

Single Oligonucleotide RNAi Technology for Gene Silencing

pLV-RNAi vector system for long-term suppression

Catalog number: SORT-B##; SORT-C0#

June 2013

Size: 20 reactions

Storage Temperature: -20°C upon arrival

Product List

pLV-RNAi Vector	Catalog #
pLV-H1-CMV-green	SORT-B01
pLV-H1-EF1 α -green	SORT-B02
pLV-H1-mPGK-green	SORT-B03
pLV-hU6-CMV-green	SORT-B04
pLV-hU6-EF1 α -green	SORT-B05
pLV-hU6-mPGK-green	SORT-B06
pLV-mU6-CMV-green	SORT-B07
pLV-mU6-EF1 α -green	SORT-B08
pLV-mU6-mPGK-green	SORT-B09
pLV-H1-CMV-red	SORT-B10
pLV-H1-EF1 α -red	SORT-B11
pLV-H1-mPGK-red	SORT-B12
pLV-hU6-CMV-red	SORT-B13
pLV-hU6-EF1 α -red	SORT-B14
pLV-hU6-mPGK-red	SORT-B15
pLV-mU6-CMV-red	SORT-B16
pLV-mU6-EF1 α -red	SORT-B17
pLV-mU6-mPGK-red	SORT-B18
pLV-H1-EF1 α -puro	SORT-B19
pLV-hU6-EF1 α -puro	SORT-B20
pLV-mU6-EF1 α -puro	SORT-B21
pLV-H1-EF1 α -bsd	SORT-B22
pLV-hU6-EF1 α -bsd	SORT-B23
pLV-mU6-EF1 α -bsd	SORT-B24
pLV-H1-EF1 α -GFP-Bsd	SORT-B25
pLV-mU6-EF1 α -GFP-Bsd	SORT-B26
pLV-H1-EF1 α -RFP-Bsd	SORT-B27
pLV-mU6-EF1 α -RFP-Bsd	SORT-B28
pLV-H1-EF1 α -GFP-Puro	SORT-B29
pLV-mU6-EF1 α -GFP-Puro	SORT-B30
pLV-H1-EF1 α -RFP-Puro	SORT-B31
pLV-mU6-EF1 α -RFP-Puro	SORT-B32
pLV-hU6-EF1 α -GFP-Bsd	SORT-B33
pLV-hU6-EF1 α -RFP-Bsd	SORT-B34
pLV-hU6-EF1 α -GFP-Puro	SORT-B35
pLV-hU6-EF1 α -RFP-Puro	SORT-B36
pLV-H1TetO-GFP-Puro	SORT-C01
pLV-H1TetO-RFP-Puro	SORT-C02
pLV-H1TetO-GFP-Bsd	SORT-C03
pLV-H1TetO-RFP-Bsd	SORT-C04

Product Description

RNA interference (RNAi) refers to a mechanism by which double-strand RNAs (e.g. siRNA, shRNA, and miRNA) inhibit gene expression via nucleotide sequences complementary to the targeted messenger RNA, leading to mRNA degradation or translational repression (for reviews, see [1-7]). The discovery that small interfering RNA (siRNA) can trigger knockdown of gene expression in mammalian systems has made RNAi a standard tool for sequence-specific gene silencing [8]. Instead of the time consuming and labor intensive knockout procedures in animal models, the RNAi-based gene knockdown method provides an alternative approach to functional genomic studies in both animal and cell line models.

Available technologies for generating small double-strand RNAs (i.e. siRNA and shRNA) for RNAi include chemical synthesis (for siRNA) and vector-based expression (for shRNA) [8-12]. While effective in triggering RNAi, the synthetic siRNAs are expensive and only mediate transient knockdown effects. In contrast, the promoter driven expression of short hairpin RNAs (shRNAs) in cells is more cost-effective, and the effect of gene silencing can possibly persist for a long period of time upon selection for the antibiotics-resistance marker encoded by the vector.

pLV-RNAi is a lentiviral-based RNAi vector system that allows efficient delivery of RNAi into cells via lentiviral transduction in some model systems where transfection is inadequate. The pLV-RNAi system provides options for different RNA pol III promoters for shRNA expression and different selectable markers for establishing stable cell lines. Moreover, the pLV-RNAi system offers choices of various RNA pol II promoters, which drive different expression levels of the selectable makers in the model system of your choice (Fig. 1). Also, our single oligonucleotide RNAi technology (SORT) for gene silencing delivers a unique feature in the construction of lenti-shRNA expression vectors. Distinct from other shRNA expression vectors currently available, only one DNA oligonucleotide is required for cloning a shRNA sequence into the pLV-RNAi vector (see “Advantages of the product” below and Fig. 2). This novelty of the pLV-RNAi vector system offers users with the advantage of lower cost, higher cloning efficiency, and less operational complexity over the other shRNA expression systems.

Inducible pLV-RNAi vector system is designed to deliver gene silencing in a tightly regulated fashion in mammalian cell lines. The shRNA expression is suppressed by Tet repressor (TetR) binding to the hybrid H1 promoter containing tetracycline operators (TetO) and RNA interference only takes place in the presence of tetracycline. The inducible RNAi system provides the option of expressing shRNA in certain period of time.

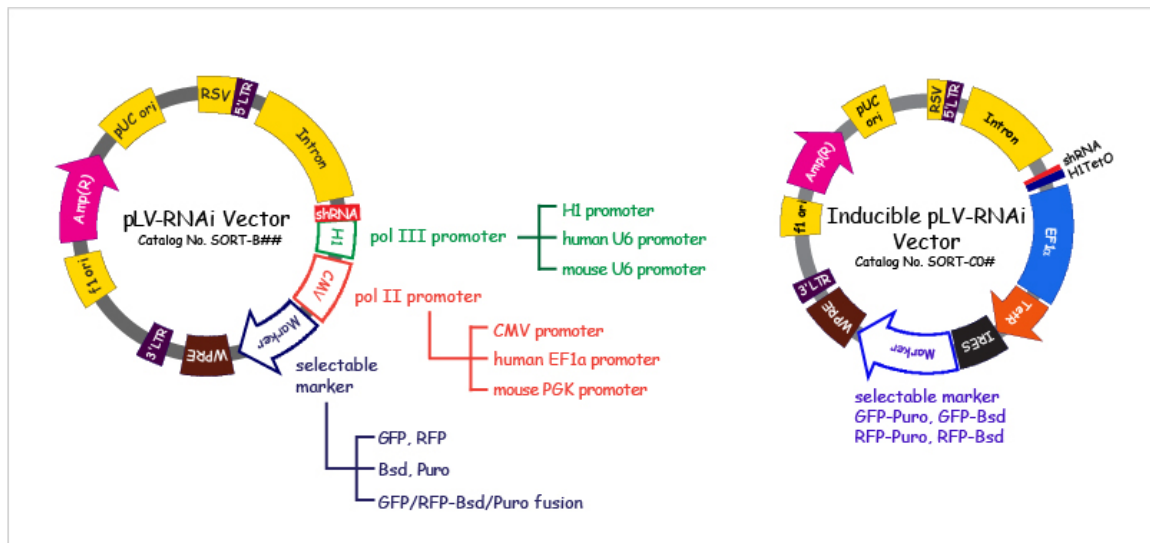


Figure 1. Structure map of pLV-RNAi vector system.

