GenTarget Inc

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Lentiviral shRNA Expression Cloning Kit

for making shRNA expression knockdown lentivectors

Cat#	Product Name	Amount	Application
LTSH-H1-GB	pEco-Lenti-H1-shRNA-(**GFP**-**Bsd**) cloning kit	1 kit	Make shRNA expression lentivector with GFP-Blasticidin selection marker
LTSH-H1-GP	pEco-Lenti-H1-shRNA-(**GFP**-**Puro**) cloning kit	1 kit	Make shRNA expression lentivector with GFP-Puromycin selection marker
LTSH-H1-RB	pEco-Lenti-H1-shRNA-(**RFP**-**Bsd**) cloning kit	1 kit	Make shRNA expression lentivector with RFP-Blasticidin selection marker
LTSH-H1-RP	pEco-Lenti-H1-shRNA-(**RFP**-**puro**) cloning kit	1 kit	Make shRNA expression lentivector with RFP-Puromycin selection marker
LTSH-H1-Puro	pEco-Lenti-H1-shRNA-(**puro**) cloning kit	1 kit	Make shRNA expression lentivector with Puromycin selection marker
LTSH-H1-Bsd	pEco-Lenti-H1-shRNA-(**Bsd**) cloning kit	1 kit	Make shRNA expression lentivector with Blasticidin selection marker

Cat#	Product Name	Amount	Application
LTSH-U6-GB	pEco-Lenti-U6-shRNA-(**GFP**-**Bsd**) cloning kit	1 kit	Make shRNA expression lentivector with GFP-Blasticidin selection marker
LTSH-U6-GP	pEco-Lenti-U6-shRNA-(**GFP**-**Puro**) cloning kit	1 kit	Make shRNA expression lentivector with GFP-Puromycin selection marker
LTSH-U6-RB	pEco-Lenti-U6-shRNA-(**RFP**-**Bsd**) cloning kit	1 kit	Make shRNA expression lentivector with RFP-Blasticidin selection marker

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LTSH-U6-RP	pEco-Lenti-U6-shRNA-(**RFP**-**puro**) cloning kit	1 kit	Make shRNA expression lentivector with RFP-Puromycin selection marker
LTSH-U6-Puro	pEco-Lenti-U6-shRNA-(**puro**) cloning kit	1 kit	Make shRNA expression lentivector with Puromycin selection marker
LTSH-U6-Bsd	pEco-Lenti-U6-shRNA-(**Bsd**) cloning kit	1 kit	Make shRNA expression lentivector with Blasticidin selection marker

Each Kit Contents	Amount
One of the pre-cut, lniear vector: pEco-Lenti-H1 / U6-(Marker) linear vector; (dependent upon the each product catalog #, the **Marker** is different).	10ul (10rxn)
10x shRNA oligo annealing solution	50ul
5X ligation buffer	25 ul
T4 DNA ligase enzyme	10ul (10rxn)
Cloning positive control insert: annealed shRNA duplex [1x]: 10ul (10rxn) 5′- AGCGtgaaacgatatgggctgaatacCGAGgtattcagcccatatcgtttca actttgctatacccgacttatgGCTCcataagtcgggtatagcaaagtAAAA	
Sequencing primer: 5'- ggatccaatatttgcatgtcgctatg (for H1 promoter) Or 5'- ggactatcatatgcttaccg (for U6 promoter)	1 tube (10ul x 25ng/ul) (10 rxn)

Note: Chemical competent cells are required for the cloning, but not included in this kit. You can use any common chemical competent cells, like DH5a, NovaBlue or others.

Storage:

shRNA Cloning Kit is shipped on dry ice. Each kit contains sufficient amounts for **10 shRNA cloning reactions**. Upon received, stored at -20°C. Products stable for 6 months.

