

HUSH ShRNA PLASMIDS AND LENTI-PARTICLES (29-MER)

APPLICATION GUIDE

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Package Contents and Storage Conditions

Lentiviral particles, TLxxxxxV (SKU) in pGFP-C-shLenti

Materials	Format	Quantity
Target specific lenti particles expressing 29-mer shRNA	Lentiviral particles	4 vials of target-specific lentiviral particles (4 unique shRNA sequences), 0.5 mL each
Scrambled 29-mer lenti particles (TR30021V)	Lentiviral particles	1 vial of scramble control, 0.5 mL

TLxxxxx (SKU) in the lentiviral vector, pGFP-C-shLenti

Materials	Format	Quantity
Gene-specific shRNA in pGFP-C-shLenti vector	Lentiviral plasmids	Four unique constructs per gene, 5 ug per vial.
HuSH 29-mer Scrambled in pGFP-C-shLenti (TR30021)	Lentiviral plasmids	5 ug plasmid DNA

TGxxxxx (SKU) in the retroviral vector, pGFP-V-RS vector

Materials	Format	Quantity
Gene-specific shRNA expression pGFP-V-RS vectors	Purified and sequence-verified expression plasmids with gene-specific shRNA cassettes	5 ug plasmid DNA per vial. Four unique constructs per gene
HuSH 29-mer Non-Effective Scrambled pGFP-VRS (TR30013)	A purified and sequence-verified plasmid containing non-effective 29-mer scrambled shRNA cassette	5 ug plasmid DNA

TFxxxxx (SKU) in the retro-viral vector, pRFP-C-RS vector

Materials	Format	Quantity
Gene-specific shRNA expression pRFP-C-RS vectors	Purified and sequence-verified expression plasmids with gene-specific shRNA cassettes	5 ug plasmid DNA per vial. Four unique constructs per gene.
HuSH 29-mer Non-Effective Scrambled pGFP-VRS (TR30015)	A purified and sequence-verified plasmid containing non-effective 29-mer scrambled shRNA cassette	5 ug plasmid DNA

TRxxxxxx (SKU) in the retro-viral vector, pRS

Materials	Format	Quantity
Gene-specific shRNA expression pRS vectors	Purified and sequence-verified expression plasmids with gene-specific shRNA cassettes	5 ug plasmid DNA per vial. Four unique constructs per gene.
HuSH 29-mer Non-Effective Scrambled pRS (TR30012)	A purified and sequence-verified plasmid containing non-effective 29-mer scrambled shRNA cassette	5 ug plasmid DNA

Storage conditions

The dried plasmids can be stored at 4°C. However, once reconstituted with dH₂O, the plasmids must be stored at -20°C. Viral particles should be stored at -80°C, avoid repeat freeze and thaws.

Related products

Positive controls: Positive control shRNA expression vectors are available for Green Fluorescent Protein (GFP), Red Fluorescent Protein (RFP) and Luciferase (Luc) in the pRS, pGFP-V-RS, and pRFP-C-RS vector. They have been validated for inhibiting the expression of their respective target genes in transient transfection experiments in HEK293 cells with co-transfected GFP, RFP or Luciferase plasmids, respectively.

- TR30001 HuSH 29mer against Enhanced GFP (in pRS vector)
- TR30002 HuSH 29mer against Luciferase Protein (in pRS vector)
- TR30009 HuSH 29mer against tGFP (in pRS vector)
- TR30016 HuSH 29mer against tGFP (in pRFP-C-RS vector)
- TR30017 HuSH 29mer against tRFP (in pGFP-V-RS vector)
- TR30021 HuSH 29mer against tGFP (in pGFP-C-shLenti vector)

PowerPrep® HP Plasmid Purification Kits: OriGene now offers its own state-of-the-art plasmid purification technology to its customers in the form of PowerPrep® HP Midi and Maxi kits. The same transfection-grade plasmid DNA available for our gene-specific products can now be achieved with PowerPrep® kits. Our plasmid purification technology offers higher yield and lower endotoxin levels than market leaders and utilizes ion-exchange columns eliminating the use of syringes. More detailed information on the PowerPrep® HP kits is available at www.origene.com/other/Plasmid_Purification.

TrueORF cDNA expression clones: OriGene has one of the largest collections of cDNA clones available in the world. TrueORF clones have

built-in C-terminal tags for easy detection with anti-tag antibodies. More detailed information on the OriGene TrueORF collection can be found at www.origene.com/ORF.

LentiViral Packaging Plasmid Mix: This plasmid mix (TR30022) is necessary for lentiviral packaging. When transfecting the HuSH plasmid into a lentiviral packaging cell line, this plasmid is to be co-transfected.

Additional materials recommended

Transfection reagent: Transfection reagents must be selected and optimized based on the cell type being used. For cells that are inherently difficult to transfect, a retroviral gene delivery system can be used. OriGene suggests transfection reagents like TurboFectin 8.0 (OriGene) or Viromers which have been shown to transfect common cell types, <http://www.origene.com/cdna/transfection.msp>

LB-kan (25 ug/ml) LB-amp (100 ug/ml) LB_Chloramphenicol (34 ug/ml) liquid media

LB-kan (25 ug/ml) or LB-amp (100 ug/ml) LB_Chloramphenicol (34 ug/ml) agar plates

Reagents and supplies for immunoblots: user preferred. OriGene has a selection of antibodies and detection reagents that are available at www.origene.com/antibody/.

Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund.

RNAi Collection Overview

As a cellular defense mechanism, host cells process double-stranded RNA into small molecules which target homologous RNAs for destruction (Hannon 2002). In mammalian cells, RNA interference (RNAi) can be triggered by siRNAs that cause strong, yet transient inhibition of gene expression on specific genes (Elbashir 2001). These siRNAs can be synthesized and transfected into mammalian cells, resulting in effective suppression of gene expression. Unfortunately, such suppression is

transient. By contrast, short hairpin RNAs (shRNA) can suppress gene expression over a prolonged period by continually expressing an RNA duplex (Brummelkamp 2002; Paddison 2002).

