

HuSH™ Validation Vector

RNAi Validation Vector - pCMV-LUC (V)

Application Guide

Package Contents and Storage Conditions	2
Other required reagents	2
Related products	2
Introduction	3
Production and Quality Assurance.....	4
General Considerations	4
Protocols	5
Subcloning your cDNA of interest into the validation vector	5
Digesting the validation vector, pCMV-LUC (V)	5
Not I digestion of cDNA clone	5
Partial digestion of cDNA	6
PCR Subcloning	6
Ligation and transformation	8
Validating the RNAi effect	9
Frequently Asked Questions	9
Appendix	13
Vector map	13
Vector features	13
Vector sequence	13
Sample results using the pCMV-LUC (V) validation vector.....	13
Limited Warranty and Licensing	15

Package Contents and Storage Conditions

Each kit comes with the following components:

Validation vector:

1 vial of pCMV-LUC (V) vector (TR30004), 5 µg lyophilized DNA

Sequencing primer

1 vial of LucVP3, 100 pmol lyophilized DNA

Controls

1 vial of pCMV-LUC(V)-CASP1, 1 µg lyophilized DNA

1 vial HuSH29mer shRNA construct against CASP1, 1 µg lyophilized DNA

1 vial of pRS (TR20003), 1 µg lyophilized DNA

The lyophilized materials are shipped at ambient temperature, and should be stored at 4°C upon receipt. Once reconstituted, the plasmid and primer must be stored at -20°C.

Other required reagents

- Competent bacterial cells
- LB agar + ampicillin plates
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH, then autoclave)
- Ampicillin
- DNA purification reagents
- Sterile deionized water
- RNAi constructs (shRNA plasmids or siRNA oligonucleotides)
- cDNA (to be cloned into the validation vector)
- Transfection reagent
- Mammalian cells and cell culture supplies
- Luciferase substrate
- Luminescent plate reader

Related products

HuSH-29: potent shRNA constructs for expression knockdown, covering 100% of human and mouse genomes

<https://www.origene.com/products/rnai>

TrueClone: expression-ready full-length cDNA clones covering 98% of the human genome

<https://www.origene.com/products/cdna-clones>

Turbofectin 8.0: new generation of transfection reagent optimized for both cDNA and shRNA delivery into eukaryotic cells

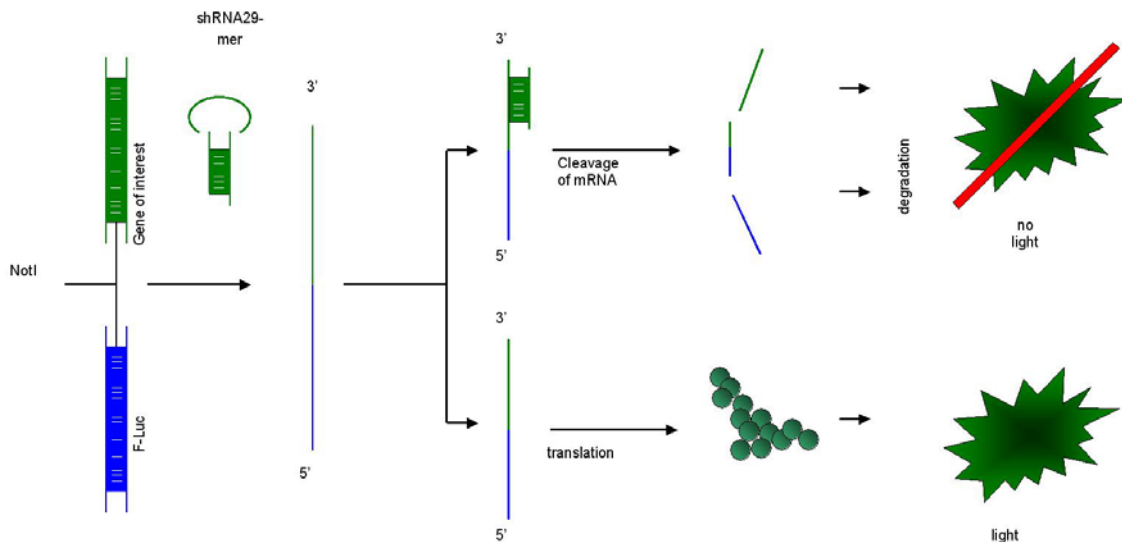
<https://www.origene.com/products/others/transfection-reagents/turbofectin>

Introduction

There continues to be some unpredictability in the use of RNA interference (RNAi) for gene silencing, which often requires time-consuming effort to determine effectiveness, select the best constructs, and optimize use for knockdown applications. Testing requires the use of antibodies (which may not be available), consumable qPCR probes, expensive equipment and/or phenotypic measurements.