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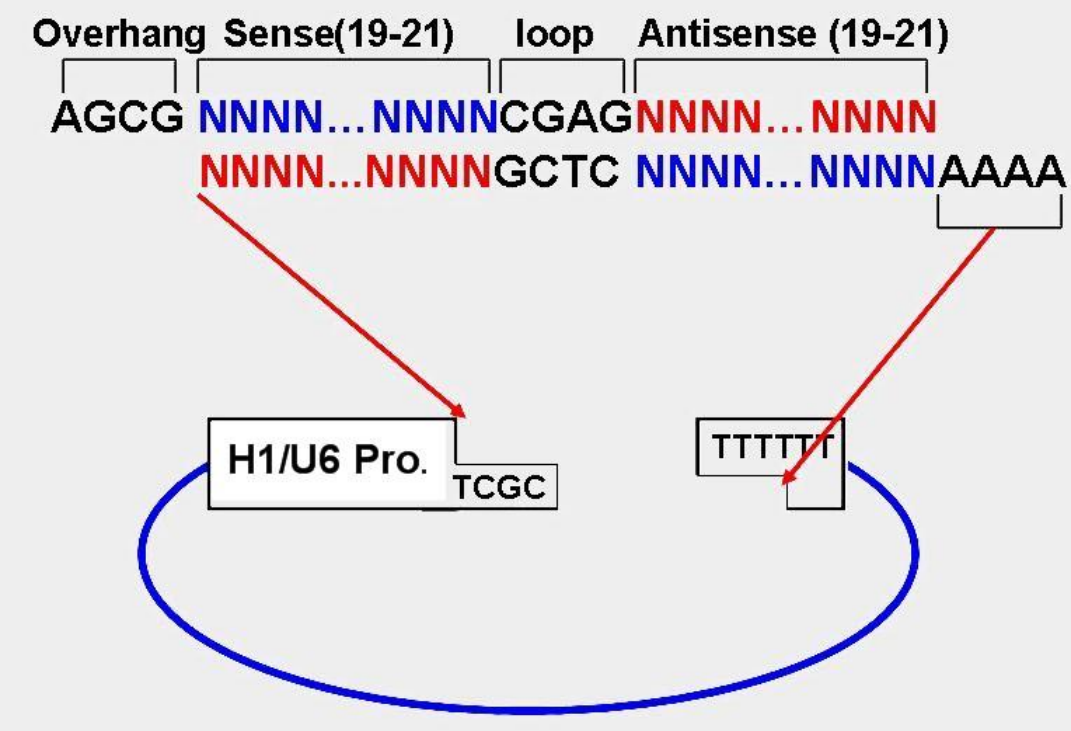
Quick protocol outlines (for experienced users):

1. Design two DNA oligonucleotides, hairpin structure encoding shRNA sequence,
2. Anneal the two oligo to generate a duplex;
3. Clone the duplex into provided linear **pEco-shRNA** vector by T4 ligation reaction;
4. transform into competent cells and grow in LB/ ampicillin plate;
5. Pick 1-2 colonies, mini prep the plasmid DNAs, confirm positive clone by sequencing;
6. knockdown analysis after transfect shRNA plasmids into mammalian cells;
7. Produce shRNA lentivirus and transduced into desired cells for knockdown analysis or generate shRNA stable cells. (**Note:** to produce the shRNA knockdown lentivirus, you require the virus production cell line (CAT#: TLV-C) and virus packaging plasmid mixture (CAT#: HT-Pack), which are not included in this cloning kit, but available for purchase from GenTaget other vendors).

Cloning Scheme



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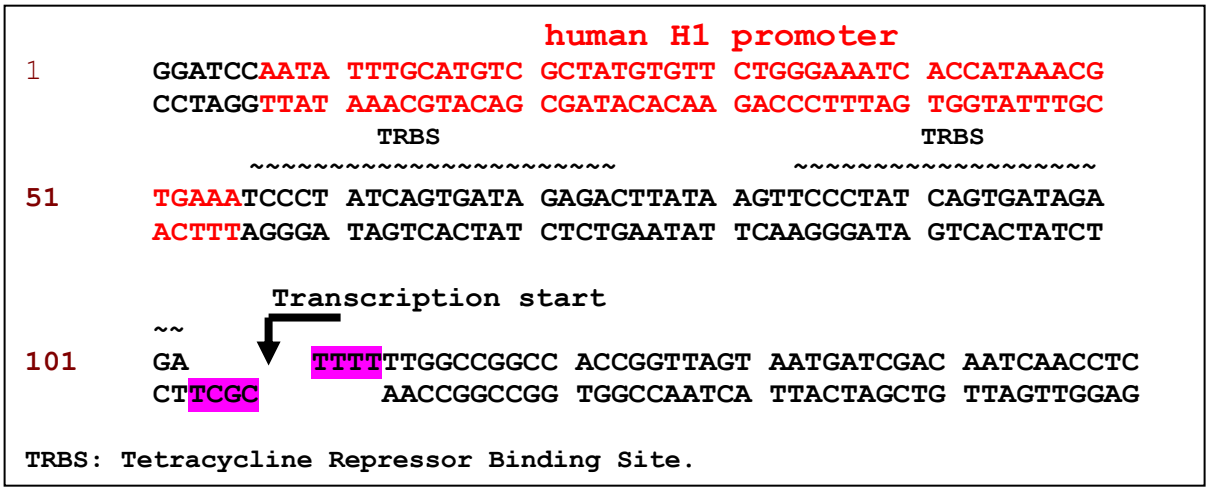