HuSH shRNA

OriGene offers genome-wide shRNA for human, mouse and rat; available in 4 different vectors (3 retroviral vectors, pRS, pGFP-V-RS and pRFP-C-RS; 1 lentiviral vector, pGFP-C-shLenti). Lentiviral particles are also available. Other shRNA vectors also available for different fluorescent marker or mammalian selection markers, please see details at:

http://www.origene.com/shRNA/vector_information.aspx .

shRNA insert description

The HuSH shRNA gene-specific expression cassettes are prepared using synthetic oligonucleotides. These oligonucleotide sequences were computer designed for optimal suppression of gene expression and minimal off-target effects. All shRNA sequences are verified through DNA sequencing analysis. The sequences are provided to the user on the OriGene website prior to purchase.

The HuSH shRNA gene-specific expression cassettes were optimized to include both the termination signal for RNA Pol III and GC content targeted at 50% to further improve the quality of the gene-specific shRNA expression vectors.

For any given gene, four (4) independent shRNA expression vectors are provided as 5 ug of purified and dried plasmid per tube. Additionally, OriGene provides customers with a pRS, pGFP-V-RS, pRFP-C-RS or pGFP-C-shLenti vector containing a non-effective (scrambled) shRNA cassette (TR30012, TR30013, TR30015 or TR30021) as a specific negative control for gene down regulation. All the expression shRNA cassettes are sequence-verified. Many of the HuSH products have been designed and annotated to have good homology to mouse sequences. Although our data suggest that the tested vectors will be able to suppress the corresponding gene expression by 70% or more, we have not validated the effectiveness of all offered shRNA constructs.

Positive control shRNA expression vectors against Green Fluorescent Protein (GFP), Red Fluorescent Protein (RFP) and Luciferase (Luc) genes and all empty vectors are available for purchase [please refer to page 3 for complete list]. shRNA-GFP and shRNA-Luc were constructed in the pRS and pRFP-C-RS vectors using the same approach. shRNA-RFP was

constructed in pGFP-V-RS vector. Each was shown to inhibit its co-transfected target gene by approximately 90% when assayed after co-transfection into HEK293 cells with the expression plasmids for GFP, RFP or Luc.

As part of the quality control process, each plasmid was transformed into E. Coli and DAN was isolated from a single bacterial clone. DNA sequence analysis was performed on each plasmid and the sequences were matched to the specific regions of the target genes through BLAST analysis.

pRS—Retroviral shRNA vector

The pRS shRNA expression vector has a number of features allowing both transient and stable transfection, as well as the stable delivery of the shRNA expression cassette into host cells via a replication-deficient retrovirus. Efficacy of the shRNA expression vectors should be determined in transient transfection experiments against the target genes. Once the suppressing function of an shRNA vector is established, that vector can be used to create stable cell lines, either through transfection or retroviral infection, via puromycin selection.

The OriGene pRS plasmid contains both 5' and 3' LTRs of Moloney murine leukemia virus (MMLV) that flank the puromycin marker and the U6-shRNA expression cassette. Upon transfection of the plasmids into a packaging cell line, replication-deficient viruses can be obtained and used to infect target cells. A puromycin-N-acetyl transferase gene is located downstream of the SV40 early promoter, resulting in resistance to the antibiotic puromycin. The shRNA expression cassette consists of a 29 nt target-gene-specific sequence, a 7 nt loop, and another 29 nt reverse complementary sequence, all under the control of the human U6 promoter. A termination sequence (TTTTTT) is located immediately downstream of the second 29 nt reverse complementary sequence to terminate the transcription by RNA Pol III. The 29 nt gene-specific sequences were sequence-verified to ensure its match to the target gene. A detailed map of the plasmid is shown on the following page and the complete DNA sequence of the plasmid without a shRNA expression cassette can be found at http://www.origene.com/shrna/vector_information.aspx.

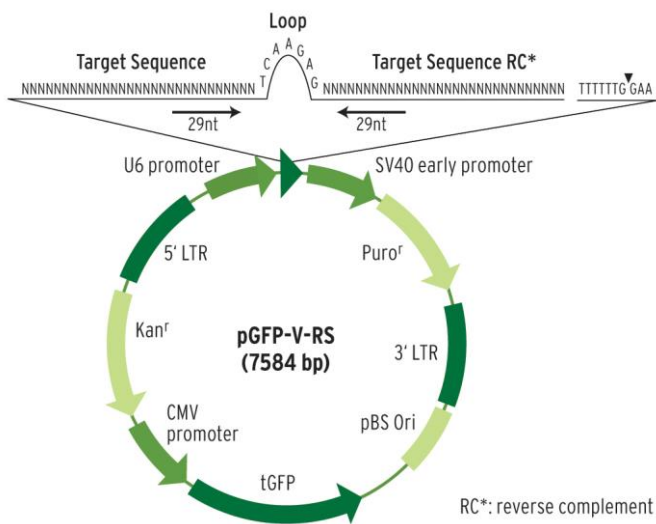
The gene-specific shRNA expression plasmids were constructed using synthetic oligonucleotides cloned into the BamHI / Hind III cloning sites of the retroviral vectors; BamHI / BsmB I are used for lenti shRNA vectors. Each of the shRNA expression plasmids has a 29 nucleotide gene-specific

pGFP-V-RS —Retroviral shRNA vector:

In addition to all the useful features of pRS, the pGFP-V-RS vector contains the CMV driven tGFP gene which expresses tGFP protein constitutively in mammalian cells. This feature makes it possible to monitor the transfection efficiency. The bacterial selection marker is kanamycin (25 ug/ml) instead of ampicillin (100 ug/ml) as found in the pRS vector. The detailed vector information can be found on the OriGene website (same URL as above).

