PROTOCOL

SMARTvector™ Lentiviral shRNA & shMIMIC Lentiviral microRNA glycerol stocks

Product description

The Dharmacon™ SMARTvector™ Lentiviral shRNA and shMIMIC Lentiviral microRNA platforms are innovative systems ideally suited for RNA interference (RNAi)-mediated studies. The purpose is to provide the researcher with the most effective tools for delivering and expressing genetic content in their cells of interest. The platforms utilize the advanced design of SMARTvector lentiviral shRNA and shMIMIC lentiviral microRNAs and employs:

- a microRNA scaffold
- enhanced rational design for efficient target gene silencing
- choice of seven constitutive RNA polymerase II promoter options* and four RNA polymerase II promoter options in a Tet-On™ 3G inducible system to enable experiments in a broad range of cell types
- choice of two fluorescent reporter options [TurboGFP™ (tGFP), TurboRFP™ (tRFP), Evrogen, Moscow, Russia] and a no-reporter option
- expanded depth of coverage per gene

SMARTvector Lentiviral shRNA and **shMIMIC Lentiviral microRNA** available in glycerol stock or lentiviral particles format. If using lentiviral particles, please refer to the SMARTvector Technical Manual for **constitutive shRNA expression** or **inducible shRNA expression.**

Important safety note

Please follow the safety guidelines for use and production of vector-based lentiviral particles as set by your institution's biosafety committee.

- For glycerol stocks of *E. coli* containing lentiviral plasmids, BSL1 guidelines should be followed
- For handling and use of lentiviral products to produce lentiviral particles, BSL2 or BSL2+ guidelines should be followed
- For handling and use of lentiviral particle products, BSL2 or BSL2+ guidelines should be followed

Additional information on the safety features incorporated in the SMARTvector and shMIMIC lentiviral vectors and the Dharmacon™ Trans-Lentiviral™ packaging system can be found on page 3.

SMARTvector lentiviral shRNA and shMIMIC lentiviral vectors are not compatible with third generation packaging systems, due to the requirement of the expression of tat, which third generation systems do not contain. We recommend the **Trans-Lentiviral shRNA packaging system** (Cat#TLP5912, #TLP5917) for use with our vectors.

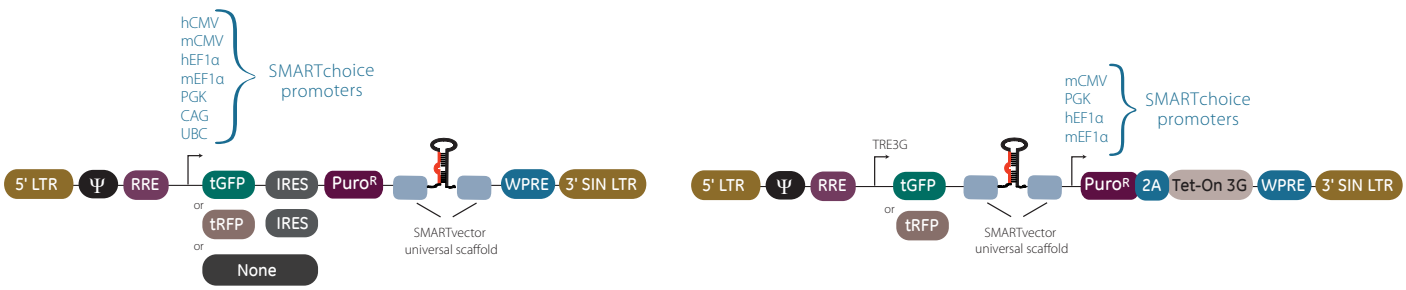

Figure 1. Elements of the SMARTvector lentiviral shRNA and shMIMIC lentiviral microRNA vectors (left) and SMARTvector inducible lentiviral shRNA and shMIMIC inducible lentiviral microRNA vectors (right). *Some promoter options may only be available as custom products or upon request.

Table 1. Description of SMARTvector and shMIMIC plasmid elements.

