

SMARTchoice lentiviral shRNA workflow: Choosing the optimal promoter for shRNA expression and improved target silencing.

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Introduction

The Dharmacon™ SMARTchoice™ combines an innovative viral vector concept with a unique and user-friendly experimental workflow to deliver rationally designed shRNA constructs into a wide variety of cells. Using the SMARTchoice shRNA Promoter Selection Plate, the researcher can determine the most active promoter in the specific cells of interest by evaluating multiple promoters simultaneously. First in the workflow, basic experimental conditions are optimized for the cells of interest. Following optimization, cells are transduced with lentiviral particles extracted from the SMARTchoice shRNA Promoter Selection Plate to assess the cell-specific activity of seven different promoters in a single experiment. Next, positive and negative shRNA controls expressed from the optimal promoter are used to further optimize transduction conditions, such as multiplicity of infection (MOI), prior to evaluating targeted silencing of the gene of interest.

Here we demonstrate the SMARTchoice workflow using two human immune suspension cell lines, IM-9 and Jurkat. Basic experimental optimization indicated the ideal cell density, serum and Polybrene (PB) concentrations for lentiviral transduction. The SMARTchoice Promoter Selection Plate was used to identify the most active promoter for each cell line using fluorescence microscopy. Transduction of SMARTchoice positive (GAPDH) and negative (non-targeting) shRNA lentiviral controls expressed from the best promoter permitted the determination of an MOI range for knockdown of the gene target. Once the ideal promoter and transduction conditions were established for the cells of interest, custom-tailored SMARTvector™ 2.0 lentiviral constructs targeting the human RHOA gene were then transduced and successful gene silencing determined using reverse transcription quantitative real-time PCR (RT-qPCR).



Choose cell line of interest

- Transduction conditions are cell-specific

Perform basic transduction optimization

- Cell density
- Polybrene concentration
- Serum conditions for transduction medium

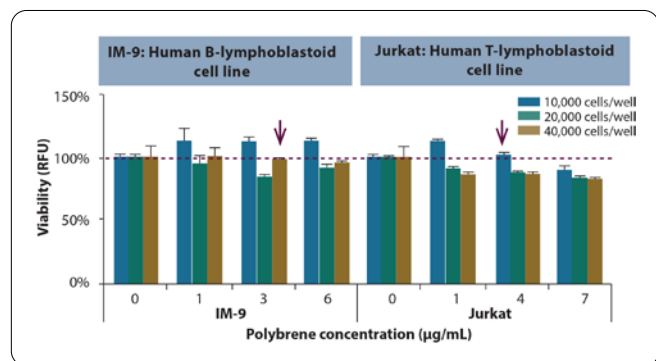


Figure 1: Viability of IM-9 and Jurkat cells. Viability was measured for three different cell plating densities using a resazurin assay 48 hours after treatment with varying concentrations of Polybrene (PB) and serum-free medium. Serum-free medium condition resulted in acceptable viability (data not shown). Results were normalized to no PB control wells to identify potential toxic effects of PB on cell viability. The highest concentration of PB tolerated with acceptable cell viability was 3 µg/mL of PB at 40,000 cells per well for IM-9 cells and 4 µg/mL PB at 10,000 cells per well for Jurkat cells.

Transduce cells using SMARTchoice promoter selection plate

- Transduce with conditions identified from basic optimization
- Incubate virus with cells for six hours or overnight and then add supplemented growth medium
- Visually inspect cells and assess TurboGFP fluorescence intensity daily for 48 to 96 hours