Note: CMV-GFP is outside of the LTR region, so it will not be packaged into the viral particles.

Start	End	Description
1	6	EcoRI
75	331	U6 promoter
335	340	BamHI
379	385	HindIII
386	391	Sall
413	604	SV40 promoter
671	1270	Puromycin-N-acetyl transferase sequence
1349	1942	3' LTR
2299	2918	pBR322 ORI
2977	3563	PolyA signal
3604	3667	MCS (PmeI (3604), NotI (3619, 3649) MluI (3630), AsiI (3667)
3648	4380	tGFP
4467	5192	CMV promoter
5205	6095	Neomycin phosphotransferase for Kanamycin resistance
6231	6701	5' LTR



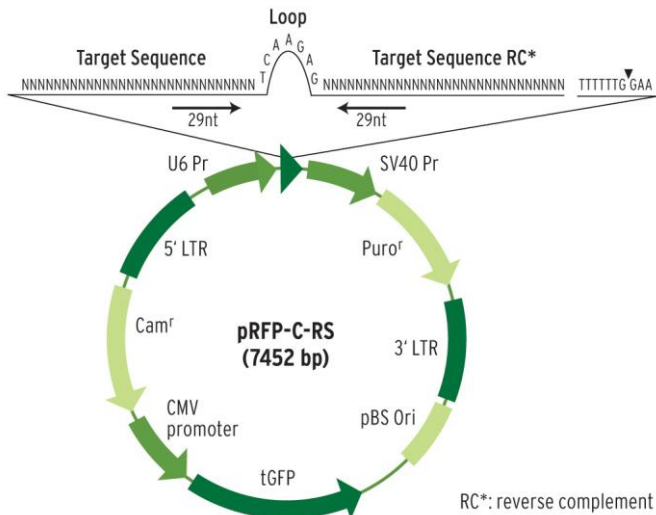
pRFP-C-RS —Retroviral shRNA Vector:

In addition to all the useful features of pRS, the pRFP-C-RS vector also contains CMV driven tRFP gene which expresses tRFP protein constitutively in mammalian cells. pRFP-C-RS also assists the user in monitoring a dual-gene knockdown efficiency when an shRNA cassette targeting gene A (expressed in RFP vector) is cotransfected along with an shRNA cassette targeting gene B (expressed in GFP vector) along with respective target transcripts. The bacterial selectable marker for the vector is Chloramphenicol instead of Ampicillin or Kanamycin. The detailed vector information can be found on the OriGene website (same URL as above).

Note: CMV-RFP is outside of the LTR region, so it will not be packaged into the viral particles.

Start	End	Description
1	6	EcoRI
75	331	U6 promoter
335	340	BamHI
379	385	HindIII
386	391	Sall
413	604	SV40 promoter
671	1270	Puromycin-N-acetyl transferase sequence
1349	1942	3' LTR
2299	2918	pBR322 ORI
2977	3563	Poly A signal

3604	3667	MCS [PmeI (3604), NotI (3619, 3649), MluI (3630) AsiSI (3667)]
3648	4380	tRFP
4467	5192	CMV Promoter
5263	5922	CAM ^r for Chloramphenicol resistance
6104	6574	5' LTR



Lentiviral shRNA vectors:

Lentiviral shRNAs particles are a powerful way to silence target gene expression dividing, non-dividing cells, primary cells, stem cells and difficult-to-transfect cell lines.

pGFP-C-shLenti --- Lenti shRNA Vector

pGFP-C-shLenti vector is a third generation lentiviral vector which requires that the viral components necessary to produce infectious viral particles be carried on multiple other vectors.

There are three major functional elements within the 5`-LTR and 3`-LTR regions:

1. an shRNA expression cassette driven by an U6 promoter
2. a puromycin resistance marker driven by a SV40 promoter
3. a tGFP driven by a CMV promoter.

All elements between the 5` and 3` LTRs are packaged to viral particles. The bacterial selection marker for the vector is Chloramphenicol (34ug/ml recommended).

Start	End	Description
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Contents and storage

Contents	Titer	Quantity	Shipping	Storage
lentiviral scramble shRNA control particles	$>1 \times 10^7$ TU/ml	500ul	Dry ice	-80°C (stable for at least 6 months)
4 vials of target-specific shRNA particles	$>1 \times 10^7$ TU/ml	500ul each	Dry ice	-80°C (stable for at least 6 months)

Note:

- The exact titer of each lentiviral particle will be reported on the Certificate of Analysis.
- The Lenti scramble shRNA negative control particles constitutively express GFP, which can be used for transduction optimization studies. GFP can be detected using fluorescence microscope or standard FACS.

Precautions and Disclaimer

Although the lentiviral transduction particles are replication-incompetent, it is highly recommended that they be treated as Risk Group Level 2 (RGL-2) organisms. Follow all published RGL-2 guidelines for handling and waste decontamination.

Suggestions:

- Wear double gloves and lab coat at all times.
- Perform work in a Class II Biosafety Cabinet (BSC) and post biohazard warning signs.
- Minimize splashes or aerosols with careful pipetting.
- Autoclave all biological wastes and decontaminate before disposal.

Lentiviral transduction Protocols

The following protocol is based on a 24-well plate. If your experiments require a different size of culture plates, just scale up or down accordingly based on the relative surface area.

Tissue Culture Vessel	Growth area, cm ² /well
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96-well plate	0.35
24-well plate	2
12-well plate	4
6-well plate	9.5
35 mm plate	8
60 mm plate	20
100 mm plate	60

Day 1, seed cells

Seed 0.5×10^5 HEK293T cells or your specific cells in each well of 24-well plate to 50% confluency upon transduction. Incubate 18–20 hours at 37°C in a humidified 5% CO₂ incubator.