Product Components

Each pLV-RNAi kit provides a sufficient amount of reagents for 20 ligation reactions:

20µl	pLV-RNAi vector (25ng/µl)
100µl	10× annealing buffer (store at room temperature)
20µl	pLV-RNAi with shRNA insertion as negative control* (1ng/µl)
30µl	Sequencing primer** (10µM)

*control shRNA sequence: GCAGTTATCTGGAAGATCAGGTTGGATCCAACCTGATCTTCCAGATAACTGC

**sequencing primer: H1, AACGCTGACGTCATCAACCCGC; hU6, ATTTGCATATACGATACAAGGC; mU6, GAAGCTCGGCTACTCCCTGCC; H1TO, AATATTTGCATGTCGCTATGTG

Advantages of the Product

1. The pLV-RNAi vector is ready-to-use. No restriction digestion or vector purification is required. Also, the pLV-RNAi is a self-inactivated (SIN) vector carrying a deletion in the U3 region of the 3' LTR, which eliminates the promoter activity of LTR. The self-inactivating deletion provides an additional level of safety by preventing the integrated viral genome from generating full-length lentiviral RNA.
2. Less cloning complexity. The single-strand DNA oligo encoding shRNA sequence is a perfect palindrome, and the same (two) palindromic oligos can anneal to each other to form a double-strand oligo. This eliminates the need to mix and anneal two different DNA oligos and reduces operational mistakes during the cloning process. The overall chance of mutations introduced during DNA oligo synthesis is also reduced by 50% (Fig. 2A).
3. Lower cost. Only one DNA oligo is required to generate an shRNA expression clone. This setup is the most economical, and is distinguishable from all the other shRNA vectors commonly used in academia and industry, for which two different oligos are needed to make a shRNA expression clone. Also, the length of the DNA oligo used for pLV-RNAi (52-56 nt) is shorter than the length of the DNA oligos required for most of the other commercially available shRNA vectors.
4. High efficiency and low background in shRNA cloning. The 5'-AAAA overhangs on annealed double-strand oligo can only be ligated to the 5'-TTTT overhangs on both ends of the linear pLV-RNAi vector. Unlike the overhangs generated by restriction digestion in other available shRNA expression vectors, these 5'-TTTT overhangs in pLV-RNAi will not self-ligate. As a result, the ligation of the double-strand oligo into the pLV-RNAi vector is highly efficient, and the contamination of empty, self-ligated pLV-RNAi vectors is greatly reduced (Fig. 2B).
5. The choice of three promoters for shRNA and selectable marker expression. The shRNAs can be expressed from human H1 (H1), human U6 (hU6), or mouse U6 (mU6) promoters in the pLV-RNAi system. Researchers are able to choose the pol III promoter most suitable to their model system. In addition, the pLV-RNAi system offers choices of various pol II promoters, which drive different expression levels of the selectable markers in the model system of your interest.
6. High efficiency in delivery and long-term suppression. Lentiviral transduction is considered to be one of the most effective delivery system for RNAi expression, especially in primary cells and some cancer cell lines that are difficult to transfect by conventional methods. The lenti-shRNA viral genome is integrated into the host chromosomes, and thus, the shRNA is stably expressed in transduced cell lines. Also, unlike the retroviral system, the lentiviral integration is cell cycle independent. The shRNA encoded by the lentivirus can be efficiently delivered into both dividing and non-dividing cells. The selectable markers (i.e. Puro, Bsd, GFP, RFP, Bsd-GFP, and Bsd-RFP) allow users to generate stable cell lines with long-term suppression of genes of interest by antibiotics selection or fluorescence-activated cell sorting (FACS).

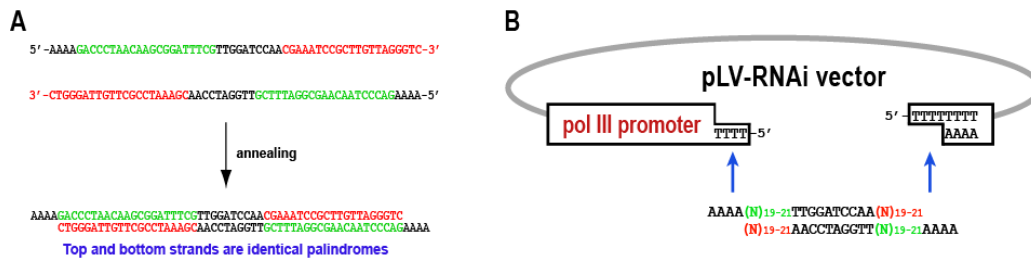


Figure 2. Overview of pLV-RNAi cloning system. (A) Example of single oligonucleotide RNAi technology: two identical DNA oligos anneal to each other to form a double-strand oligo with 5'-AAAA overhangs. The single-strand oligo encodes 21-nt sense target sequence (in green), 10-nt loop sequence (TTGGATCCAA), and 21-nt antisense target sequence (in red). (B) The 5'-AAAA overhangs of an annealed double-strand oligo can only ligate with the 5'-TTTT overhangs on the pLV-RNAi vector, which greatly increases the ligation efficiency and reduces the background of cloning.

Protocol

Designing oligonucleotide encoding shRNA (sense-loop-antisense) sequence

The single-strand oligonucleotide encoding shRNA sequence includes the following features (Fig. 3):

1. The stem-loop sequence. It consists of 19-21 nucleotides (nt) of the target (sense) sequence, followed by a 10-nt loop sequence (TTGGATCCAA) and 19-21 nt of target antisense sequence. This sense-loop-antisense sequence is a perfect palindrome with 48-52 nt in length. Therefore, only one oligonucleotide has to be ordered since both strands in the annealed double-strand oligonucleotide are identical (Fig. 2A).
2. The overhang sequence. The 5'-AAAA sequence is complementary to the overhang of the pLV-RNAi vectors and restores the promoter and terminator sequences in the vector (Fig. 2B).

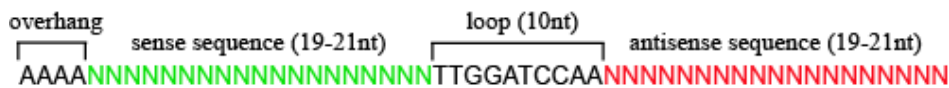


Figure 3. Structure of oligonucleotide encoding shRNA sequence.

There are no standard but only general guidelines for selecting the target sequences [13-17]. However, following these guidelines does not guarantee efficient knockdown. The effectiveness of shRNA sequence has to be determined by gene suppression analysis. In general, we suggest selecting 3 to 5 target sequences per gene of interest to identify at least two functional shRNAs with over 70% knockdown efficiency in target gene expression.

