiviral shRNA



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