Note: While determining the plate density, please consider both the growth rates of cells and the length of time the cells will be growing before transduction.

Day 2, transduction

1. Calculate the amount of viral particles to be added according to the desired multiplicity of infection (MOI).

Multiplicity of Infection (MOI):

Multiplicity of Infection (MOI) is the number of transducing lentiviral particles per cell

To calculate:

$(\text{Total number of cells per well}) \times (\text{Desired MOI}) = \text{Total transducing units needed (TU)}$

$(\text{Total TU needed}) / (\text{TU/mL}) = \text{Total mL of lentiviral particles to be added to each well}$

Note: When transducing a cell line for the first time, a range of volumes or MOI should be tested. The transduction controls (cat# TR30021V or TR30033V) can be used for MOI optimization. MOIs of 1, 2, 5 and 10 or higher should be used to determine the optimal transduction efficiency and knockdown for each cell line.

2. Thaw the lentiviral shRNAs stock on ice. Gently spin down before opening. Keep them on ice. Mix gently before use.

3. Remove medium from wells and add appropriate amount of Lentiviral particles, culture medium, polybrene (final concentration is 8 $\mu\text{g}/\text{mL}$) to the total volume of 500 μL . Gently swirl the plate to mix.

Polybrene (Hexadimethrine bromide):

Polybrene is a small, positively charged molecule that binds to cell surfaces, neutralizes surface charge, increases binding between pseudoviral capsid and the cellular membrane.

Polybrene (Hexadimethrine bromide) has been proved to greatly enhance transduction efficiency. Some cells, like primary neurons, are sensitive to polybrene. Do not add polybrene to these types of cells. If working with a cell type for the first time, a polybrene control only well should be used to determine cell sensitivity.

4. Incubate 18–20 hours at 37°C in a humidified 5% CO₂ incubator. Overnight incubation may be avoided when toxicity of the lentiviral particles is a concern. Cells may be incubated for as little as 4 hours before changing the medium containing lentiviral particles.

Day 3, change media

Remove the medium containing lentiviral particles from wells and replace with 500 μL fresh pre-warmed complete culture medium.

Day4.

- a. For transient knockdown analysis: Do not disturb the cells until next day.
- b. For stable cell line generation: Split transduced cells 1:10 and apply complete medium containing the appropriate amount of puromycin (if you want to isolate single cell colonies, plate cells in a larger dish, such as 10 cm dish). Keep the cells cultured at 37°C in a humidified 5% CO₂ incubator.

Note: Perform a kill curve experiment for right dose of puromycin in stable cell selection using the following guidelines:

- i. Plate 1.6×10^4 cells into wells of a 96-well plate with 120 μL fresh media.
- ii. The next day, add puromycin ranging from 0.5 to 10 $\mu\text{g}/\text{mL}$ to selected wells.
- iii. Examine viability every 2 days.
- iv. Culture for 10-14 days. Replace the media containing puromycin every 3 days.

- v. The minimum concentration of puromycin that causes complete cell death after 4-7 days should be used for that cell type.

Day 5 and forward.

- a. For transient knockdown analysis: Harvest the cells and perform qRT-PCR or Western blot (observe transduction efficiency using GFP)

For stable cell line generation: replace medium with fresh, puromycin-containing medium every 3-4 days until resistant colonies can be identified (generally, 10-12 days after drug selection). Pick a minimum of 5 puromycin-resistant colonies and expand each clone to assay for knockdown of the target gene.

Transfect gene-specific shRNA into mammalian cells

1. Add 50 uL of dH₂O into each of the tubes containing shRNA expression plasmids. Vortex the tubes briefly to resuspend the DNA. The concentration of this solution is 100 ng/uL.
2. Plate the appropriate easily transfected cell line (e.g. HEK293 for human, NIH3T3 for mouse or OLN-93 for rat shRNA validation) cells at 3×10^5 in 2 ml into a well of a 6-well plate. Grow the cells overnight in a 5% CO₂ incubator to achieve 50% confluence.
3. Complex formation (perform this step immediately before transfection)

In a small sterile tube, combine the following reagents in the prescribed order. The order of reagent addition is important to achieve the optimal results.

 - a. Dilute 1 µg of DNA in 250 uL of Opti-MEM I (Gibco 51985). Vortex gently.
 - b. Add 3 uL of Turbofectin 8.0 to the diluted DNA (not the reverse order) and Pipette gently to mix completely.
 - c. Incubate for 15 minutes at room temperature.

cDNA expression plasmid for the target gene 0.01 ug to 1.0 ug
(optional, available at OriGene)

Note: Add TurboFection 8.0 (or equivalent) directly into the serum-free media. DO NOT let transfection reagent touch any plastic other than the pipette tip.

For Dual-gene knockdown experiment, add 500ng of each shRNA expression plasmids (both pGFP-V-RS vector and pRFP-C-RS vector together) with 500 ng each of target cDNAs

4. Transfection in complete culture medium
 - a. Add the mixture prepared in Step 2 dropwise to the cells. Gently rock the plate back-and-forth and from side-to-side to distribute the complex evenly.
 - b. Incubate the cells in a 5% CO₂ incubator for 48 hrs before harvesting for RNA analysis and 72 hrs before harvesting for protein analysis.