Vector element	Utility
5' LTR	5' Long Terminal Repeat is necessary for lentiviral particle production and integration into host cell genome
Ψ	Psi packaging sequence allows viral genome packaging using lentiviral packaging systems
RRE	Rev Response Element enhances titer by increasing packaging efficiency of full-length viral genomes
tGFP or tRFP	TurboGFP or TurboRFP reporter for visual tracking of transduction and expression upon doxycycline induction
TRE3G	Inducible promoter with Tetracycline Response Elements which is activated by the Tet-On 3G protein in the presence of doxycycline
None	No reporter option for use in applications where fluorescence is not required
IRES	Internal Ribosomal Entry Site allows expression of fluorescent reporter and puromycin resistance gene in a single transcript
Puro^R	Puromycin resistance permits antibiotic-selective pressure and propagation of stable integrants
SMARTvector universal scaffold	microRNA-adapted shRNA or microRNA for gene knockdown or expression of a mature microRNA, respectively
Tet-On 3G	Encodes the doxycycline-regulated transactivator protein, which binds to P_{TRE3G} only in the presence of doxycycline
WPRE	Woodchuck hepatitis Post-transcriptional Regulatory Element enhances transgene expression in the target cells
3' SIN LTR	3' Self-inactivating Long Terminal Repeat for increased lentiviral safety

Antibiotic resistance

SMARTvector Lentiviral shRNA plasmids contain two antibiotic resistance markers (Table 2).

Table 2. Antibiotic resistances conveyed by SMARTvector and shMIMIC

Antibiotic	Concentration	Utility
Ampicillin (carbenicillin)	100 µg/mL	Bacterial selection marker (outside LTRs)
Puromycin	Variable	Mammalian selection marker

Plasmid preparation

Culture conditions for individual plasmid preparations

For plasmid preparation, grow all SMARTvector or shMIMIC clones at 37 °C in LB broth medium plus 100 µg/mL carbenicillin only.

1. Upon receiving your glycerol stock(s) containing the shRNA or microRNA of interest, store at -80 °C until ready to begin.
2. To prepare plasmid DNA, first thaw your glycerol stock culture and pulse vortex to resuspend any *E. coli* that may have settled to the bottom of the tube.
3. Take a 10 µL inoculum from the glycerol stock into 3-5 mL of LB broth medium with 100 µg/mL carbenicillin. Return the glycerol stock(s) to -80 °C.

> If a large culture volume is desired, incubate the 3-5 mL culture for 8 hours at 37 ˚C with shaking and use as a starter inoculum. Dilute the starter culture 1:500-1:1000 into the larger volume.

4. Incubate at 37 °C for 18-19 hours with vigorous shaking.
5. Pellet the culture and begin preparation of plasmid DNA. Plasmid DNA can be isolated using a plasmid miniprep kit of your choice.

> Due to the tendency of lentiviral vectors to recombine, we recommend keeping the incubation times as short as possible and avoid subculturing. Return to your original glycerol stock for each plasmid preparation.

Packaging lentiviral particles

The SMARTvector lentiviral shRNA and shMIMIC lentiviral microRNA constructs are Tat dependent, therefore you must use a packaging system that expresses the tat gene. For packaging our lentiviral shRNA constructs, we recommend the Trans-Lentiviral shRNA packaging kit (Cat #TLP5912 or TLP5917). The Trans-Lentiviral shRNA packaging system allows creation of replication-incompetent, HIV-1-based lentiviral particles that can be used to deliver and express your shRNA in either dividing or non-dividing mammalian cells. The Trans-Lentiviral shRNA packaging system uses replication-incompetent lentiviral particles based on the trans-lentiviral system developed by Kappes (Kappes 2001). For protocols and information on packaging SMARTvector lentiviral shRNA or shMIMIC lentiviral microRNA constructs with the Trans-Lentiviral shRNA packaging system, please see the product manual available on our website.

Transfection

Quantities and volumes should be scaled up according to the number of cells/wells to be transfected (Table 3). This example is for 24-well plate format.

1. In each well, seed ~ 5×10^4 adherent cells or ~ 5×10^5 suspension cells in 0.5 mL of growth medium 24 hours prior to transfection.

> The recommended confluency for adherent cells on the day of transfection is 70-90%. Suspension cells should be in logarithmic growth phase at the time of transfection.

2. Dilute 1 µg of DNA in 50 µL of DMEM or other serum-free growth medium.
3. Gently mix DharmaFECT kb transfection reagent and add 3 µL to the diluted DNA. Mix immediately by pipetting.
4. Incubate 10 minutes at room temperature. Remove medium from wells and replace with 0.45 mL fresh growth medium.
5. Add 50 µL of the DharmaFECT kb reagent/DNA mixture gently to each well.