Listed below are a few rules we recommend for selecting shRNA sequences:

- Unique 19-21 nt target sequence with 30%-70% GC content. A 35%-55% GC content is preferred.
- Avoid target sequences with significant homology to other genes, unless the shRNA is intended to knockdown a gene family.
- A target sequence within an open reading frame is preferred. Targeting 3' UTR is ok.
- No more than 4 consecutive T in the shRNA sequence, because it may cause early transcription termination.
- GC-rich in the 5' end and AT-rich in the 3' end of target sequences are preferred.
- Choose a target sequence that starts with guanosine (G) if the U6 promoter is used.

The target sequence can be designed from BioSettia's shRNA designer:

<http://biosettia.com/support/shrna-designer>

Note: If you would like to use other shRNA design tools, please make sure the 5' overhang sequence is AAAA and the loop sequence is TTGGATCCAA.

Suggestion: Ordering oligonucleotide with gel purification and 5' phosphorylation is not required.

As a negative control for knockdown analysis, use a nonspecific target sequence or a scrambled sequence. For example, shRNA oligonucleotide targeting luciferase or β -galactosidase gene could be used as negative control.

Cloning double-strand oligo into pLV-RNAi vector: annealing, ligation, transformation.

1. In a 1.5 ml microcentrifuge tube, set up the annealing reaction in total 20 μ l mixture as following:

10 μ l	100 μ M single-strand oligo
2 μ l	10 \times annealing buffer
8 μ l	distilled water

2. Incubate the DNA oligo at 95°C for 5 minutes.

3. Remove the tube from the water bath and allow the reaction mixture to cool to room temperature for 10 minutes.

4. Dilute 1 μ l annealed double-strand oligo in 499 μ l distilled water, vortex for 5sec.

Suggestion: Load 20 μ l of diluted oligo onto 4% agarose gel (FMC Bioproducts, NuSieve GTG Agarose Catalog No. 50082) to determine annealing efficiency (example in Fig. 4).

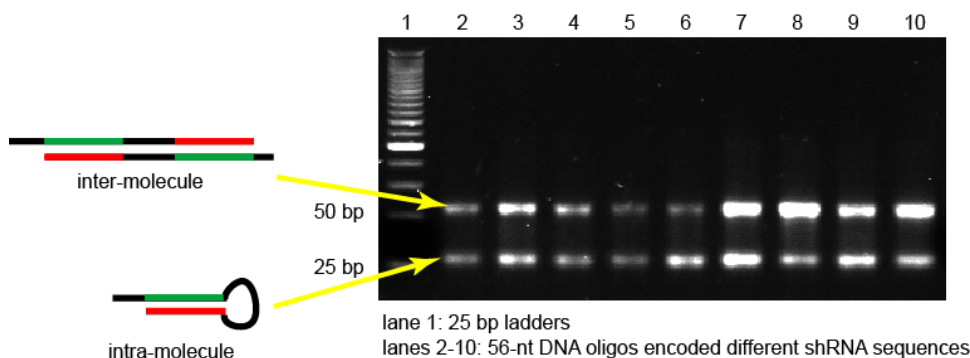


Figure 4. Example of annealing DNA oligonucleotides encoding shRNA sequences.

5. Store the remainder of the annealed oligo mixture at -20°C.

6. Mix 1 μ l (25 ng) pLV-RNAi vector with 2 μ l 500 \times diluted (50 nM final) double-strand oligo.

Note: Make sure to mix pLV-RNAi vector with annealed oligo before adding other ligation components.

7. Add ligase and other ligation components to total 20 μ l.

8. Incubate the ligation reactions at room temperature for ≥ 3 hours or incubate the reactions overnight at 4°C.

9. Transfer 3-5 µl ligation reaction mixtures to competent cells with a transformation efficiency $\geq 1 \times 10^8$ pfu/µg DNA according to the supplier protocol or the transformation protocol routinely used in your laboratory.

Note: Due to high frequency of homologous recombination occurred in lentiviral vector in some bacterial strains (e.g. TOP10, XL-1 Blue and XL-10 Gold), we recommend using LV101 (Biosettia), DH5α or Stbl3 (Invitrogen) for lentiviral vector transformation.

Suggestion: Keep the rest of ligation mixture at 4°C for overnight to obtain higher number of colonies in another transformation if necessary.

10. Plate all transformation reaction on LB plate with 100 µg/ml ampicillin and incubate at 37°C for overnight.

Plasmid DNA preparation and screening for oligo insertions.

1. Inoculate single colonies from each pLV-RNAi plate into 6 ml of LB medium with 100 µg/ml of ampicillin in a 15-ml tube. Grow bacterial culture at 37°C for 20 hours in a floor shaker with 300 rpm.

Suggestion: The single oligonucleotide shRNA cloning strategy is very efficient; therefore picking 2-3 colonies for each shRNA clone for plasmid DNA purification should be fine.

2. Spin down whole 6-ml bacterial culture in a desktop centrifuge (e.g. Sorvall RT6000) at 4,000 rpm for 10 min. Discard the supernatant and start plasmid DNA purification.

Note: We have tested that growing bacteria in 6 ml of LB medium and shaking in a floor shaker with 300 rpm at 37°C for 20 hours gives a better yield of plasmid DNA. The bacteria strain LV101 (Biosettia) or Stabl3 (Invitrogen) and Favorgen nucleic acid purification kits (<http://biosettia.com/php/products-nucleic-acid-purification>) are highly recommended for preparation of high-yield and good-quality plasmid DNA for future experiments such as transfection and lentiviral production. In general, the DNA yield is around 30-50 µg for each miniprep. The yield and quality of miniprep plasmid DNA purified by using Favorgen plasmid DNA extraction mini kit (Catalog # FAPDE 001) is sufficient for generating 30 ml of lentivirus.

3. The shRNA sequence containing a *Bam*H I (GGATCC) site inside the loop sequence. Therefore, restriction digestions by *Bam*H I can be used to confirm the insertion of double-strand oligo in the pLV-RNAi vector. We recommend that the diagnostic digestion be performed with *Bam*H I + *Sac* I. The patterns and expected sizes of the restriction fragments from each pLV-RNAi vector containing shRNA insertion are provided in Figure 5 and Table 1, respectively. The appearance of the fragment labeled as red in Table 1 in the restriction digestion indicates the presence of a shRNA insert in the vector.

Suggestion: DNA sequencing is recommended to ensure no mutations are present in the oligo insertion.