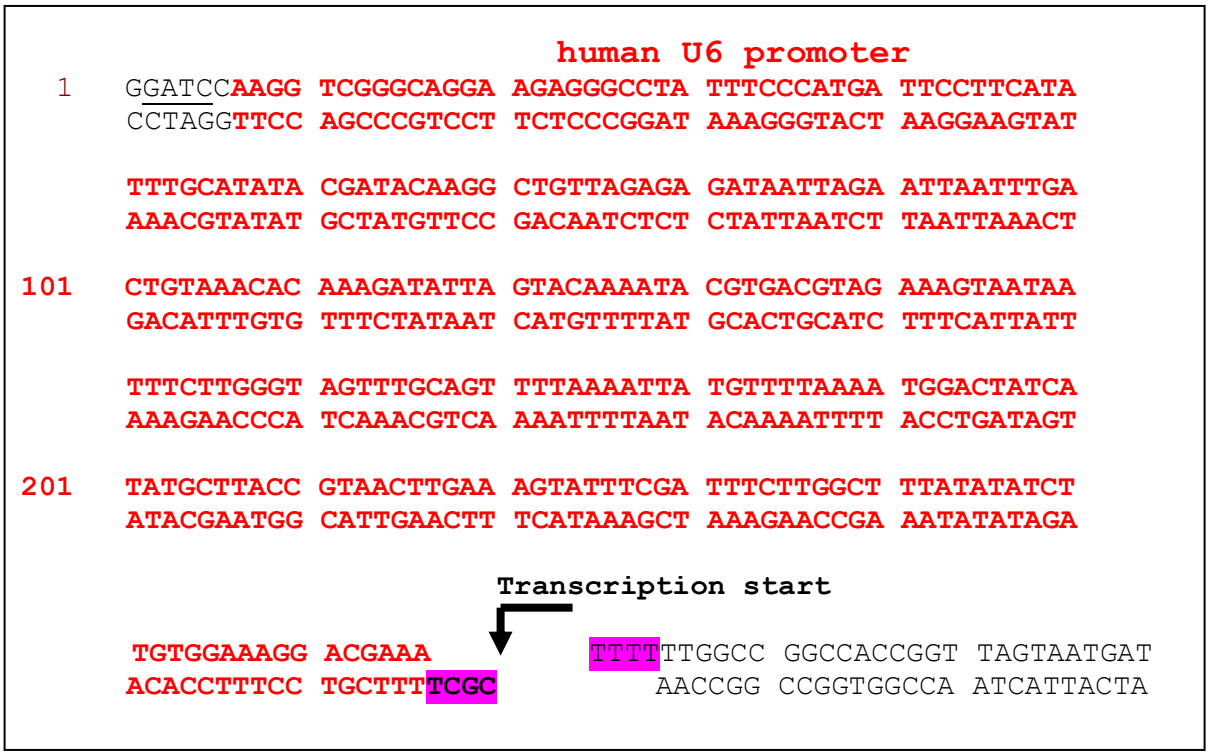

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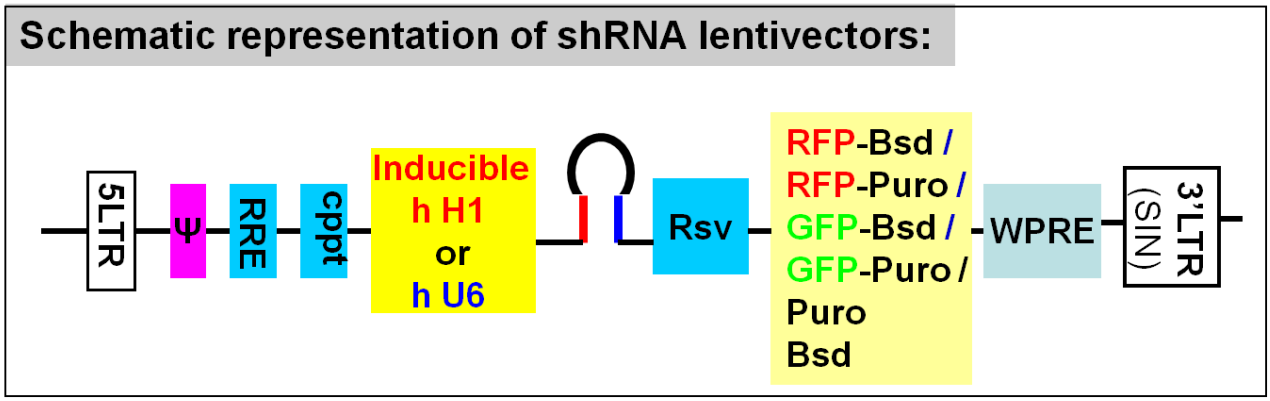
Vector Schematic maps



Note: The vector's full sequences can be **downloaded** from our website. To make the final clone's map, simply paste shRNA hairpin insert sequence (not include the 4bp overhangs at both ends) at position between 106 and 107 for H1 promoter vector, or between 270 and 271 for U6 promoter vector.