> The transfection efficiency with **DharmaFECT kb™ transfection reagent** (Cat #T-2006-01) is equally high in the presence of serum. This is not the case with other transfection reagents.

6. Gently rock the plate to achieve even distribution of the complexes.
7. Incubate at 37 °C in a CO_2 incubator.
8. Analyze transgene expression 24-48 hours later. For stable transfection, cells should be grown in selective medium for 10-15 days (see Puromycin selection below).

Table 3. Scale-up ratios for transfection of adherent and suspension cells with DharmaFECT kb transfection reagent.

Tissue culture vessel	Growth area, cm^2/well	Volume of medium, mL	Adherent (suspension) cells to seed the day before transfection*	Amount of DNA		Volume of DharmaFECT kb, µL	
				µg**	µL***	Recommended	Range
96-well plate	0.3	0.1	$0.5\text{-}1.2 \times 10^4$ (2.0×10^4)	0.2	10	0.6	0.4-1.0
48-well plate	0.7	0.25	$1.0\text{-}3.0 \times 10^4$ (5.0×10^4)	0.5	25	1.5	0.8-2.2
24-well plate	2.0	0.5	$2.0\text{-}6.0 \times 10^4$ (1.0×10^5)	1.0	50	3.0	2.0-5.0
12-well plate	4.0	1.0	$0.4\text{-}1.2 \times 10^5$ (2.0×10^5)	2.0	100	6.0	3.9-9.0
6-well plate	9.5	2.0	$0.8\text{-}2.4 \times 10^5$ (4.0×10^5)	4.0	200	9.0	6.0-12.0
60 mm plate	20	3.0	$2.0\text{-}6.3 \times 10^5$ (1.0×10^6)	6.0	300	18.0	12.0-24.0

* These numbers were determined using HEK293T and U2OS cells. Actual values depend on the cell type.
** Amount of DNA and DharmaFECT kb transfection reagent used may require optimization.
*** The volume of DNA should be 1/10 of the volume of the culture medium used for dilution of the DNA.

Puromycin selection

If adding antibiotic for selection, use the appropriate concentration as determined based on a kill curve (to determine antibiotic dose response, see short protocol).

1. Add medium containing antibiotic 24 hours post-transfection to begin selection.
2. Cells can be harvested for transgene expression 24-72 hours after starting selection.

It is important to wait at least 24 hours after transfection before beginning selection.

3. If longer selection is required for cells to be confluent, replace selective medium approximately every 2-3 days.
4. Monitor the cells daily and observe the percentage of surviving cells. Cells surviving selection will be expressing the transgene.
5. If generating stable cell lines (optional), select and grow for 10-15 days.
6. Once untransfected cells are eliminated and/or you have selected for stably transfected cell lines if desired, you can proceed to assay for transgene expression. RT-qPCR, western blot analysis or other appropriate functional assay can be used; compare treated samples to untreated, reporter alone, non-targeting control or other controls as appropriate.

Additional safety information

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 SMARTvector lentiviral shRNA and shMIMIC lentiviral microRNA platforms minimize these hazards to the greatest degree by combining a disabled lentiviral genome with the proprietary Trans-Lentiviral packaging process. Starting with the HXB2 clone of HIV-1 (GenBank, Accession #K03455), the lentiviral backbone has been modified to eliminate all but the most essential genetic elements necessary for packaging and integration (such as 5' LTR, Psi sequences, polypurine tracts, Rev responsive elements and 3' LTR). The resultant self-inactivating (SIN) vector greatly reduces the probability of producing recombinant particles and limits cellular toxicity often associated with expression of HIV genes.

Additional safety features can be incorporated by the packaging process itself. Generation of SMARTvector lentiviral shRNA or shMIMIC lentiviral microRNA particles requires a packaging step during which the expression construct containing the silencing sequence is enclosed in a lentiviral capsid. Gene functions that facilitate this process (such as those encoded by the structural genes gag, pol, env, etc.) are distributed amongst multiple helper plasmids that do not contain significant regions of homology. This tactic further minimizes the probability of recombination events that might otherwise generate lentiviral particles capable of autonomous replication. Among commercially available lentiviral vector systems, the Trans-Lentiviral packaging system offers a superior safety profile as the packaging components are separated onto five plasmids. Additionally, expression of gag-pro and tat-rev are under the control of the conditional tetracycline-responsive promoter element (TRE), limiting expression of these lentiviral components strictly to the packaging cell line. A detailed description of the Trans-Lentiviral packaging system can be found in (Wu 2000).