To solve this problem, OriGene has developed the RNAi Target Validation Vector, a construct designed to incorporate a cDNA clone and a luciferase reporter gene as a chimeric transcript. This new tool will measure the effectiveness of RNAi constructs using nothing more than a luminescence plate reader. With the RNAi Target Validation Vector, one can quickly 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.

Using unique restriction sites, the gene of interest is cloned into the validation vector 3' to the firefly luciferase gene. The construct is then transfected into the mammalian cell of choice where a chimeric mRNA is transcribed. Cotransfection of HuSH shRNA or another RNAi construct will initiate the RNA interference process. If the RNAi is effective, the chimeric luciferase-target gene mRNA will be degraded, reducing the luciferase levels. If the shRNA or siRNA has a low affinity for the target, the chimeric transcript will not be targeted and luciferase activity will be high. Reduction of the luciferase signal indicates specificity for the target and effective gene silencing by the shRNA construct or siRNA oligonucleotides.



Production and Quality Assurance

The firefly luciferase coding region was cloned into OriGene's [pCMV6-XL5](#) vector using the Xba I cloning site. The 5' Not I site on the original pCMV6-XL5 vector was mutagenized, leaving a unique Not I cloning site downstream of the luciferase cDNA. The resulting vector, pCMV-LUC (V), has been fully sequenced and large scale DNA prep was generated prior to aliquotting and lyophilizing the product.

The sequencing primer LucVP3 has been used by OriGene for the detection of successful insertion and proper orientation of cDNA 3' to the luciferase cDNA.

The pCMV-LUC(V)-CASP1 construct contains the cDNA for CASP1 cloned into the Not I site of the pCMV-LUC(V) validation vector. This construct was sequenced to confirm the orientation of the CASP1 insert, which is 5' to 3' with respect to the CMV promoter. Large scale DNA prep was generated prior to aliquotting and lyophilizing the product.

The pCMV-LUC(V)-CASP1 construct and the HuSH 29mer construct targeting CASP1 (provided controls) were cotransfected into HEK293 cells at a ratio of 15:100 using the protocol provided in this manual, *validating the RNAi effect*. After 24-48 hours, luciferase activity was measured and compared to the luciferase activity obtained from cells transfected with pCMV-LUC(V)-CASP1 and empty pRS vector (TR20003). The luciferase activity was reduced by 50-60% due to knockdown of the chimeric luciferase/CASP1 transcript.

General Considerations

For any shRNA experiment, it is important to demonstrate that the effect of a targeting construct is gene specific, and not due to non-specific effects such as the interferon response or off-target silencing. Negative control plasmids should always be included in your experiments to exclude these non-specific responses.

All OriGene HuSH-29 products include two negative controls, the empty pRS vector (TR20003) and the non-effective shGFP construct (TR30003) containing a shRNA cassette cloned into the same pRS vector. The non-effective shGFP plasmid expresses a hairpin shRNA with no homology to any human gene, and serves as a negative control for gene specific knockdown and excludes effects due to the interferon response.

When using the validation vector to determine the effectiveness of shRNA constructs, it is important to cotransfect TR20003 or TR30003 with pCMV-LUC (V)+cDNA construct separately as background controls. Studies have shown that the pRS vector might enhance expression of luciferase from pCMV-LUC (V), so it is important to determine the background expression level of luciferase in the presence of a pRS vector. If siRNA is used with the validation vector, it is advisable to cotransfect a

scrambled control siRNA oligonucleotide with pCMV-LUC (V)+cDNA construct to determine background expression levels of luciferase for those experiments.