Introduction:

RNA interference (RNAi) technology is a powerful tool for loss-of-function (knockdown silencing) research in mammalian cells. Originally observed to inhibit gene expression *in vivo* through short double-stranded RNAs, RNAi works through a series of enzymatic reactions mediated by short RNAs having sequences complementary to those of the silenced target. These reactions result in target mRNA degradation or translational repression.

RNAi knockdown can be introduced by synthetic short double-strand RNA (siRNA) or by vector expressed stem-hairpin RNA (shRNA) which is further processed by the Dicer enzyme to produce double-stranded short RNAs. Chemically synthesized double-stranded RNA (siRNA) has a transient silencing effect only; in contrast, selection of clones for stable vector-expression of RNAi can provide long term silencing.

Vector expressed RNAi for gene silencing provides a convenient method to functional studies in both animal and cell line models. Variety RNAi vectors are commercially available now in the market.

shRNA expression lentivectors:

GenTarget provides cloning kits for making shRNA lentiviral expression vectors with different selection markers (blasticidin, puromycin, GFP-

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blasticidin, GFP-puromycin, RFP-blasticidin, and RFP-puromycin). The shRNA expression may be driven by the RNA polymerase III promoter, the optional inducible H1 promoter, **or the constitutive U6 promoter.** These promoters are positioned to ensure the production of precise shRNA transcripts.

About promoter selection: U6 and H1 are poly III promoters, and are best suited for transcription of short RNA molecules. Both are active in the majority of mammalian cell types, but one could be stronger than the other in some cell types. Unless you already knew or have validated the better promoter for your cells, you can select either promoter for shRNA expression. The H1 promoter is also an optional inducible promoter, which means it can be used for regular constitutive expression (like the U6 promoter) or optionally used for tetracycline-inducible expression when the repressor protein (TetR) is present in advance. With **inducible expression,** transcription of shRNA is **repressed in the presence of TetR** alone **and induced** by the addition of **tetracycline. The** presence **of TetR can be achieved by using** the **Tet-repressor stable cell line (Cat# SC005)**, by **using GenTarget's pre-made** Tet-repressor lentiviral particles**, or** by **co-transfection with TetR expression vectors**.

The Lentiviral shRNA vector allows generation of shRNA lentiviral particles that can be transduced into cell lines. The resulting shRNA stable expressing cells can then be selected by antibiotic resistance or, if one of the fluorescent-antibiotic fusion markers is used, sorted by fluorescent signal. (**Note:** to produce the shRNA knockdown lentivirus, you will need the virus production cell line (CAT#: TLV-C) and virus packaging plasmid mixture (CAT#: HT-Pack), which are not included in this cloning kit, but are available for purchase from GenTarget.

Each kit contains a pre-cut, ready-to-use linear vector for ligation of shRNA duplex sequence. The linear vector was designed for cloning of double-strand DNA encoding a short hairpin RNA. Once transcribed, the shRNA is processed into short RNA *in vivo* for RNAi analysis. To make a shRNA expressing vector, two synthetic oligonucleotides are first annealed to form the DNA duplex which is then cloned into the ready-to-use, linear vector via T4 enzyme ligation.

Each kit provides enough material for 10 cloning reactions.

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Key Features:

1. **Ready-to-use linearized vector**--no need for tedious preparation of vector backbone
2. **Precisely directional cloning** of your DNA duplex-encoded shRNA structure
3. **Rapid, highly efficient cloning** with low background. Cloning can be done at room temperature for 30-60 min. Clones are >90% positive.
4. **Internal fluorescent reference**: the vector encodes a fluorescent protein (**GFP** or **RFP**), allowing real-time monitoring of transfection or virus transduction efficiency.
5. **Long-term stable silencing effect**: the vector encodes an antibiotic marker or a **dual marker** (a fluorescent-antibiotic fusion marker) allowing generation of stable cell lines for long-term knockdown.
6. **Generated lentiviral shRNA particles can be transduced into your cells of interest.** The lentivector can produce lentivirus for transduction into hard-to-transfect cells for long term knockdown studies. Or, the lentivector can be transfected into cells for gene expression knockdown.
7. **Optional inducible knockdown**: The shRNA lentivector with the H1 promoter can be used for constitutive high expression of shRNA without the need for induction. Optionally, the vector's human H1 promoter allows inducible expression of shRNA when the tetracycline repressor protein (TetR) in present in advance (see inducible expression link for its mechanism).
8. **Insert compatible:** the same annealed shRNA duplex can be readily cloned into all other linear shRNA lentivectors with different promoters or different selection markers.