BamH I + Sac I digestion map of pLV-RNAi vectors with 52-bp oligo insertion

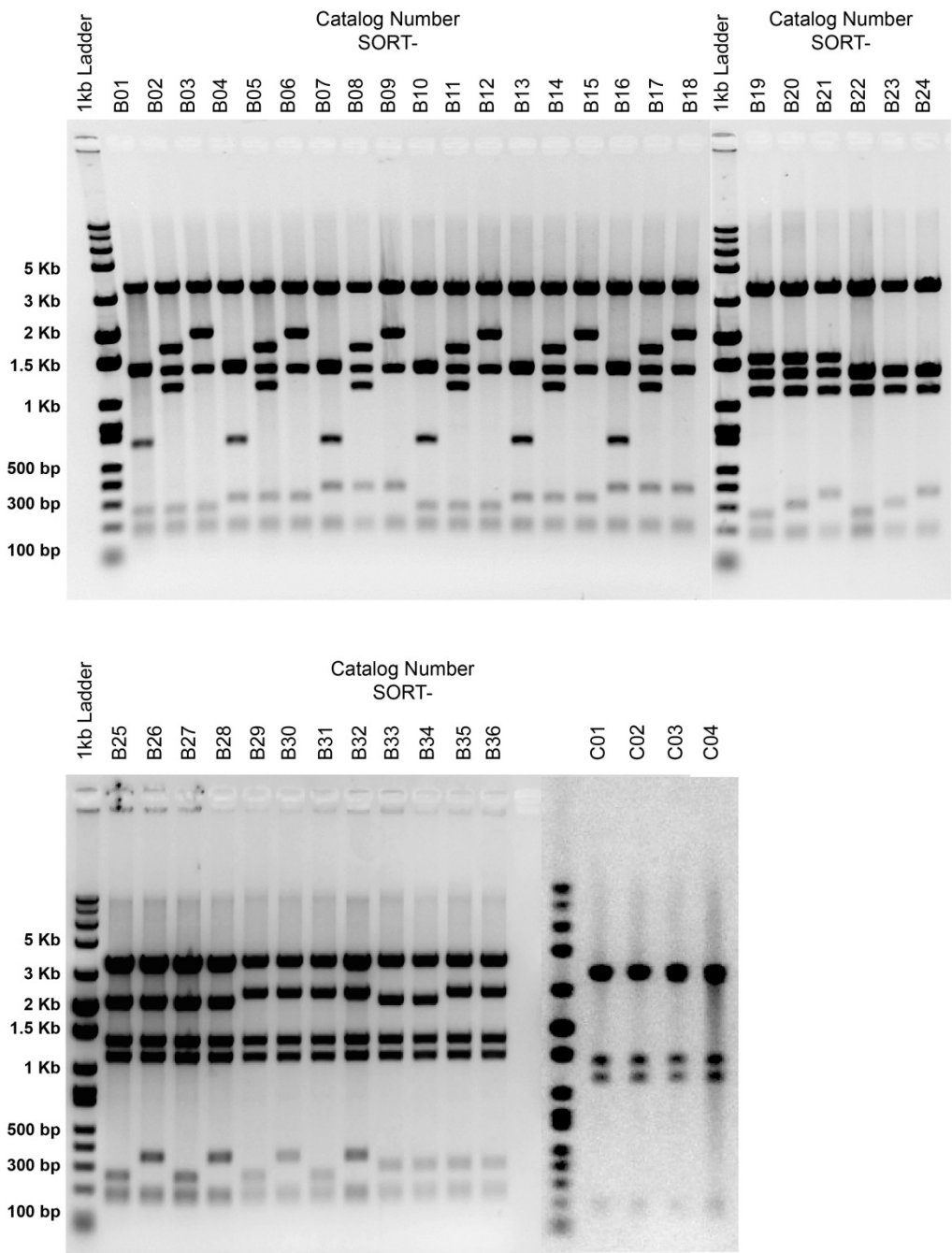


Figure 5. Patterns of fragments from *Bam*HI + *Sac* I restriction digestion of the pLV-RNAi vectors with a 52-bp shRNA insertion. The 56-nt oligo (AAAAGCAGTTATCTGGAAGATCAGGTTGGATCCAACCTGATCTTCCAGATAACTGC) as negative control was annealed and cloned into different pLV-RNAi vectors available from Biosettia (catalog # SORT-B01 to B32) as the protocol described above. The recombinant plasmids were digested with restriction enzymes *Bam*H I and *Sac* I. 0.5 µg of digested DNA was separated on 1% agarose gel and detected by staining with ethidium bromide. The expected sizes of fragments for each vector are listed in Table 1 below. The ones in red designate the diagnostic fragments for the presence of shRNA inserts in each vector.

Table 1. Expected sizes of restriction fragments generated by *Bam*H I+*Sac* I digestion of each pLV-RNAi vector with a 52-bp shRNA insertion.

BamH I + Sac I Digestions		Catalog Number SORT-								
		B01	B02	B03	B04	B05	B06	B07	B08	B09
Size of Fragments	3.66 kb	✓	✓	✓	✓	✓	✓	✓	✓	✓
	2.00 kb			✓			✓			✓
	1.72 kb		✓			✓			✓	
	1.43 kb	✓			✓			✓		
	1.38 kb	✓	✓	✓	✓	✓	✓	✓	✓	✓
	1.16 kb		✓			✓			✓	
	655 bp	✓			✓			✓		
	354 bp							✓	✓	✓
	303 bp				✓	✓	✓			
	262 bp	✓	✓	✓						
	191 bp	✓	✓	✓	✓	✓	✓	✓	✓	✓
	163 bp	✓	✓	✓	✓	✓	✓	✓	✓	✓

BamH I + Sac I Digestions		Catalog Number SORT-								
		B10	B11	B12	B13	B14	B15	B16	B17	B18
Size of Fragments	3.66 kb	✓	✓	✓	✓	✓	✓	✓	✓	✓
	1.99 kb			✓			✓			✓
	1.71 kb		✓			✓			✓	
	1.42 kb	✓			✓			✓		
	1.38 kb	✓	✓	✓	✓	✓	✓	✓	✓	✓
	1.16 kb		✓			✓			✓	
	655 bp	✓			✓			✓		
	354 bp							✓	✓	✓
	303 bp				✓	✓	✓			
	262 bp	✓	✓	✓						
	191 bp	✓	✓	✓	✓	✓	✓	✓	✓	✓
	163 bp	✓	✓	✓	✓	✓	✓	✓	✓	✓

BamH I + Sac I Digestions		Catalog Number SORT-					
		B19	B20	B21	B22	B23	B24
Size of Fragments	3.66 kb	✓	✓	✓	✓	✓	✓
	1.60 kb	✓	✓	✓			
	1.40 kb				✓	✓	✓
	1.38 kb	✓	✓	✓	✓	✓	✓
	1.16 kb	✓	✓	✓	✓	✓	✓
	354 bp			✓			✓
	303 bp		✓			✓	
	262 bp	✓			✓		
	191 bp	✓	✓	✓	✓	✓	✓
	163 bp	✓	✓	✓	✓	✓	✓