Creation of stable cell lines without retroviral infection

1. Transfect the cells with the HuSH plasmid DNA using your standard protocol for transient transfection. After transfection, do not change the medium until the cells are ready to be passaged.
2. Passage the transfected cells (1:10 split) into a fresh vessel containing growth medium and 0.5-10 ug/ml puromycin (determined by killing curve using un-transfected cells).
3. Continue to grow and passage the cells as necessary, maintaining selection pressure by keeping 0.5-1.0 ug/mL puromycin in the growth medium. After 4-7 days, a large number of the cells will be killed by the antibiotic, indicating that they did not take up or have lost the plasmid with the puromycin resistance cassette. The cells that remain growing in the puromycin-containing medium have retained the HuSH plasmid, which stably integrates into the genome of the targeted cells.
4. Select clonal populations of cells by transferring a well-isolated single clump of cells (the clonal ancestor and cells divided from it) into a well of a 24-well plate; repeat to select 5-10 clonal populations. Continue growing these cells in selection medium for 1-2 additional passages. At this time, each well contains a clonal population of stably transfected cells, which can be maintained in normal growth medium without the selection pressure of puromycin (although you may wish to grow the cells under "light pressure", 0.2 ug/mL puromycin). These populations can be used for experiments or stored under liquid nitrogen in growth medium with 10% DMSO and 20% FBS for future use.

Retroviral transduction shRNA into mammalian cells

Production of retrovirus by transient transfection

1. Plate the packaging cells at 40% confluency the day before transfection. Cells should reach 70-80% confluency in 24 hours.
2. Transfect the packaging cells as described above in the transient transfection protocol.
3. The next day, feed the cell culture with fresh growth media.
4. On day 2 post-transfection, collect the media from the culture, and centrifuge at 2000 x g for 5 minutes or pass through a 0.45 um filter (use low protein binding filter, e.g., cellulose acetate or polysulfonic filter, not a nitrocellulose filter) to remove cell debris. Freeze the supernatant at -80° C or directly use it as viral stock for viral titering through infection of NIH3T3 cells.
5. pGFP-V-RS vector conveys tGFP expression and pRFP-C-RS vector conveys tRFP expression upon transfection. However the tGFP and tRFP expression cassettes are located outside of the retroviral packaging region. The virus does not pass the tGFP or tRFP into the transduced cell.

Production of retrovirus by stable transfection of packaging cells

1. Plate the packaging cells at 40% confluency the day before transfection. Cells should reach 70-80% confluency in 24 hours.
2. Transfect the packaging cells as described above in the transient transfection protocol.
3. At 24-36 hours post-transfection, replat the transfected cells in selection media (complete growth medium with the selection agent added at its optimal concentration [e.g., puromycin at a concentration of 0.5 to 1 ug/mL for HEK293T cells]).
4. Culture the cells for one week using the drug selection medium. Many of the cells will die due to negative selection, leaving only drug-resistant cells alive. Select 10-20 large, healthy-looking drug-resistant colonies and transfer each into a well of a new 6-well plate.

5. Expand these colonies into large cultures, and compare their viral yield by virus titering, if desired. Choose the cells with the highest titer to use for virus production. Otherwise, collect the media from the culture, and centrifuge at 2000 x g for 5 minutes or pass through a 0.45 μ m filter (use low protein binding filter, e.g., cellulose acetate or polysulfonic filter, not a nitrocellulose filter) to remove cell debris. Freeze the supernatant at -80°C or directly use it as viral stock for viral titering through infection of NIH3T3 cells.

Stable retroviral transduction

1. Plate the target cells at a concentration that will produce approximately 50% confluency in 24 hours.
2. Add entire amount of viral stock (or, if virus has been titered, the chosen cfu/mL of virus) and 4 μ g/ml polybrene in growth medium directly onto target cells. Incubate at 37°C in 5% CO₂.
3. At 24 hours post-infection, replace the medium with fresh growth medium containing 0.5-1 μ g/ml puromycin (or the optimal concentration as determined for your conditions). Passage as needed, and maintain selection pressure for 1-2 weeks. Most uninfected cells should be killed by the puromycin within 1 week.
4. Select clonal populations of cells by transferring a well-isolated single clump of cells (the clonal ancestor and cells divided from it) into a well of a 24-well plate; repeat to select 5-10 clonal populations. Continue growing the cells in selection medium for 1-2 additional passages. At this time, each well contains a clonal population of stably transfected cells, which can be maintained in normal growth medium without the selection pressure of puromycin. These populations can be used for experiments or stored in liquid nitrogen tank in growth medium with 10% DMSO and 20% FBS for future use.

Production of lentiviral particles using the lenti-packaging kit

The lenti-packaging kit (cat# [TR30022](#)) is optimized for high efficient packaging. The protocol is based on 12-well plate format, the production size can be scaled up or down accordingly.

1. Plate HEK293T cells in a 12-well plate to approximately 40% confluency the day before transfection. Cells should reach 70-80% confluency within 24 hours.
2. Label 5 sterile 1.5 mL eppendorf tubes as scrambled shRNA control and four gene-specific shRNA constructs. Add to each tube the corresponding shRNA and packaging component DNAs according to the following the recipe: 0.5 ug shRNA, 0.6 ug of lenti packaging mix. Add Opti-MEM (Life Technology) to a final volume of 50 ul.
3. Prepare 300 ul of 2X transfection mixture by mixing 20 ul of MegaTran transfection reagent (OriGene Cat#TT200002) and 280 ul of Opti-MEM.
4. Transfer 50 ul of the 2x transfection mixture to each of the eppendorf tubes and mix the solution by gentle pipetting. Incubate the tube at room temperature for 20 minutes.
5. Transfer each DNA-transfection reagent mixture to a well in the plate prepared the day before.
6. Incubate the plate for 16-24 hours in a CO2 incubator. Examine the GFP expression under a fluorescence microscope to check the transfection efficiency.
7. Replace the growth medium with fresh medium; grow the cells for additional 48 hours.
8. Transfer the growth medium from each well to a 1.5 ml centrifuge tube.
9. Centrifuge the tubes and filter each supernatant through a syringe filter (0.45 micron) and collect the viral solution to a new sterile tube.
10. The viral particles are ready to use. It can be stored at 4°C for 2 weeks or -80°C for a long-term storage.

