

HuSH™

shRNA Plasmids (29-mer)

APPLICATION GUIDE

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Revision 03.18TS

PACKAGE CONTENTS AND STORAGE CONDITIONS

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 shRNA tGFP Cloning Vector (pGFP-V-RS) (TR30007)	A purified and sequence-verified plasmid without shRNA cassette insert	5 ug plasmid DNA
HuSH 29-mer Non-Effective Scrambled pGFP-V-RS (TR30013)	A purified and sequence-verified plasmid containing non-effective 29-mer scrambled shRNA cassette	5 ug plasmid DNA

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.
pRFP-C-RS vector (TR30014)	A purified and sequence-verified plasmid without shRNA cassette insert	5 ug plasmid DNA
HuSH 29mer Non-Effective (Scrambled) pRFP-C-RS (TR30015)	A purified and sequence-verified plasmid containing a non-effective 29-mer scrambled shRNA cassette	5 ug plasmid DNA

pRS vector

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.
pRS Vector (TR20003)	A purified and sequence-verified plasmid without shRNA cassette insert	5 ug plasmid DNA
HuSH 29-mer Non-Ef-fective (Scrambled) pRS (TR30012)	A purified and sequence-verified plasmid containing a 29-mer scrambled 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.

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)]

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.

RNA validation vector (TR30004): A convenient product to measure the effective-

ness of HuSH-29 or other RNAi constructs. The vector is designed to incorporate a cDNA clone and a luciferase reporter gene as a chimeric transcript and can be used to identify the most effective knockdown construct as well as optimal transfection conditions. High throughput application of this reporter vector can be used to optimize experiments involving multiple genes and cell lines. More information can be found at http://www.origene.com/rna/validation_vector.msp.

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.

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 MegaTran 1.0 (OriGene), TurboFectin 8.0 (OriGene) or FuGENE 6 (Roche), which have been shown to transfect common cell types.
- Cell line and cell culture supplies: user preferred
- Reagents for cell lysis: user preferred
- LB-kan (25 ug/ml) LB-amp (100 ug/ml) LB_Chloramphenicol (34 ug/ml) liquid culture
- 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 tran-

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

OriGene has created a retroviral silencing plasmid (pRS) that contains retroviral long terminal repeats (LTR) from the murine moloney leukemia virus, the puromycin resistance gene, and a U6 small nuclear RNA gene promoter (Hannon 2002; Elbashir 2002) to effectively express the inserted hairpin sequence and to achieve RNA interference upon introduction into and subsequent processing by mammalian cells. This plasmid has been validated for transient transfection and for the ability to inhibit targeted gene expression with GFP, luciferase and HER2 oncogene specific hairpin DNA inserts. Moreover, our vector has been validated for down regulation of overexpressed GFP using retroviral infection. Four (4) designed and sequence-verified shRNA vectors are offered for each targeted gene.

The gene-specific shRNA expression plasmids were constructed using synthetic oligonucleotides cloned into the BamHI / Hind III cloning sites of the pRS vector. Each of the shRNA expression plasmids has a 29 nucleotide gene-specific sequence insert immediately downstream of a U6 promoter in plus (+) orientation, a 7 nucleotide loop, and the 29 nucleotide sequence in reverse complement, followed by a TTTTTT termination sequence. All inserts have the sequence structure shown below:

U6 promoter - GATCG -- 29 nt sense -TCAAGAG - 29 nt reverse complement -- TTTTTT (termination) - GAAGCT

Insertion of the shRNA cassette into the pRS vector destroys the BamHI restriction site by changing the sequence from GGATCC to GGATCG.

Approximately 40 shRNA vectors were tested against their target genes in co-transfection experiments where the shRNA expression plasmids are introduced into HEK293 cells with their corresponding cDNA expression plasmids. Twenty-five to thirty percent of the shRNA expression vectors were able to inhibit target gene expression by at least 70%. By providing four shRNA constructs targeting a given gene, we expect that at least one of the four constructs should provide >90% gene expression inhibition.

The OriGene HuSH product line contains shRNA expression vectors covering most known genes, such as protein kinases, phosphatases, oncogenes, tumor suppressor genes and other signaling molecules or structural proteins. For any given gene, four (4) independent shRNA expression vectors are provided as 5 ug of purified and dried plasmid per tube. The original vector plasmid (pRS vector, TR20003; pGFP-V-RS vector TR30007; or pRFP-C-RS TR30014) is also included in the same package. Additionally, OriGene provides customers with a pRS, pGFP-V-RS or pRFP-C-RS vector containing a non-effective (scrambled) shRNA cassette (TR30012, TR30013 or TR30015) 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 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 DNA 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. More information is available on the OriGene website <http://www.origene.com/rnai/quality.msp>.