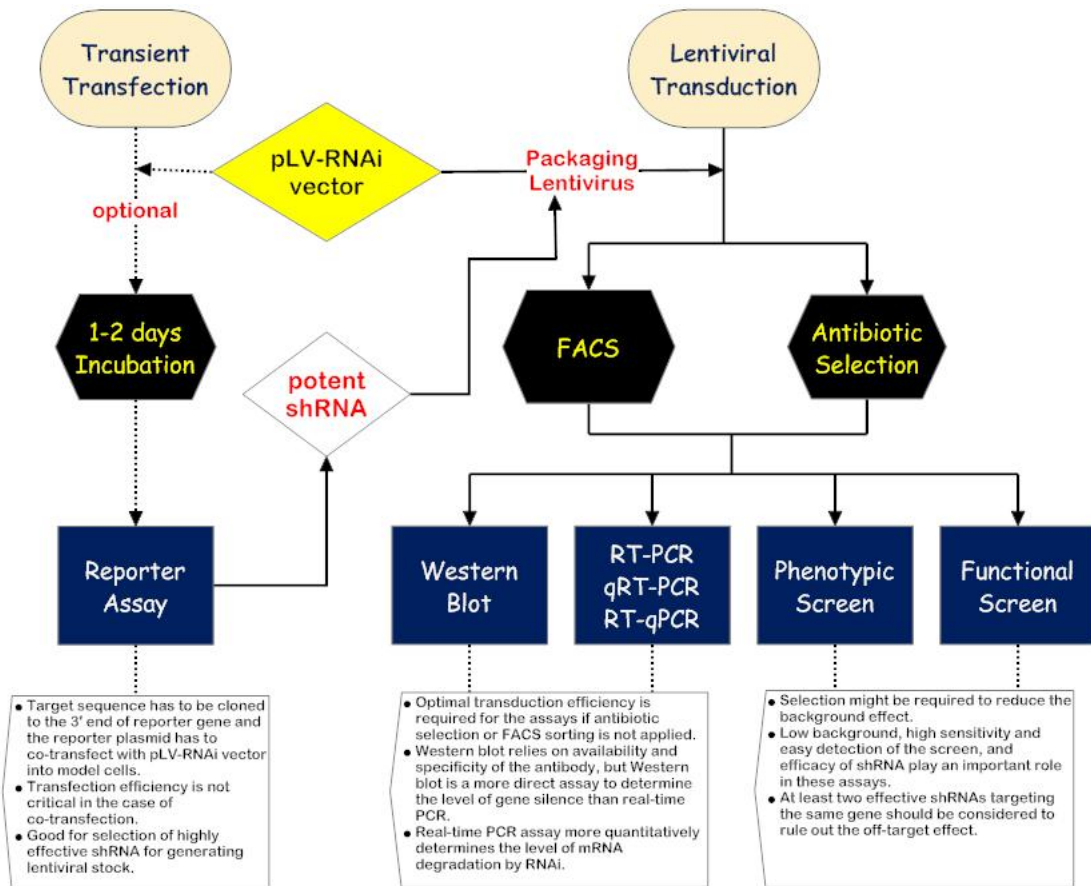
BamH I + Sac I Digestions		Catalog Number SORT-							
		B25	B26	B27	B28	B29	B30	B31	B32
Size of Fragments	3.66 kb	✓	✓	✓	✓	✓	✓	✓	✓
	2.33 kb					✓	✓	✓	✓
	2.14 kb	✓	✓						
	2.13 kb			✓	✓				
	1.38 kb	✓	✓	✓	✓	✓	✓	✓	✓
	1.16 kb	✓	✓	✓	✓	✓	✓	✓	✓
	354 bp		✓		✓		✓		✓
	303 bp								
	262 bp	✓		✓		✓		✓	
	191 bp	✓	✓	✓	✓	✓	✓	✓	✓
	163 bp	✓	✓	✓	✓	✓	✓	✓	✓

BamH I + Sac I Digestions		Catalog Number SORT-							
		B33	B34	B35	B36	C01	C01	C03	C04
Size of Fragments	3.66 kb	✓	✓	✓	✓	✓	✓	✓	✓
	3.60 kb					✓	✓		
	3.40 kb							✓	✓
	2.33 kb			✓	✓				
	2.14 kb	✓	✓						
	2.13 kb								
	1.38 kb	✓	✓	✓	✓	✓	✓	✓	✓
	1.16 kb	✓	✓	✓	✓	✓	✓	✓	✓
	354 bp								
	303 bp	✓	✓	✓	✓				
	262 bp								
	191 bp	✓	✓	✓	✓	✓	✓	✓	✓
	163 bp	✓	✓	✓	✓	✓	✓	✓	✓
	127 bp					✓	✓	✓	✓

Confirmation of knockdown and RNAi analysis

Confirmation of knockdown of the specific target gene is an essential step to ensure the success of an RNAi experiment. The approaches to validating effective shRNAs include the demonstration that the expression level of the target mRNA and/or gene product is substantially reduced while a negative control shRNA shows no effect. Optionally, a reporter assay can be applied to screen potent shRNAs before packaging pLV-RNAi recombinants into lentiviruses.

The flow chart below illustrates an overview of the options and strategies to analyze RNAi efficacy with the pLV-RNAi vector.



Production of shRNA lentiviruses from recombinant pLV-RNAi vectors

Production of lenti-shRNA viral stocks requires packaging of the lentiviral genomic RNA transcribed from pLV-RNAi vector with HIV-1 *gag*, *pol*, and *rev* gene products and vesicular stomatitis virus G (VSV-G) protein encoded by helper plasmids. In general, at least two helper plasmids are required, with one plasmid expressing the Gag-Pol polyprotein and an accessory protein Rev and the other expressing VSV-G as envelop protein to increase cell tropism. Nevertheless, Gag-Pol and Rev proteins can be expressed from separated plasmids as well. Therefore, a three-plasmid system or a four-plasmid system can be used to generate shRNA lentiviral stocks, depending on the source and nature of the helper plasmids. In the three-plasmid system, pLV-RNAi is cotransfected with two helper plasmids (Gag-Pol + Rev and VSV-G) into cells, while in the four-plasmid system, pLV-RNAi is cotransfected with three helper plasmids (Gag-Pol, Rev and VSV-G). It is generally considered to be safer to produce the lentiviral stocks with more helper plasmids, due to the reduced chance of recombination among all vectors that generates replication-competent viruses.

The manufacturers of transfection reagents, the suppliers of lentiviral packaging constructs, and many academic laboratories have provided protocols for producing lentiviral stocks. The following procedure is provided as an example only. We produce pLV-RNAi lentiviral stocks with Biosettia's packaging mix (Catalog# pLV-PACK-500) in Lenti-293T cells (Catalog # celine-01) using the transfection conditions summarized in a table below.

Using Biosettia's packaging plasmid mix

	10-cm plate	6-well plate
	Cat# pLV-PACK-500	Cat# pLV-PACK-500
Lentiviral vector	9 µg	1.5 µg
pLV-PACK packaging mix	9 µg	1.5 µg
Total plasmid DNA	18 µg	3.0 µg
Lipofectamine™ 2000	45 µl	7.5 µl
Total Opti-MEM	3 ml	0.5 ml
Lenti-293T cells / vol. of medium	1.0×10^7 /5ml	1.7×10^6 /1ml

Using other packaging plasmids

	10-cm plate		6-well plate	
	3-plasmid system	4-plasmid system	3-plasmid system	4-plasmid system
pLV-RNAi vector	9 µg		1.5 µg	
Gag-Pol + Rev expression vector ¹	6 µg		1.0 µg	
Gag-Pol expression vector ²		4.5 µg		0.75 µg
Rev expression vector ³		1.8 µg		0.3 µg
VSV-G expression vector ⁴	3 µg	2.7 µg	0.5 µg	0.45 µg
Total plasmid DNA	18 µg		3.0 µg	
Lipofectamine™ 2000	45 µl		7.5 µl	
Total Opti-MEM	3 ml		0.5 ml	
293T cells / vol. of medium	1.0×10^7 /5ml		1.7×10^6 /1ml	

Below we have listed the commonly used vectors for lentiviral packaging.