Protocols:

Note: Chemically competent cells are required for cloning, but not included in this kit. You can use any common chemical competent cells, such as DH5a, NovaBlue, or others.

- **Quick protocol (*for experienced users*):**

8. Design two DNA oligonucleotides with hairpin structures encoding the shRNA sequence. **Note:** The vector's full sequence may be downloaded from our website. To make the final clone map,

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simply paste the shRNA hairpin insert sequence (not including the 4bp overhangs at both ends) between positions 106 and 107 for the H1 promoter vector, or between 270 and 271 for the U6 promoter vector.

9. Anneal the two oligos to generate a duplex.

10. Clone the duplex into the provided linear pEco-shRNA vector by T4 ligation.

11. Transform into competent cells and grow in LB/ ampicillin plate.

12. Pick 1-2 colonies; perform mini prep isolation of the plasmid DNAs; confirm positive clone by sequencing.

13. Perform knockdown analysis after transfecting the shRNA plasmids into mammalian cells.

14. Produce shRNA lentivirus and transduce into the desired cells for knockdown analysis or generation of shRNA stable cells. **Note:** to produce shRNA knockdown lentivirus, you will need the virus production cell line (CAT#: TLV-C) and virus packaging plasmid mixture (CAT#: HT-Pack), which are not included in this cloning kit, but available for purchase from GenTarget.

- **Full Protocol:**

1. **Design single-stranded DNA oligonucleotides**:

   - **Design** two DNA oligonucleotides, a top strand and a bottom strand according to the following structure: The top strand requires a AGCG overhang at its 5'-end, followed by the selected target sequence (sense sequence) of 19-21 nucleotides, a CGAG loop (or use your own loop), and the reverse-complementary sequence of the target sequence (antisense). The bottom strand requires an AAAA-overhang at its 5'-end with the remainder complementary to the top strand.

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- **Loop length** has little or no effect on knockdown. Four nucleotides (CGAG) have been determined to be the minimal length for effective RNAi knockdown. You may design your own loop sequence, such as a restriction enzyme (RE) recognition sequence; however, most RE sequences are palindromes which form a continuous hairpin structure with the RNAi sequence which may not be processed correctly into RNAi by Dicer.

- Two overhangs ensure the **directional cloning** of the annealed double stranded oligonucleotide into the linear vector provided.

- The **transcription start site** is at the first nucleotide of the target sequence (sense) on the top strand. Native H1 RNA initiates at an A, so A is recommended as the first base in the sense target sequence.

- **shRNA target sequence (sense) selection**:
  There are some general guidelines for selecting an shRNA sequence, but the effectiveness of an RNAi target sequence must be verified empirically. To avoid off-target effects, design a scrambled sequence from the selected shRNA sequence or a universal Null sequence as a negative control for knockdown analysis. Many online tools or designers can help your selecting your shRNA sequence. Please see the following links.

  - **Promega's siRNA Target Designer**
  - **Clontech's RNAi Target Sequence Selector**
  - **Gene Link shRNA designer**
  - **Invitrogen's BLOCK-iT™ RNAi Designer**
  - **katahdin RNAi Central**
  - **WI siRNA selection program**

2. **Clone shRNA expression plasmids**:
   - **Anneal** the two single-stranded DNA oligonucleotides:

Set up the annealing reaction as follows:

100 µM Top strand oligo:	10 µl
100 µM Top strand oligo:	10 µl
10X oligo annealing buffer:	3 µl
DNase-free water:	7 µl
Total volume:	30 µl

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Incubate the reaction mixture at 95°C for 5 minutes (can be done in PCR machine). Leave the mixture on the PCR machine to gradually cool down for **30 minutes**. The, put tube on ice. Make 1: 1000 dilution (add 1µl annealed mixture in 99 µl Cold-DNase free water, and then take 2 µl, added into 18µl of 1x annealing solution on ice). Final diluted annealed duplex is ready for ligation. Save undiluted duplex at -20oC for long term storage. (**Note:** always put diluted, annealed duplex on ice to avoid double strand DNA melt.)

- **Ligate** the two single-stranded DNA oligonucleotides into the vector:
  Set up the ligation reaction as follows:

pEco-Lenti-H1-shRNA linear vector:	1 µl
Annealed duplex (1: 1000 dilution):	1 µl
5X T4-ligase buffer:	2 µl
DNase free water:	5 µl
T4 ligase:	1 µl
Total volume:	10 µl

Mix reaction well and incubate for 30-60 minutes at room temperature (incubate for a longer time to generate more colonies). Place reaction on ice and ready for transformation.