VECTOR INFORMATION

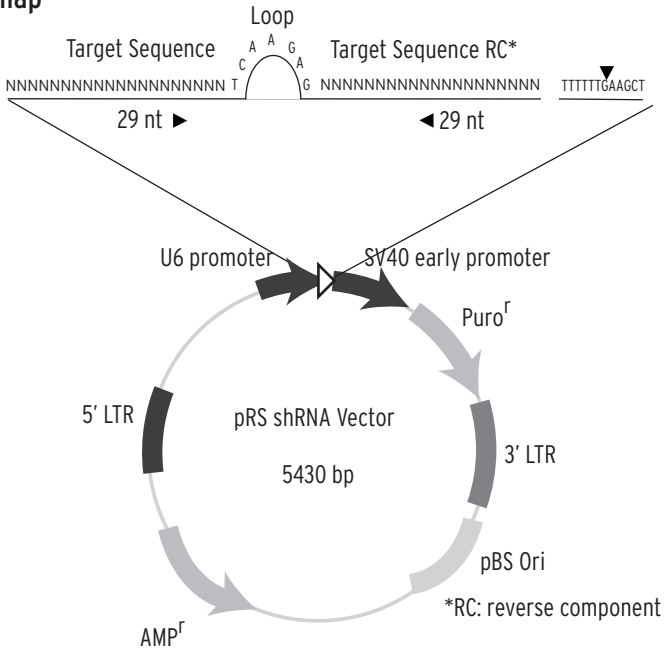
The pRS shRNA expression vector has a number of features allowing both transient and stable transfection, as well as the stable delivery of the shRNA expression cassette into host cells via a replication-deficient retrovirus. Efficacy of the shRNA expression vectors should be determined in transient 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 sequence was 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 an shRNA expression cassette can be found at http://www.origene.com/rna/vector_information.msp.

Features for pRS vector:

Start	End	Description
1	6	EcoRI
75	331	U6 promoter
335	340	BamHI
379	385	HindIII
386	391	Sall
413	681	SV40 promoter
743	1342	Puromycin-N-acetyl transferase sequence
1441	2034	3' LTR
2391	3010	pBR322 ORI
3172	4032	Beta-lactamase for ampicillin resistance
4168	4639	5' LTR

pRS vector map



Features for pGFP-V-RS vector:

In addition to all the useful features of pRS, the pGFP-V-RS vector contains the pCMV 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).

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.

PRODUCT APPLICATION

Introduction of gene-specific shRNA into mammalian cells via transfection

1. Add 50 μ L 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/ μ L.
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. 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.

Serum-free DMEM	100 μ L
TurboFection 8.0 solution	3 μ L
shRNA expression plasmid DNA	1 μ g
cDNA expression plasmid for the target gene (optional, available at OriGene)	0.01 μ g to 1.0 μ g

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 50ng of each shRNA expression plasmids (both pGFP-V-RS vector and pRFP-C-RS vector together) with 50ng each of target cDNAs

- Mix the tube contents gently. Do NOT vortex!
- Incubate at room temperature for 15-45 minutes.
- Add the DNA-TurboFectin 8.0 mix to the 6-well plate directly without removal of the culture media. Mix by gently swirling the plate.
- 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 into a fresh vessel containing growth medium and 0.5-1.0 ug/ml puromycin. 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 1-2 weeks, 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.
3. 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.

Introduction of gene-specific shRNA into mammalian cells via retroviral infection

Production of retrovirus by transient 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. 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, replate 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 $\mu\text{g}/\text{mL}$ 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 μm 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 $\mu\text{g}/\text{ml}$ polybrene in growth medium directly onto target cells. Incubate at 37°C in 5% CO_2 .
3. At 24 hours post-infection, replace the medium with fresh growth medium containing 0.5-1 $\mu\text{g}/\text{ml}$ 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.

Determining shRNA functions through immunoblotting

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 H₂O 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.

¹2X 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.

RNAi construct validation (Optional)

The most up to date protocol for this process can be found on the OriGene website at <http://www.origene.com/assets/Documents/HuSH/ValidationVectorProtocol.pdf>

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 based HuSH vectors, the E. coli selection marker is chloramphenicol (34 ug/ml)

1. If desired, customers can first amplify the shRNA vectors. 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 DH5alpha) 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 5 ml of LB-Kan, LB-Chloramphenicol or LB-Amp media and grow them overnight.
4. Purify the DNA plasmids from the culture using a miniprep DNA isolation kit.
5. Resuspend the DNA in 50 uL of TE solution and determine the concentration of the samples. Store the solution at -20°C.

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.

More Quality Control information is available at the OriGene web-site <http://www.origene.com/rna/quality.msp>.

FAQ: pRS MAMMALIAN SHRNA EXPRESSION PLASMIDS

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

Answer: The pGFP-V-RS plasmid contains tGFP driven by a CMV promoter. The pRFP-C-RS plasmid contains tRFP driven by a CMV promoter. Both plasmids provide an easy way to identify cells that have been transfected. The pGFP-V-RS vector is kanamycin resistant, pRFP-C-RS vector is chloramphenicol resistant while the pRS vector is ampicillin resistant.

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 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 www.origene.com/rnai/exact-HuSH.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, so long as the transfection efficiency of our pRS. pGFP-V-RS and pRFP-C-RS vector is high, ~70% or greater for transient transfections. Afterwards, the effective construct(s) can be used for retroviral 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/rnai/exact-HuSH.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.

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 functionality of the shRNA vectors, 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.

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 (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, retroviral packaging cell lines can be used to generate retroviruses for stable cell line generation. For example, the plasmid can be transiently transfected into the Phoenix helper-free amphotropic packaging cells line. Two days after the transfection, the cell culture supernatant is collected, filtered and used to infect the target cell lines. Stable cell lines can be established following drug selection.

What use does the empty vector serve?

Answer: For any experiment involving the introduction of foreign DNA into cells,

it is important to eliminate any non-specific effects by using an empty vector negative control. The vectors can also be used for cloning your own shRNA construct(s).

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' CACAAGCTGGAGTACAACAGCCA 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 TR30016 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* are 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 for 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. However, we are in the process of creating a lentiviral HuSH vector. Please check our website for its release.

Do I have to use retroviral 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 gene expression 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.

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