¹ For example: pCMV-deltaR8.91 (TRC), psPAX2 (Addgene)

² For example: pMDLg/pRRE (Addgene), pLP1 (Invitrogen), pPACKH1-GAG (SBI)

³ For example: pRSV-REV (Addgene), pLP2 (Invitrogen), pPACKH1-REV (SBI)

⁴ For example: pMD.G (TRC), pMD2.G (Addgene), pCMV-VSV-G (Addgene), pVSV-G (SBI), pLP/VSVG (Invitrogen)

Note: The transfection reagent Lipofectamine™ 2000 (LF2000, Invitrogen) is preferred for transfection. The average lentiviral titers in our preparations are around 5×10^6 - 5×10^7 infection units per ml (IU/ml) when titered with 293T cells.

Suggestion: We recommend using Biosettia Pre-mixed Lentiviral Packaging System (Catalog # pLV-PACK-500) for optimal lentiviral production.

Day 0: Seed 6.0×10^6 (1.0×10^6) 293T cells in a 10-cm plate (6-well plate), so that the cell density will be around 1.0×10^7 (1.7×10^6) at the time of transduction.

Day 1: Gently mix 45.0 (7.5) μ l LF2000 and 1.5 (0.25) ml Opti-MEM medium and incubate at room temperature for 5 minutes. Meanwhile, gently mix 18.0 (3.0) μ g in total of pLV-RNAi vector and helper plasmids mixture into 1.5 (0.25) ml Opti-MEM medium (Invitrogen).

Gently mix DNA and LF2000, incubate at room temperature for 20 minutes to allow DNA and lipid to form complexes. In the meantime, replace the overnight culture medium with 5.0 (1.0) ml DMEM + 10% FBS without antibiotics. Add the 3.0 (0.5) ml DNA-LF2000 complexes to 293T cells.

Note: We have noticed that fetal bovine serum purchased from different manufacturers may affect the attachment of 293T cells to the bottom of tissue culture plates, resulting in variation in the efficiency of lentiviral production. We recommend switching to a different brand of FBS if 293T cells disattach from plates during lentiviral production.

Day 2: Replace the media containing the DNA-LF2000 complexes with 10.0 (2.0) ml complete medium at 12-16 hours post-transfection.

Day4: Collect supernatants at 48 hours post-transfection and transfer media to a polypropylene storage tube. Spin the virus-containing media at 1300 rpm for 5 minutes to pellet any 293T cells that were carried over during collection. Carefully transfer the supernatant to a sterile polypropylene storage tube.

Note: Lentiviral stock may be stored at 4 °C for up to 5 days, but should be aliquoted and frozen at -80 °C for long-term storage.

Suggestion: To reduce the number of freeze and thaw cycles, aliquot lentiviral stock to smaller tubes before storage at -80 °C.

Titering the lentiviral stocks

It is important to titer the lentiviral stocks in the cell line of interest to produce consistent results using the equivalent multiplicity of infection (MOI) in experiments. Knowing MOI will help you to control the copy number of shRNA lentiviruses integrated into the chromosomes of the cells of interest. If you are using pLV-RNAi vectors with antibiotic selection genes, the titering procedure includes selection of stably transduced cells with the corresponding antibiotics and counting the antibiotic-resistant cell colonies. For pLV-RNAi vectors with fluorescent markers, a flow cytometry should be used to determine the viral titer by measuring the number of fluorescent cells. If you are using both methods to titer your lentiviruses, please keep in mind that the titers may be different due to sensitivities of FACS machine and cell lines resistant to drug selection.

A. Determination of lentiviral titers by antibiotics selection.

Day 0: Seed the cells of your choice in a 6-well plate so that the cell density that will be ~25-50% confluent at the time of transduction.

Day 1: Thaw lentiviral stock, gently mix virus and then prepare 2 ml 10-fold serial dilutions ranging from 10^{-3} to 10^{-7} in complete medium containing 5-8 μ g/ml polybrene.

Remove the medium from previous day and add 2 ml fresh dilutions into each well.

Suggestion: Leave one well uninfected for mock control. Spin transduction in a desktop centrifuge (e.g. Sorvall RT6000) at $1,000 \times g$ for 30-60 min at room temperature may be necessary if titering virus in cell lines other than 293T.

Day 2: Replace the medium containing virus and polybrene with 2 ml of complete medium.

Day 3-4: Replace medium with fresh medium containing puromycin or blasticidin to select for stably transduced cells.

Note: At least 48 hours of transduction allows lenti-shRNA to integrate into the host genome. The optimal puromycin (range 0.5-5 $\mu\text{g/ml}$) and blasticidin (range 2-50 $\mu\text{g/ml}$) concentration varies from cell line to cell line.

Suggestion: A pilot experiment should be performed to determine the minimum concentration of antibiotics required to kill the untransduced cells before this experiment.

Day 5-6: Replace medium with 2 ml fresh medium containing puromycin or blasticidin every 2 days.

Day 7-8: Allow puromycin-resistant or blasticidin-resistant colonies to form in dilution wells. No live cells should be growing in the mock control well.

Note: The number of days required for the formation of visible colonies may vary among different cell lines.

Wash wells twice with 2 ml PBS

Stain cells with 1 ml 0.5% crystal violet solution in 20% ethanol and incubate for 30 minutes at room temperature.

Wash wells with distilled water by submerging the plate in a tray full of water, and repeat the wash one more time.

Dry the plate and count the number of blue-stained colonies.

The titer should be the average colony number times the dilution factor.

Note: The transduction efficiency varies from cell line to cell line. The lentiviral titer in the cell line of your interest may be lower (sometimes more than 10-fold) or higher than the virus titer in commonly used cell lines such as 293T cells.

B. Determination of lentiviral titers by flow cytometry.

Day 0: Seed the cell of your choice in a 6-well plate so that the cell density that will be ~25-50% confluent at the time of transduction.

Note: The number of cells seeded in the well is required to calculate lentiviral titer later.

Day 1: Thaw lentiviral stock, gently mix virus and then prepare 2 ml 10-fold serial dilutions ranging from 10^{-1} to 10^{-4} in complete medium containing 5-8 $\mu\text{g/ml}$ polybrene.

Remove the medium from previous day and add 2 ml fresh dilutions into each well.

Suggestion: Leave one well uninfected for mock control. Spin transduction in a desktop centrifuge (e.g. Sorvall RT6000) at $1,000 \times g$ for 30-60 min at room temperature may be necessary if titering virus in cell lines other than 293T.

Day 2: Replace the medium containing virus and polybrene with 2 ml of complete medium.

Day 3-4: Follow your lab protocol to collect and resuspend cells for flow cytometry to determine the percentage of green or red fluorescent cells.