Analyzing shRNA knockdown via WB

Preparing cell lysates

1. Remove the culture media by aspiration. Wash the cells in the dish once with ice-cold PBS and aspirate off PBS.
2. Add ice-cold RIPA* with freshly added protease inhibitors to cells (1 ml per 10 cm dish; 0.2 ml per well/six-well plate). For adherent cells, rock the cells in the presence of lysis buffer in plates in a cold room or on ice for 15 minutes. For suspension cells, pellet the cells, then resuspend in lysis buffer. Transfer the cell lysis solution into eppendorf tubes.

3. Centrifuge the lysate at 14,000 x g in a pre-cooled centrifuge for 15 minutes. Immediately transfer the supernatant to a fresh centrifuge tube and discard the pellet.
4. Determine the protein concentration by any commercially available reagent or kit. At this step, the sample can be divided into aliquots and stored at -80°C for long-term.

*RIPA buffer

Stock: 50 mM Tris-HCl pH 7.4, 1% NP-40; 0.25% Na-deoxycholate¹, 150 mM NaCl, 1 mM EDTA

Add fresh: 1 mM PMSF², 1 ug/ml Aprotinin, 1 ug/ml Leupeptin

¹ Do not add Na-deoxycholate when preparing lysate for kinase assays, as it may denature the protein and cause it to lose activity.

² PMSF is made as a 200 mM stock solution in isopropanol and stored at room temperature. The vapor is hazardous. It is important to work with it in a chemical hood. PMSF is not stable in H₂O as it has a half-life of approximately 30 minutes.

Protein blotting

1. Prepare 3 ug of cell lysate in 1X Laemmli sample buffer¹ in a volume of 20 uL (for a mini-gel, up to 15 ug of protein can be loaded per lane). Heat the sample to 70°C for 10 min. Prepare a pre-stained protein standard as well.
2. Run the samples on a pre-cast SDS polyacrylamide gel with Tris-Glycine SDS running buffer at 125V for 90 minutes until the dye reaches the bottom the gel. Remove the gel and soak in protein transfer buffer for 15 minutes.
3. Prepare the PVDF membrane by pre-wetting it in 100% methanol, washing once in dH₂O for 5 min and equilibrating it in the protein transfer buffer for 10 minutes.
4. Assemble the electroblotting cassette and place the electrodes in the blotting unit, according to the manufacturer's instructions.
5. Transfer in Tris-Glycine transfer buffer at 25 V (100 mA) for 1.5 hours.
6. Following transfer, remove the membrane from the blotting cassette and mark the orientation of the gel with a pencil. Rinse briefly with PBS and trim the membrane. The membrane may be stored at 4°C for several weeks. However, once the membrane is dried, it needs to be wetted by methanol followed by PBS.

1X Laemmli sample buffer: 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue

Protein detection with specific antibodies

1. Wash PVDF membrane with TBST² once for 5 min. at room temperature.
2. Block non-specific binding on the membrane with freshly prepared 5% nonfat dried milk for 1 hour on a shaking platform at room temperature.
3. Wash three times for 5 minutes each with TBST.
4. Incubate the membrane with a specific primary antibody diluted in TBST and 5% BSA at the manufacturer's recommended dilution with gentle agitation at 4°C overnight or for several hours at RT.
5. Wash three times for 5 min each with TBST.
6. Incubate with HRP-conjugated secondary antibody at 1:20,000 (or manufacturer's recommended dilution) in TBST-5% BSA for 1 hour at room temperature.
7. Wash three times again for 5 minutes each with TBST.
8. For detection, use the enhanced chemiluminescence reagent from OriGene (Western Blotting Luminol Reagent (TA10006)) or other commercially available detection system and prepare according to the manufacturer's directions.
9. Lay the membrane on a plastic surface with the protein side up. Add the mixed detection solution to the membrane. Incubate for 3 minutes. Remove the excess solution and cover the membrane with transparent plastic.
10. Place the wrapped blot with protein side up in an X-ray film cassette. Place a sheet of X-ray autoradiography film on the top of the membrane. Close the cassette for 1 min. Remove the film for development. Add additional films if needed for longer or shorter exposures.

²TBST: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20.

Plasmid DNA amplification (Optional)

For pRS based HuSH vectors, the E. coli selection marker is ampicillin (100 ug/mL). For pGFP-V-RS based HuSH vectors, the E. coli selection marker is kanamycin (25 ug/mL). For pRFP-C-RS & pGFP-C-shLenti based HuSH vectors, the E. coli selection marker is chloramphenicol (34 ug/ml).

1. Add 50 uL of dH₂O into each tube. Vortex the tubes briefly to resuspend the DNA. Pipette 1 uL of this solution to another tube and add 99 uL dH₂O. The concentration of the DNA solution should be around 1 ng/uL. The plasmid solution should be stored at -20°C.

2. Thaw transformation competent *E. Coli* cells (standard laboratory DH5 alpha) on ice. Perform transformation with 1-2 uL of the diluted shRNA plasmid. Plate out the transformants on LB-Kan, LB-Amp or LB-Chloramphenicol plates and incubate overnight at 37°C, until colonies appear.
3. The following day, inoculate single bacterial colonies into 200 ml of LB-Kan, LB-Chloramphenicol or LB-Amp media and grow them overnight.
4. Purify the DNA plasmids from the culture using a midiprep DNA isolation kit and follow the corresponding protocol.

Note: due to the nature of shRNA structure, it might be difficult for *E. Coli* to break it apart during amplification; therefore treat shRNA construct as low copy plasmid.