With these safety measures in place, SMARTvector lentiviral shRNA or shMIMIC lentiviral microRNA particles can be employed in standard Biosafety Level 2 tissue culture facilities.

Any investigator who purchases lentiviral vector products is responsible for consulting with their institution's health and biosafety group for specific guidelines on the handling of lentiviral vector particles. 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) here.
- See also: NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines), downloadable here.
- For Biosafety Considerations for Research with Lentiviral Vectors, see.

Frequently asked questions (FAQs)

Question	Answer
Where can I find the sequence of an individual shRNA construct?	The 19 nt antisense sequence for any individual shRNA can be found online at dharmacon.horizondiscovery.com
What packaging cell line should I use for making lentiviral particles?	You can use any HEK293T cell line to package lentiviral particles. The key element that your cell line needs to contain is the Large T antigen.
Can I use any 2nd generation packaging system with the SMARTvector Lentiviral shRNA vector?	The SMARTvector and shMIMIC lentiviral vectors are tat dependent, so a packaging system that expresses the tat gene is required. We recommend the Trans-Lentiviral shRNA packaging kit (Cat #TLP5912 or TLP5917), which utilizes HEK293T cells.
Where can I purchase puromycin?	Puromycin is available from Fisher Scientific Cellgro (Cat #BP2956-100) or Invivogen (Cat #ant-pr-1).
How many transfections are available in each volume size of DharmaFECT kb transfection reagent?	The number of transfections that can be performed depends on the size of the culture dish used and the volume size of DharmaFECT kb transfection reagent purchased. For example, if you purchase 1 mL of DharmaFECT kb transfection reagent, then you can perform 333 transfections wells in 24-well plate format. See Table 3 for additional information.

For additional Frequently Asked Questions (FAQs), please visit here.

Troubleshooting

For help with transfection or transduction of your lentiviral constructs, please email technical support at ts.dharmacon@horizondiscovery.com with the answers to the questions below, your sales order or purchase order number and the catalog number or clone ID of the construct with which you are having trouble.

1. Are you using direct transfection or transduction into your cell line?
2. What was the 260/280 ratio of DNA? Over 1.8?
3. What was the transfection efficiency if you used direct transfection? What transfection reagent was used?
4. Were positive and negative knockdown controls used (such as our GAPDH validated positive control and the validated non-targeting negative control)?
5. What were the results of the controlled experiments?
6. How was knockdown measured (for example RT-qPCR or western blot analysis)?
7. What is the abundance and the half-life of the protein? Does the protein have many isoforms?
8. What packaging cell line was used if you are using transduction rather than transfection?
9. What was your lentiviral titer in your cells?
10. What was your MOI?
11. Did you maintain the cells in puromycin selection medium after transfection or transduction?
12. How much time elapsed from transfection/transduction to puromycin selection?

If transfection into your cell line is unsuccessful, you may need to consider the following list of factors influencing successful transfection.

1. Concentration and purity of plasmid DNA and nucleic acids—determine the concentration of your DNA using 260 nm absorbance. Avoid cytotoxic effects by using pure preparations of nucleic acids.
2. Insufficient mixing of transfection reagent or transfection complexes.
3. Insufficient mixing of transfection reagent or transfection complexes.
4. Transfection in serum containing or serum-free medium — our studies indicate that the transfection efficiency with DharmaFECT kb transfection reagent is equally high in the presence of serum. This is not the case with other transfection reagents.
5. We do not recommend antibiotics (e.g., pen-strep) in the transfection complexing medium.
6. Cell history, density, and passage number—it is very important to use healthy cells that are regularly passaged and in growth phase. The highest transfection efficiencies are achieved if cells are plated the day before; however, adequate time should be given to allow the cells to recover from the passaging (generally > 12 hours). Plate cells at a consistent density to minimize experimental variation. If transfection efficiencies are low or reduction occurs over time, thawing a new batch of cells or using cells with a lower passage number may improve the results.

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Label licenses

If you have any questions, contact

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