Note: At least 48 hours of transduction allows lenti-shRNA to integrate into the host genome.

The titer should be the average number of live fluorescent cells times the dilution factor.

Note: The transduction efficiency varies from cell line to cell line. The lentiviral titer in the cell line of your interest may be lower (sometimes more than 10-fold) or higher than the virus titer in commonly used cell lines such as 293T cells.

Example: 1×10^5 cells were seeded on Day 0 and the cell doubling time is around 24 hours. The FACS data showed 5% and 40% of green fluorescence cells in the 10^{-3} and 10^{-2} dilution wells, respectively. The lentiviral titer is calculated by multiplying the fraction of transduced cells by 2×10^5 (the expected number of cells in the well on Day 1, the time of transduction), and by the dilution factor.

$$0.05 \times (2 \times 10^5) \times 10^3 = 1 \times 10^7$$

$$0.4 \times (2 \times 10^5) \times 10^2 = 8 \times 10^6$$

The average lentiviral titer is 9×10^6 IU/ml

Lentiviral transduction for RNAi analysis.

Day 0: Seed cells at appropriate density.

Suggestion: Plate cells so that cell density will be ~10-25% confluent at the time of transduction.

Day 1: Transduction. Remove the medium from the tissue culture plate by aspiration and replace it with fresh complete medium containing 5-8 µg/ml polybrene. Gently mix lentivirus with pipette tip, and add appropriate amount of virus to each well.

Note: (1) Polybrene may be toxic to some cell lines. (2) The non-concentrated and non-purified lentiviral stock collected from 293T supernatant may contain substances affecting the target cell growth, especially when a large volume of low-titer lentiviral stock is added. In case the lentiviral stock inhibits cell growth, concentration and purification of the viruses may be required. (See below for a protocol of virus concentration) Alternatively, incubation time for transduction can be shortened to hours. For example, the virus-containing medium may be replaced with fresh medium after one hour of transduction.

Suggestion: Transduce cells at multiplicity of infection (MOI) = 1, 5, 10, 25, and 50 to determine the optimal knockdown condition. Spin transduction in a desktop centrifuge (e.g. Sorvall RT6000) at $1,000 \times g$ for 30-60 min at room temperature helps increase of transduction efficiency.

Day 2: Replace the transduction medium with fresh complete medium to remove lentivirus and polybrene.

Day 3-4: Select transduced cells (>50% confluence is recommended) with medium containing appropriate antibiotics or by flow cytometry to sort out fluorescence-positive cells if necessary.

Note: The optimal antibiotic concentration varies from cell line to cell line.

Suggestion: A pilot experiment should be performed to determine the minimum concentration of antibiotic required to kill the untransduced cells before this experiment.

Day 6+: Analysis of transduced cells.

Suggestion: Expand the culture of cell lines stably expressing shRNA and store the cell line stocks in liquid nitrogen before analyzing the cells.

Day 6-10: Doxycycline is required for inducing gene silencing in cells transduced with the inducible pLV-RNAi vector system. Make a solution of 1 mg/ml in water. Filter sterilize, aliquot, and store at -20°C . To induce

shRNA expression, adding Doxycycline to a final concentration of 1 µg/ml in complete medium is recommended. If desired, you may vary the concentration of Doxycycline from 0.01 to 1 µg/ml to modulate RNAi effect.

Note: Some basal expression of shRNA may be observed if the FBS contains tetracycline.

Optional: Concentration of lentivirus by ultracentrifugation

1. Filter lentivirus through a 0.45 µm filter.
2. Centrifuge at 25,000 rpm for 90 minutes in a SW-28 or SW-41 rotor.
3. Discard the supernatant and use a Pasteur pipette with an attached P100 tip to carefully remove the remaining medium.
4. Gently resuspend viral pellet in 1/100 volume of DMEM. Let the virus suspension sit for overnight at 4° C.
5. On the following day, mix gently, aliquot and freeze virus.

Optional: Validation of effective shRNA by reporter assay

For validating effective shRNA via a reporter assay, please visit the following web sites for further information:

www.promega.com (psiCHECK vector system)

www.invitrogen.com (BLOCK-iT RNAi targeting system)

www.clontech.com (ProLabel Detection system)

Safety guidelines for working with lenti-shRNA.

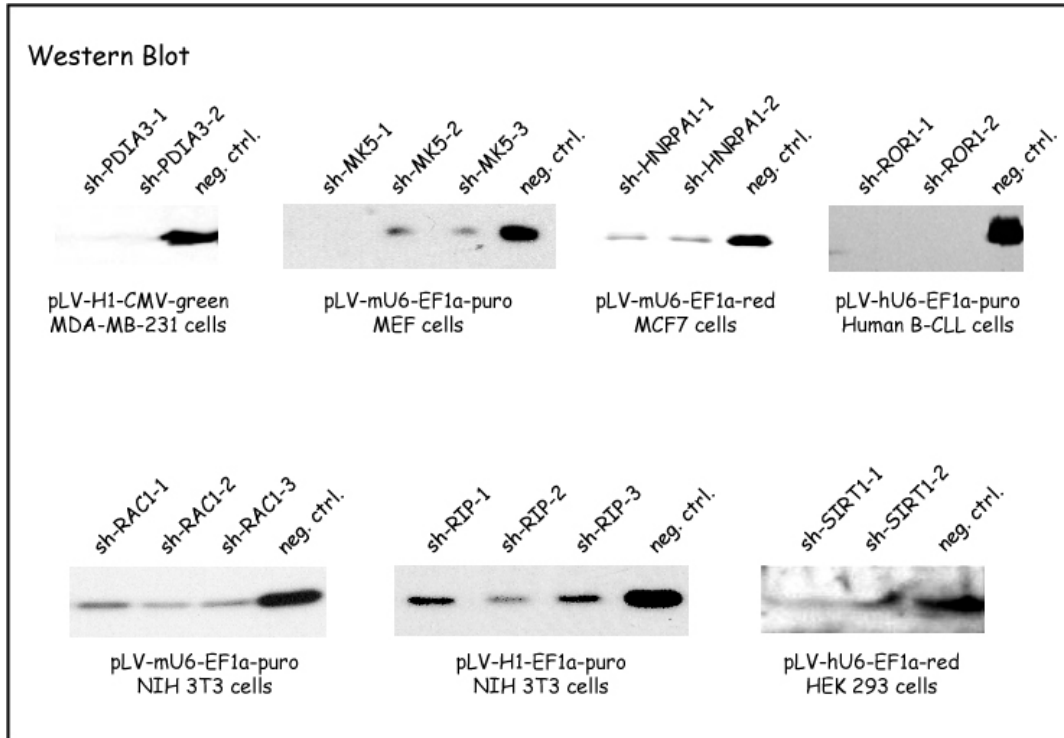
The recombinant lentiviruses have been designated as Level 2 organisms by NIH and CDC. A Biosafety Level 2 (BSL-2) facility is required in order to work with lentiviruses. The information of Biosafety in Microbiological and Biomedical Laboratories (BMBL) can be downloaded from the following link:

http://www.cdc.gov/od/ohs/biosfty/bmbl5/BMBL_5th_Edition.pdf

Please be aware that you are working with media containing lentiviral particles which could transduce human cells.