Quality control and quality assurance

Plasmid validation

All plasmid products with shRNA expression cassettes have been isolated from single colonies. The purified plasmids were examined on an agarose gel to ensure the presence of the plasmids and to verify quantity.

Sequence validation

The final products have been re-sequenced for confirmation.

Transformation validation

The dried plasmids were resuspended in dH₂O, then used for transforming *E. Coli* cells. The efficiency of transformation is identical to that before drying.

Troubleshooting Guide for lenti shRNA particles

Problem	Cause	Solution
No GFP is observed	Viral stock stored incorrectly	Store stocks at -80°C. Do not freeze/thaw. Aliquot the viral stocks.
	MOI is too low	Transduce at a higher MOI.
	Polybrene not included during transduction	Transduce in the presence of polybrene.
	CMV promoter is not	Go head to perform qRT-PCR to

	functional in target cells	check target gene knockdown; At same time, check GFP expression in HEK293T cells to make sure lentivirus is functional.
	Too early to check	Check GFP expression 48-72 hours after transduction.
No gene knockdown is observed	Viral stock stored incorrectly	Store stocks at -80°C. Do not freeze/thaw. Aliquot the viral stocks.
	MOI is too low	Transduce a higher MOI.
	Cell were harvested and assayed too soon after transduction	The shRNA must be permitted to accumulate in cells. Harvest 72 hours after transduction. Alternatively, placing cells under puromycin selection to kill untransduced cells.
Cytotoxic effects observed after transduction	Target gene is essential for cell viability	Reduce the MOI for transduction.
	Polybrene is toxic to cells	Use less polybrene or no polybrene during the transduction.
	Lentivirus is toxic to cells	Incubate virus 4 hours instead of overnight
	Too much puromycin was used for selection	Determine the puromycin sensitivity of the cells by performing a kill curve and use the minimum concentration required to kill the un-transduced cells.

Negative Lenti Particle Controls

negative Controls	Objective
Untreated Cells	Untreated cells will provide a reference point for comparing all other samples.
Transduction with non-targeting shRNA: pGFP-C-shLenti (Cat# TR30021V) pRFP-CB-shLenti (Cat#	This non-targeting shRNA is a useful negative control that will activate RISC and the RNAi pathway, but does not target any genes. this lenti scramble shRNA control particles constitutively expressing GFP, it is a useful for transduction optimization studies.

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FAQ:

What are the differences between the pRS, pGFP-V-RS, pRFP-C-RS & pGFP—C-shLenti expression plasmids?

	pRS	pGFP-V-RS	pRFP-C-RS	pGFP-C-shLenti
Catalog #	TRxxxxxx	TGxxxxxx	TFxxxxxx	TLxxxxxx
Bacterial selection marker	Amp (100ug/ml)	Kanamycin (25ug/ml)	Chloramphenicol (34ug/ml)	Chloramphenicol (34ug/ml)
Viral packaging	Retro	Retro	Retro	Lenti
Mammalian selection	Puromycin	Puromycin	Puromycin	Puromycin
Scramble Control vector	TR30012	TR30013	TR30015	TR30021
Empty Vector	TR20003	TR30007	TR30014	TR30023
Reporter for transfection	None	GFP	RFP	GFP
Reporter for Infection	None	None	None	GFP

OriGene also provides more shRNA vector with different selection marker or fluorescent proteins for use in our ExactHuSH service. See http://www.origene.com/shRNA/vector_information.aspx for details.

I am doing retroviral packaging and infection, will my infected cells express tGFP or tRFP?

Answer: No. The CMV-tGFP and the CMV-tRFP elements are outside of the LTR region that gets packaged by retrovirus. Thus, you cannot use tGFP or tRFP expression to monitor transduction but you can use puromycin selection to generate stable cell lines.

Your HuSH product is stated to be “locus-specific”. How do I know that it will knock down the expression of my variant or isoform?

Answer: Unless stated otherwise, shRNA constructs were designed to be effective against most transcriptional variants at a particular gene locus. If you would like a knockdown construct against a specific transcriptional variant(s), OriGene can generate a custom HuSH product that will selectively knockdown only the specified variants. Please review the options on our ExactHuSH website at <http://www.origene.com/shRNA/Custom-shRNA.aspx>

Can I screen all the constructs provided in HEK293 cells and then pick the most effective one for subsequent studies?

Answer: Absolutely. We recommend that you screen all constructs individually to identify the most effective constructs. HEK293 cells are a convenient and easily transfectable system for screening all human shRNA constructs (use NIH3T3 for mouse shRNA constructs or OLN-93 for rat shRNA constructs). Other cell lines can also be used for this purpose, as long as the transfection efficiency of ~80% or greater for transient transfections. Afterwards, the effective construct(s) can be used for viral infection of or for direct transfection into your target cells.

Can I get a HuSH construct against any species other than those on your web site?

Answer: OriGene can do any custom HuSH design and construction, regardless of species specificity. The only information you need to provide is the accession number of sequence you wish to target. We can offer you assistance in identifying the particular species' sequences to be used for gene knockdown studies or you can follow the link to our ExactHuSH page <http://www.origene.com/shRNA/Custom-shRNA.aspx>

Will your HuSH products work with any retroviral packaging cell line?

Answer: Our pRS, pGFP-V-RS and pRFP-C-RS vectors have been designed for viral packaging in most commercially available packaging cell lines. However, please make sure that the packaging line has not been previously transfected with plasmids containing a puromycin resistance cassette. Furthermore, you need to ensure that the chosen packaging line's viral particles are able to infect your target cell line (some cell lines have restricted species specificity). We suggest packaging lines such as PT67 (Clontech) or Phoenix (Orbigen) for use with our constructs for cell line infection. The pGFP-C-shLenti vectors are designed for lentiviral packaging.

How should I use the products?