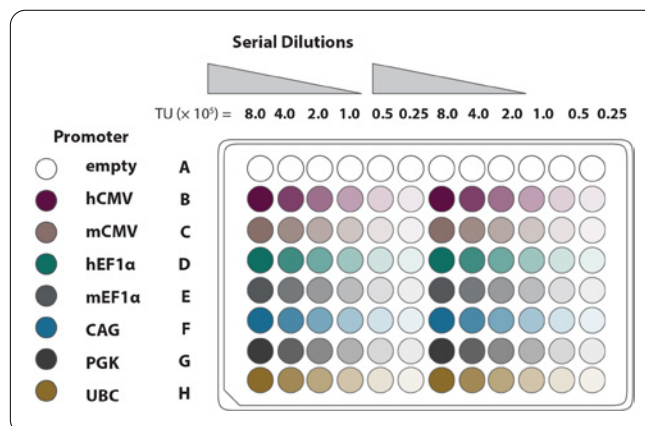


Figure 2: SMARTchoice Promoter Selection Plate. The Promoter Selection Plate contains transduction-ready lentiviral particles for a streamlined evaluation of promoter functionality. SMARTvector 2.0 lentiviral particles represent constructs with one of seven different promoters expressing TurboGFP and a Non-targeting Control (NTC) shRNA, and are arrayed in duplicates in a 96-well plate at five 2-fold dilutions starting from 8×10^5 transducing units (TU)/well. Empty = no virus (DMEM only), hCMV = human cytomegalovirus, mCMV = mouse cytomegalovirus, hEF1α = human elongation factor-1 alpha, mEF1α = mouse elongation factor-1 alpha, CAG = chicken α-actin hybrid, PGK = mouse phosphoglycerate kinase, UBC = human ubiquitin C.

Determine optimal promoter for cells of interest using one of these methods

- Fluorescence microscopy
- Fluorescence Activated Cell Sorting (FACS)
- High-content imaging

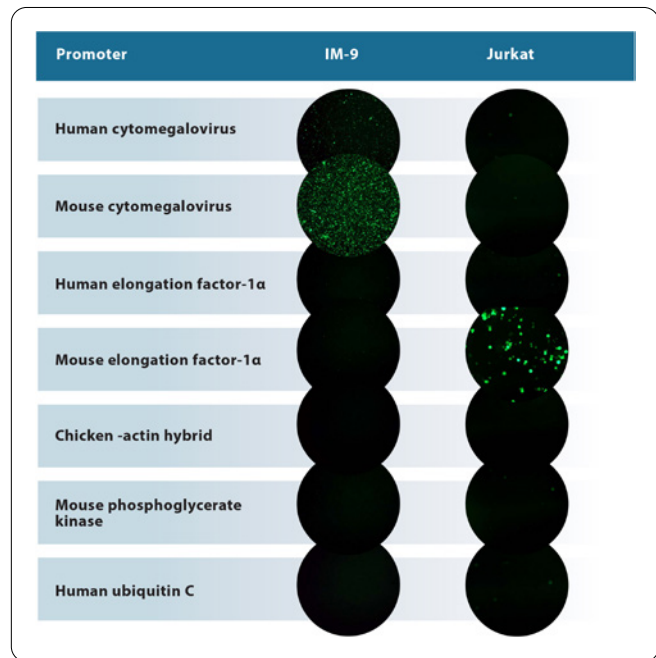


Figure 3: Fluorescence microscopy of IM-9 and Jurkat cells transduced with Dharmacon SMARTvector 2.0 Non-targeting Control Particles from the Dharmacon SMARTchoice Promoter Selection Plate. Images taken from the 8×10^5 TU/well column of the Promoter Selection Plate 96 hours after transduction. mCMV promoter is the optimal promoter for IM-9 cells and mEF1α is best for Jurkat cells.

Further optimize transduction conditions for range of MOI with matched control particles containing the promoter and fluorescent reporter of choice

- Attain lowest toxicity
- Enhance gene silencing

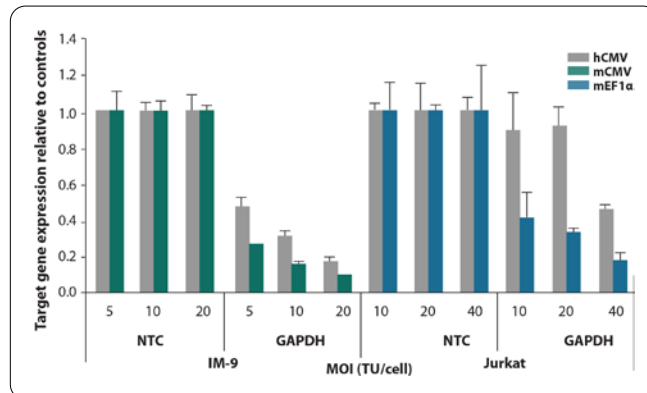


Figure 4: Optimization of MOI for gene knockdown in IM-9 and Jurkat cells using SMARTvector 2.0 positive and negative control lentiviral particles containing optimal promoters. Following the SMARTchoice workflow, cells were transduced with SMARTvector shRNA particles containing shRNA expressed from mCMV, mEF1α or hCMV promoter targeting GAPDH or a NTC. Lentiviral particles were transduced at predetermined optimized conditions for transduction medium and PB concentration at three MOIs: 5, 10, 20 (IM-9) or 10, 20, 40 (Jurkat). Gene expression was normalized to the NTC shRNA of the corresponding MOI and promoter. For both cell lines, greater knockdown was achieved using the optimal promoter compared to the hCMV promoter. The ranges of MOIs for these cell lines resulted in acceptable viability (not shown) and gene knockdown in a dose-dependent manner. These MOIs can be used for subsequent shRNA-mediated knockdown experiments for the genes of interest.