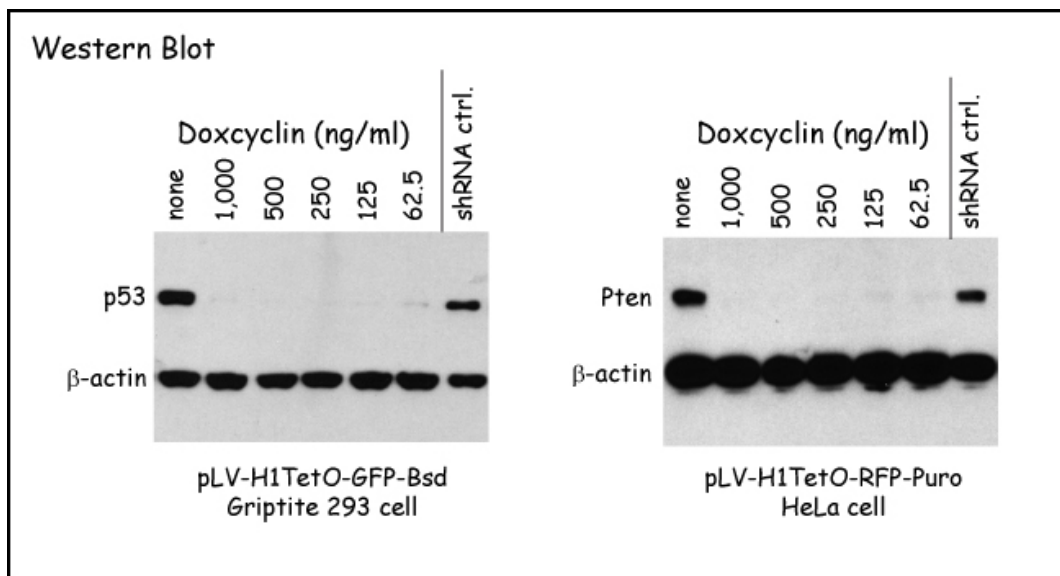
Examples of Gene Silence with pLV-RNAi Vectors

The shRNAs targeting genes of interest were cloned into pLV-RNAi vector system to generate lenti-shRNA viral stocks. The lentiviruses were then used to transduce the cell lines of choice. The cell lysates were collected at 2 days post-transduction, separated on SDS-PAGE gels, and subjected to Western blot analyses. The negative control (neg. ctrl.) is an shRNA inhibiting LacZ gene expression.



Examples of Gene Silence with Inducible pLV-RNAi Vectors

The shRNAs targeting genes of interest were cloned into the inducible pLV-RNAi vectors to generate lenti-shRNA viral stocks. The lentiviruses were then used to transduce the cell lines of choice. Doxycycline (Dox) was added to the transduced cells at 4 days post-transduction. The cell lysates were collected at 3 days after Dox induction, separated on SDS-PAGE gels, and subjected to Western blot analyses. The negative control (neg. ctrl.) is an shRNA inhibiting LacZ gene expression.



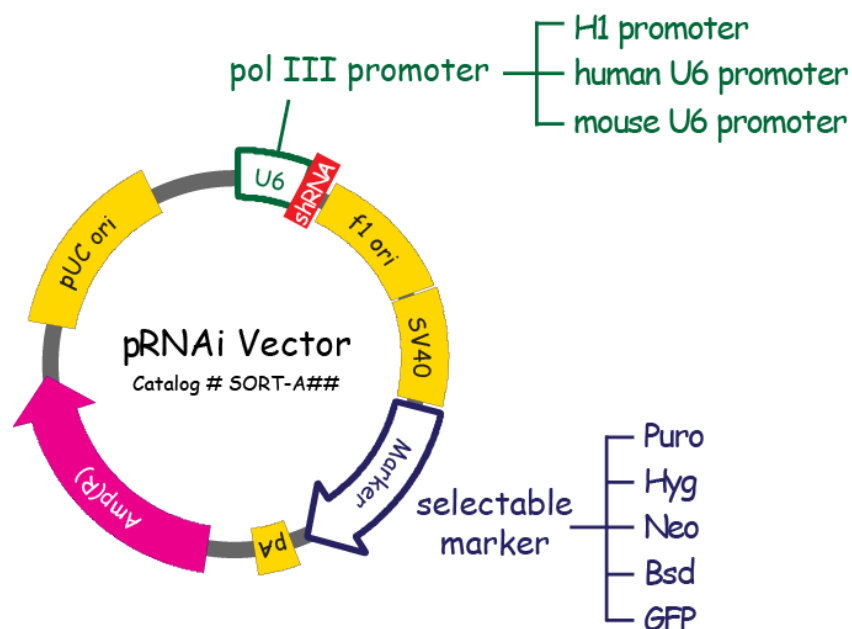
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Related Products

Plasmid-based RNAi Vector System (pRNAi)

The pRNAi is a regular RNAi vector system designed for introduction of shRNA into cells by transfection. Same as the pLV-RNAi vectors, the pRNAi system provides options of different RNA pol III promoters for expressing shRNA and different selectable markers used to establish stable cell lines if desired.



pRNAi Vector	Catalog #
pRNAi-H1-puro	SORT-A01
pRNAi-hU6-puro	SORT-A02
pRNAi-mU6-puro	SORT-A03
pRNAi-H1-hyg	SORT-A04
pRNAi-hU6-hyg	SORT-A05
pRNAi-mU6-hyg	SORT-A06
pRNAi-H1-neo	SORT-A07
pRNAi-hU6-neo	SORT-A08
pRNAi-mU6-neo	SORT-A09
pRNAi-H1-bsd	SORT-A10
pRNAi-hU6-bsd	SORT-A11
pRNAi-mU6-bsd	SORT-A12
pRNAi-H1-green	SORT-A13
pRNAi-hU6-green	SORT-A14
pRNAi-mU6-green	SORT-A15

Lentiviral Packaging Mix.

Pre-mixed Plasmids for Lentiviral Packaging	Catalog #
Lentiviral Packaging Mix. 500 µg	pLV-PACK-500
Lentiviral Packaging Mix. 1000 µg	pLV-PACK-1000

Lentiviral Packaging Cell Line.

Lentiviral Packaging Cell Line	Catalog #
Lenti-293T Cell Line (1 vial)	celine-01A

Nucleic Acid Purification Kits

Plasmid DNA Purification Kits	Catalog #
Plasmid DNA Extraction Mini Kit (100)	FAPDE 001
Plasmid DNA Extraction Mini Kit (300)	FAPDE 001-1
Plasmid DNA Extraction Midi Kit (50)	FAPDE 002-1
Plasmid DNA Extraction Maxi Kit (20)	FAPDE 003-1
EndoToxinFree Plasmid DNA Extraction Maxi Kit (10)	FAPDE 003-EF

User Notification

pLV-RNAi vector system is shipped to customers at ambient temperature to reduce shipping and handling costs without affecting product quality and effectiveness.

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