Set up a cloning **positive control reaction** by using **1 µl** of annealed control-shRNA duplex (provided) and thaw on ice. The positive clone generated from the control shRNA duplex is capable of silencing the firefly luciferase gene (see "Example of knockdown" below in this manual).

- **Transform Cells:**
  1. Transfer 2µl of the ligation reaction into a vial of DH5a chemically competent *E. Coli* cells, mix gently. (**Note**: chemically competent cells are not included in this kit)
  2. Place cells on ice for 5 minutes, and then transfer cells into 42 ^{o}C water bath; incubate for 30 seconds without shaking. Immediately transfer cells to ice.
  3. Add 250 µl of SOC medium; incubate at 37 ^{o}C for 1 hour with shaking.

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4. Spread all 250 µl of cultured cells onto a pre-warmed LB plate containing 100 µg/ml **ampicillin** and incubate overnight at 37 °C

Note: In general, 30-100 colonies will be produced from your reaction and 0 to 5 colonies from the no-insert control reaction.

3. **Confirm positive clones**:

- Pick 1-2 colonies, grow in LB/ampicillin medium, isolate plasmid DNAs by miniprep column, and sequence using the sequencing primer provided. **Note:** The provided primer is at a ready-to-use concentration of 25 ng/µl; simply use 1µl per reaction. Sequencing of the stem hairpin structure may require a special solution for best results.

- Purified positive plasmid DNAs are ready for transfection into cells for knockdown analysis, or for use in producing lentiviral particles in packaging cell lines. The generated lentiviral particles can be used to transduce a cell line.

4. **Production of shRNA lentiviral particles**:

Note: GenTarget's pEco-Lenti-shRNA vectors are fully compatible with most commercially available lentiviral systems including ViraPower / Block-it (Invitrogen), MissionShRNA (Sigma), Lent-X (Clontech), GIPZ Lentiviral ShRNAmir (Open Biosystems), etc. The following protocol is recommended for the highest virus titer using GenTarget's lentiviral reagents.

- **Cells**: seed 293T packaging cells (cat# **TLV-C**) in plate or flask according virus production scale; incubate overnight in 5% CO2.

- **Transfection**: at the time for transfection, cells should grow to 90% confluence. Use your favorite transfection protocol according to the transfection reagent manual to co-transfection of shRNA lentivector and packaging plasmid mixture.

- The next day, remove the medium and replace it with complete culture medium.

- Harvest viral supernatants at 48-72 hours after transfection.

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- Centrifuge virus particles at 3000 rpm x 15 minutes at 4 °C to pellet cellular debris. Filter through a sterile 0.45 µm filter.

- Store virus at -80 °C.

5. **Transduction of shRNA lentivirus and selection of stable clones**:

   - **Cells**: Plate the host cells at 10%-20% confluence, and culture at 37°C overnight.

     Note: For inducible shRNA expression, the host cells must be a Tet-repressor stable cell line. GenTarget provides a Tet-repressor expression cell line with Blasticidin selection (Cat# **SC005**).

   - On the 2nd day, thaw the lentiviral stock. Change medium to complete medium containing 6 µg/ml polybrene, and add the appropriate amount of lentiviral particles to achieve an MOI range of 1 to 10. Incubate overnight at 37°C.

   - At 24 hours after transduction, remove the viral-containing medium and replace with complete medium. Incubate overnight at 37 °C.

   - At 72 hours after transduction, remove the medium and replace with complete medium containing the appropriate amount of antibiotic to select for stably transduced cells.

     Note: the amount of antibiotic added is dependent upon cell type. A kill curve must be made to determine the correct amount of antibiotic. In general, 0.5 - 10 µg/ml of blasticidin and 10-100 µg/ml puromycin is used.

   - Change medium containing puromycin every 3-4 days.

   - As soon as the mock well has no live cells, trypsinize the antibiotic-resistant colonies and make a series of dilutions, and seed into each well of a 24-well plate; continue to grow cells.

   - Inspect cells under a fluorescence microscope; select the wells that exhibit GFP signal from all cells and grow up in a large flask.

   - Collect cells and freeze in cryogenic vial.

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Validation of shRNA knockdown:

In general, most RNAi designers can obtain a greater than 50% success rate with a greater than 75% knockdown level. However, there is no "holy-grail" for an ultimate RNAi design; the effectiveness of an RNAi sequence must be empirically determined. Different approaches, including Q-PCR and western blot, are used to validate shRNAs by measuring mRNA levels or protein products. Alternatively, reporter assays can be used to screen shRNAs.