Order tailored Dharmacon SMARTvector 2.0 Lentiviral shRNA constructs for your gene of interest

- Select optimal promoter
- Choose desired fluorescent reporter

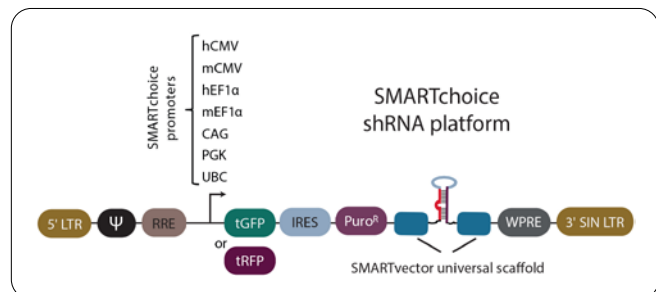


Figure 5: SMARTchoice lentiviral shRNA platform. Lentiviral vector particles contain one of seven promoter options and express TurboGFP (tGFP) or TurboRFP (tRF) fluorescent reporter along with a puromycin resistance selectable marker. A NTC or targeting shRNA is embedded within a proprietary microRNA scaffold, which is an endogenous substrate of the RNAi pathway.

Receive transduction-ready, high-titer lentiviral particles specific for your cells and gene targets

- Minimum titer of $1 \times 10^8 \pm 20\%$

Evaluate successful gene silencing using three custom SMARTvector 2.0 Lentiviral shRNA constructs

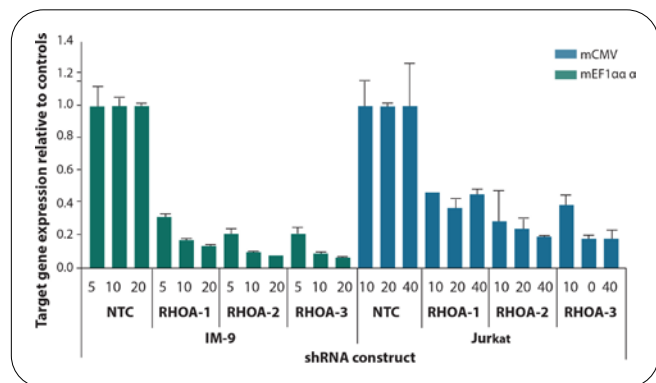


Figure 6: Targeted gene silencing in IM-9 and Jurkat cells using custom Dharmacon SMARTvector 2.0 Lentiviral shRNA constructs containing the optimal promoter and reporter. Cells were transduced with SMARTvector shRNA particles targeting human RHOA at the predetermined optimized transduction conditions. Gene expression was normalized to the NTC shRNA at the corresponding MOI. Construct RHOA-3 gave the greatest knockdown for both cell lines at 94% for IM-9 and 82% for Jurkat, followed by RHOA-1 and RHOA-2 resulting in 86% and 92% for IM-9 and 63% and 80% for Jurkat knockdown, respectively.