Answer: Customers can use the 5 ug shRNA DNA plasmid directly for transfection and gene-knockdown studies. After transfection, cell lysates can be obtained and used for Western blot analysis with an antibody against the target protein to verify the knockdown efficiency, or RNA can be harvested from transfected cells and used in quantitative RT-PCR to determine the loss of gene expression. If desired, the shRNA plasmids can be re-transformed for unlimited supplies of the plasmids. If viral infection is preferred, follow the viral packaging protocol and infect your target cells of interest.

Can I select for the plasmid-transfected cells?

Answer: Yes. One day after transfection, the cells can be selected with culture media containing 0.5 - 1.0 ug/mL puromycin or blasticidin (Sigma).

How can I create a stable cell line with a functional shRNA expression vector?

Answer: Stable cell lines can be generated by two different methods. First, target cells can be transfected with the shRNA plasmid. One day after transfection, the transfected cells can be selected with 0.5 - 1.0 ug/ml puromycin for 1-2 weeks with passages as needed. Alternatively, viral particles can be used to transduce cells. Ready-to-transduce Lenti shRNA particles are offered. For retroviral shRNA vectors, retroviral packaging cell lines can be used to generate retroviruses for stable cell line generation.

What use does the scrambled non-effective plasmid serve?

Answer: To specifically rule out the potential non-specific effect induced by expression of the HuSH product, OriGene provides customers with a negative control (TR30012, TR30013 or TR30015), that was constructed by cloning a scrambled sequence cassette (5' GCACTACCAGAGCTAACTCAGATAGTACT3') into our pRS, pGFP-V-RS, or pRFP-C-RS vectors respectively. The plasmid should serve as a negative control for gene-specific knockdown experiments and exclude any potential interferon response.

Can you tell me the sequence of your control constructs?

Answer: The 29mer sequence used to target firefly luciferase in TR30002 is 5' GGATTTTCAGTCGATGTACACGTTTCGTAC 3'. The 29mer sequence used to target eGFP in TR30001 is 5' CACAAGCTGGAGTACAACACTACAACAGCCA 3', the 29mer sequence used to target tGFP in TR30009 and TR30016 is 5' GCTACGGCTTCTACCACTTCGGCACCTAC 3', and the 29mer sequence used to target tRFP in TR30017 is: 5' CTTCAAGACCACATACAGATCCAAGAAAC 3'. The non-effective control sequence is: 5' GCACTACCAGAGCTAACTCAGATAGTACT 3'.

Do I need to use a special strain of E. Coli to amplify my HuSH constructs?

Answer: Special E. Coli is not required for HuSH amplification but we do not recommend using JM109. We routinely use DH5alpha from New England Biolabs.

Are the HuSH plasmids high copy or low copy number?

Answer: Although the plasmids technically contain a high-copy number Ori of replication, the hairpin slows replication and thus, we recommend using a low-copy number method for plasmid DNA amplification.

Can the pRS, pGFP-V-RS or pRFP-C-RS vectors be packaged by lentivirus?

Answer: No, our current RS system is strictly retroviral. The pGFP-C-shLenti is designed for lenti systems.

Do I have to use viral packaging and infection to create stable cell lines?

Answer: If your transfection efficiency is very high (e.g. 80% or greater), it is not necessary to use retroviral packaging. Simply split your cells 24hrs. post-transfection and add puromycin (0.5 - 1ug/ml) to the fresh growth medium.

Will 0.5ug/ml-1ug/ml concentration of puromycin work for my cell line?

Answer: We strongly recommend that a kill-curve be performed on each batch of cells to ensure that the optimal puromycin concentration is employed.

What is the OriGene guarantee on the shRNA expression plasmids?

Answer: OriGene guarantees that the sequences in the shRNA expression cassettes are verified to correspond to the target gene with 100% identity. One of the four constructs at minimum are guaranteed to produce 70% or more knock-down provided a minimum transfection efficiency of 80% is achieved. Western Blot data is recommended over qPCR to evaluate the silencing effect of the shRNA constructs 72 hrs post transfection. To properly assess knockdown, the gene expression level from the included scramble control vector must be used in comparison with the target-specific shRNA transfected samples.

For non-conforming shRNA, requests for replacement product must be made within ninety (90) days from the date of delivery of the shRNA kit. To arrange for a free replacement with newly designed constructs, please contact Technical Services at techsupport@origene.com. Please provide

your data indicating the transfection efficiency and measurement of gene expression knockdown compared to the scrambled shRNA control (Western Blot data preferred).

What if the gene is not expressed in HEK293 cells and the transfection efficiency of my target cells is below 80%? How do I screen my shRNA constructs to pick the most effective one?

Answer: If your gene is not expressed in HEK293, you can do a co-transfection with an expression construct and the shRNA construct at a 1:1 ratio transiently into HEK293 cells. You can also select stable cells in your target cell line. It is well known that if a gene that is vital for cell growth is silenced, it will be difficult to get stable cells for that particular cell line.

Which method do you recommend for assessing the knockdown efficiency of my gene-specific shRNA constructs?

Answer: Western Blot is recommended over qPCR to evaluate the silencing effect of the shRNA constructs 72 hrs post transfection. To properly assess knockdown, the gene expression level from the included scramble control vector must be used in comparison with the target-specific shRNA transfected samples.

Can I use transfection methods to introduce the Lenti constructs to my target cells?

Answer: Yes, you can use any transfection method directly for your cells so long as the transfection is efficient enough for your assay. Infection, however, usually works better.

Does OriGene provide a packaging kit for producing Lentiviral particles?

Answer: Yes, the part number is TR30022

Can I use other transfection reagents other than MegaTran1.0 for the transfection?

Answer: Yes, OriGene's TurboFectin 8.0 or other commercial transfection reagents with high transfection efficiency can be used.

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