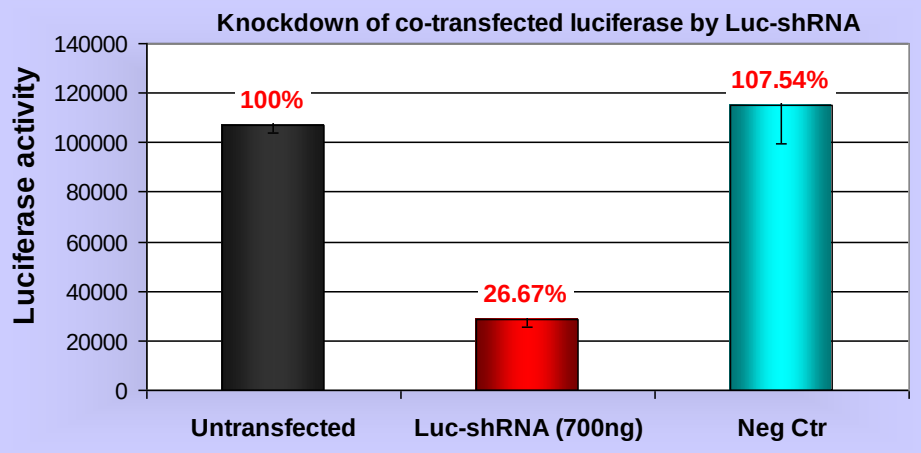
In order to screen for the so called "off target effect," we have designed a negative shRNA sequence for use as a universal negative control. In order to minimize non-specific knockdown, this sequence was designed against the entire human and mouse transcripts with minimal sequence homology to any human or mouse ORF sequences. The **negative-control shRNA lentiviral particles containing this sequence** may be purchased from GenTarget, or you can design and clone your own negative control shRNA using this kit.

Examples of knockdown using pEco-H1-shRNA-(GFP-Bsd) vectors:

Example A: Luc-shRNA: (measure the luciferase activity by luciferase assay kit)

Luc-shRNA top strand:

5'- AGCGatgaaacgatatgggctgaatacCGAGgtattcagcccatatcgtttca



Knockdown of co-transfected luciferase expression in 293-HEK cells by pEco-H1-luc-shRNA-(GFP-Bsd) plasmid.
The Luc-shRNA duplex was cloned into the pEco-H1-shRNA-(GFP-Bsd) vector, then the Luc-shRNA plasmid (700ng) was co-transfected along with the pcDNA3.1-luciferase (firefly) plasmid (100ng) into 293HEK cells in a 24-

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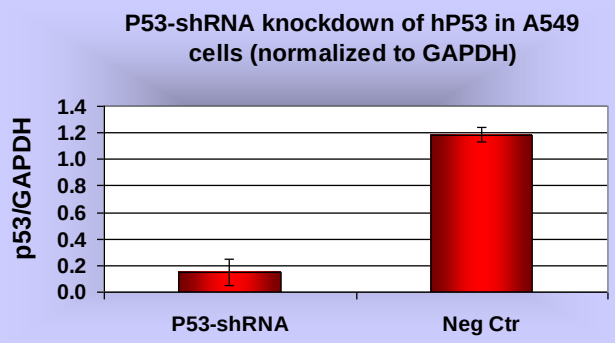
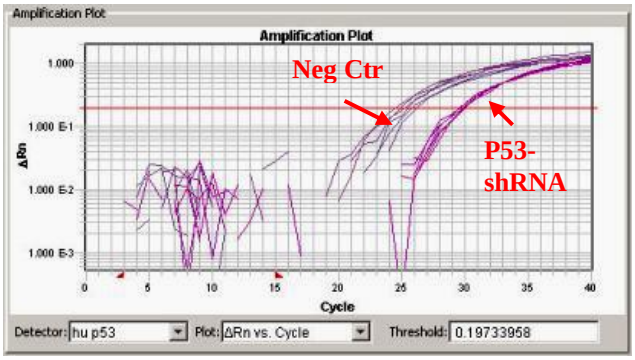
well plate. Cells were harvested at 3 days post- transfection. Luciferase activity was measured from cell lysate (10 µl/ea.) using GenTarget's luciferase reporter assay kit on an LMax microplate luminometer. Null-shRNA plasmid served as the Neg-Ctr plasmid.