Summary and conclusions

Successful gene silencing using lentiviral vector-based RNA interference (RNAi) depends on efficient delivery of the virus into the cell combined with robust and stable expression of the shRNA. The SMARTchoice Promoter Selection Plate enables the identification of the optimal promoter for your cells of interest leading to increased target mRNA knockdown at a lower MOI by shRNAs. Here, IM-9 and Jurkat cells, immune suspension cell lines, were used in a workflow demonstration of successful target silencing using SMARTvector 2.0 Lentiviral shRNA constructs configured with the most active SMARTchoice promoter. After basic optimization of transduction conditions (Figure 1), the SMARTvector 2.0 Promoter Selection Plate (Figure 2) was used to evaluate the strength of the seven different promoters through the expression level of TurboGFP reporter (Figure 3). Cells transduced with control shRNAs driven by the optimal promoter (mCMV for IM-9 and mEF1a for Jurkat) demonstrated increased gene silencing compared to shRNAs expressed from the hCMV promoter (Figure 4). Cells were then transduced with three custom SMARTchoice shRNA constructs targeting human RHOA. All three shRNAs with the preferred promoter resulted in efficient target knockdown, with RHOA-3 shRNA resulting in the strongest silencing of greater than 90% for IM-9 and 80% for Jurkat cells (Figure 5). The SMARTchoice shRNA platform enables straightforward identification of an optimal promoter to achieve increased gene silencing with rationally designed lentiviral shRNAs in a broad range of cells.

Materials and methods

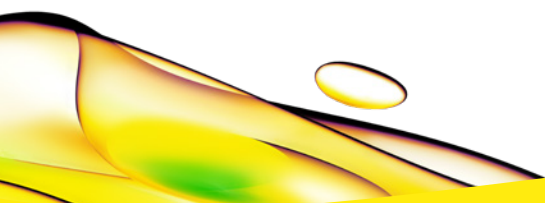
Initial optimization of transduction conditions: IM-9 and Jurkat cells were acquired from ATCC and grown per supplier's recommendations. Varying concentrations of PB (American Bioanalytical, Cat #AB01643) were tested in serum-free medium, and cells were cultured for six hours before the addition of growth medium containing appropriate amounts of serum and other supplements. All cells were cultured for 48 hours before assaying for viability with a resazurin assay. Treatments were normalized to the intensity of the serum-free medium containing no PB for each cell density.

SMARTchoice promoter selection plate: Transduction Ready Mix was prepared and SMARTchoice Promoter Selection Plate was used per manufacturer's protocol. IM-9 cells were transduced at 40,000 cells per well with 3 µg/mL PB and Jurkat cells were transduced at 10,000 cells per well with 4 µg/mL PB. Cells were cultured 6 hours, gently rocking the cell plate once every hour. Following transduction, supplemented growth medium was added to each well of the cell plate. At 96 hours, fluorescent images were taken and the promoter with the highest TurboGFP expression was selected for each cell line.

Transduction of custom SMARTvector 2.0 Lentiviral shRNA particles: IM-9 and Jurkat cells were transduced as above in triplicate wells per manufacturer's protocol. A non-targeting control construct and a construct targeting human *GAPDH* for each promoter was used, as well as three different constructs targeting human *RHOA* for each promoter at MOIs of 5, 10, and 20 for IM-9 or 10, 20 and 40 for Jurkat cells. IM-9 and Jurkat cells were cultured 96 and 72 hours, respectively, before harvesting for knockdown analysis.

RNA isolation: Promega™ SV 96 Total™ RNA Isolation System (Cat #Z3505) was used for total RNA purification according to the supplier's protocol. After isolation, total RNA was frozen at -80 °C for storage until further use.

RT-qPCR: Maxima™ Universal First Strand cDNA Synthesis Kit (Cat #AK1661) was used for the reverse transcription step. cDNA reactions were set up according to the supplier's RT-qPCR protocol in 20 µL reactions. RNA (5 µL) with oligo(dT)18 and random hexamer primers (1:1) were used in reverse transcription reactions with the following protocol: 25 °C for 10 minutes, 50 °C for 15 minutes and an inactivation step of 85 °C for 5 minutes. No reverse transcriptase enzyme and no template controls were used for each cDNA synthesis and each qPCR assay to determine presence of contaminating genomic DNA or reagent contamination. Solaris™ qPCR Gene Expression Assays (ALDOA Cat #AX-010376; GAPDH Cat #AX-004253; RHOA Cat #AX-003860; PPIB Cat #AX-004606) and Master Mix (Cat #AB-4350) were used for detection and relative quantification of gene knockdown. Reactions (12 µL for 384-well format and 15 µL for 96-well format) were set up per manufacturer's instructions and run on LightCycler™ 480 or Stratagene™ Mx3005p. Relative gene expression was calculated using a $\Delta\Delta Cq$ method www.revvy.com ALDOA was used as a reference gene for *GAPDH* and *RHOA* expression in IM-9 and PPIB as reference gene for Jurkat cells. Targeted gene knockdown was normalized to the corresponding NTC for each MOI and promoter type.



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