Example B: P53-shRNA: (Measure the mRNA level by real-time qPCR)

P53-shRNA top strand:
5'- AGCGccactacaactacatgtgtaaCGAGttacacatgtagttgtagtgg





Knockdown of endogenous human P53 in A549 cells by pEco-H1-p53-shRNA-(GFP-Bsd) plasmid. P53-shRNA duplex was cloned into the pEco-H1-shRNA-(GFP-Bsd) vector, transfected into A549 cells, and grown in medium containing 10 µg/ml Blasticidin. Cells were harvested at 3 days post-transfection. P53 levels were assayed from extracted total RNAs by real-time Q-PCR. Data were normalized to internal levels of GAPDH. The Null-shRNA plasmid served as the negative control.

Conclusion: RNAi gene silencing can be effectively carried out via pEco-H1-shRNA-(GFP-Bsd) vectors.

Trouble shooting:

Problems	Solution
Few or no colonies	1. Make freshly annealed duplex and dilute for ligation reaction 2. Extend ligation time or leave at 4 °C overnight 3. Use more duplex--add 5µl diluted duplex in ligation reaction 4. Use different competent cells

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Lentiviral shRNA cloning service:

GenTarget offers a cost-effective shRNA cloning services. Simply tell us the target you want to knock down or provide us with your own sequences, and we will design the shRNA for your target and clone its sequence into our shRNA expression vectors with the promoter and marker of your choice. We will deliver sequence-verified shRNA plasmids and the pre-made lentiviral shRNA particles. Our service has the fastest turnaround time and lowest costs available. Please **contact us** for quote or email us.

References:

1. Lee, R. C., et al, The C-elegans Heterochronic Gene lin-4 Encodes small RNAs with antisense complementarily to lin-14. Cell, 75(843-854), 1993.
2. Hannon, G.J., RNA interference. Nature, 418(6894): p. 244-51, 2002.
3. Bosher, M., et al, RNA interference, Nature Cell Biol. 2 E31-E36, 2000.
4. Meister, G. and T. Tuschl, Mechanisms of gene silencing by double-stranded RNA. Nature, 2004. 431(7006): p. 343-9.
5. Paddison, P.J., A.A. Caudy, and G.J. Hannon, Stable suppression of gene expression by RNAi in mammalian cells. Proc Natl Acad Sci U S A, 2002. 99(3): p. 1443-8.

Related products: GenTarget's Pre-made lentivirus Products:

Product Category	Product Description (please click category name to see product's pages)
Human, mouse or rat ORFs	Premade lentivirus expressin a **human, mouse or rat** gene with RFP-Blastididin fusion dual markers.
Fluorescent markers	Preamde lentivirus express human codon optimized fluorescent protein, **GFP / RFP/ CFP/ BFP / YFP**.
Luciferase expression	Premade lentivirus for all kinds of luciferase protein expression**: firefly and Renilla** with different antibiotic selection markers.
CRE recombinase	Premade lentivirus for expressing **nuclear permeant CRE** recombinase with different flurescent and antibiotic markers.
LoxP ColorSwitch	Premade lentivirus expressing "LoxP-GFP-Stop-LoxP-RFP" cassette, used to monitor the CRE recombination event in vivo.
CRISPR /hu CAS9	Preamde lentivirus express humanzied wild-type **Cas9** endonuclease for genomic editing with **CRISPR**
TetR inducible expression repressor	Premade lentivirus expressin **TetR** (tetracycline regulator) protein, the repressor protein for the inducible expression system.
iPS factors	Premde lentivirus for human and mouse iPS **(Myc, NANOG, OCT4, SOX2, FLF4**) factors with different fluorescent and antibitoic markers

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T-antigen Expression	Express **SV40 large T antigen** with different selection markers
Cell Organelle imaging	Premade lentivirus for cell organelle imaging. The fluorescent marker **GFP/RFP/CFP was sub-cellular localized** in different cell organelle for living cell imaging.
LacZ expression	Express different full length **β- galactosidase (lacZ)** with different selection markers
Anti-miNA lentivirus	Pre-made lentivirus expression a specific **anti-miRNA** cassette.
Fluorescent-ORF fusion	Pre-made lentivirus expression a "**GFP/RFP/CFP-ORF**" fusion target.
Pre-made shRNA lentivirus	Premade shRNA lentivirus for knockdown a specific genes (**P53, LacZ, Luciferase** and more).
microRNA and anti-microRNA lentivirus	Premade lentivirus expression human or mouse **precursor miRNA**. And **anti-miRNA** lentivector and virus for human and mouse miRNA.
Negative control lentiviruses	Premade **negative control lentivirus with different markers**: serves as the negative control of lentivurs treatment, for validation of the specificity of any lentivirus target expression effects.
Other Enzyme expression	Ready-to-use lentivirus, expressing **specific enzymes** with different selection markers.