Protocols

Subcloning your cDNA of interest into the validation vector

The full-length cDNA inserts in all OriGene TrueClones are flanked by two Not I sites. Not I specifically recognizes an uncommon eight basepair sequence; therefore, these inserts can be released through Not I digestion without cutting the insert. If an internal Not I site exists in the TrueClone insert, a complete Not I digestion will result in multiple fragments, which may not be suitable for subcloning directly. This situation would require either a partial digestion strategy or PCR subcloning (see instructions below).

Any cDNA clone can be used in the RNAi Target Validation Vector. If the restriction sites surrounding your clone aren't compatible with Not I subcloning, a PCR subcloning strategy utilizing the Not I, Sac II, and/or Sma I restriction sites for directional cloning will be necessary. Perform restriction analysis of the cDNA to be cloned into the vector to exclude the existence of internal cutting sites.

Digesting the validation vector, pCMV-LUC (V)

1. Set up a digestion reaction using 1 µg DNA with Not I (or other enzymes as necessary) using conditions recommended by the restriction enzyme provider.
2. Incubate at 37°C for 90 min, or as recommended by the manufacturer.
3. If digesting with a single enzyme, add 0.5 µL DNase-free calf intestinal phosphatase (CIP; Promega #9PIM182) to the digestion reaction, and incubate at 37°C for an additional 30 minutes.
4. Run the digestion reaction on a 1% agarose gel. Cut out the band corresponding to the 6.2 Kb vector.
5. Use a gel purification kit to purify the linearized vector.
6. Estimate the recovered DNA quantity by A₂₆₀ spectrophotometric analysis or by running 1 µL of the purified eluate on an agarose gel and comparing the band intensity to a reference such as DNA Quanti-Ladder (cat #QLD200).
7. Continue with the ligation and transformation protocol, below.

Not I digestion of cDNA clone (insert)

1. Digest 0.5 - 1 µg donor plasmid DNA (such as an OriGene TrueClone) with Not I using the conditions recommended by the restriction enzyme provider.
2. Run the digestion reaction on a 1% agarose gel to separate the insert and vector fragments. Cut out the band corresponding to the cDNA insert.
3. Use a gel purification kit to purify the insert fragment.
4. Estimate the recovered DNA quantity by A₂₆₀ spectrophotometric analysis or by running 1 µL of the purified eluate on an agarose gel and comparing the band intensity to a reference such as DNA Quanti-Ladder (cat #QLD200).
5. Continue with the ligation and transformation protocol, below.

Partial digestion of cDNA (insert)

Partial digestion uses limiting enzyme and incubation time to produce vector/clone fragments that are not completely digested. Some of the DNA will be cut at only one of the Not I sites, some at multiple but not all Not I sites, and some will be cut at all Not I sites. In this case, you will be looking for digestion at two sites only - specifically, the two sites in the MCS but not internal to the insert.

To do a partial digestion, set up the following master mix:

- 10 μL 10X buffer
 - 1 μL 100X BSA
 - 1-4 μg DNA
 - up to 100 μL H_2O
1. Aliquot this master mix to five tubes, aliquotting 30 μL , 20 μL , 20 μL , 20 μL and 10 μL respectively.
 2. To the first tube containing 30 μL , add 0.5 μL Not I enzyme (10U/ μL). Pipet up and down to mix well.
 3. Remove 10 μL from this tube, and add it to the next tube in the series. Pipet up and down to mix well.
 4. Repeat steps 2 and 3 with all remaining tubes. Now you have 5 tubes of 20 μL digestion reactions with a serial dilution of enzyme.
 5. Incubate for 15 minutes at 37°C (instead of the usual 1-2 hours).
 6. Run each digestion reaction separately on a 1% agarose gel, and look for the reaction in which you see a band of the full size of the insert (a partial digestion product which has left the internal Not I sites intact). Purify that fragment as the intact cDNA clone. Continue with the ligation and transformation protocol, below.

PCR Subcloning (if using enzymes other than Not I to subclone)

Primer design

Design gene specific primers with restriction sequences at the ends. Consider the following recommendations when designing primers.

- Use sequence complementary to your cDNA for 18-22 bases.
- Introduce the sequence for the 5' restriction site at the 5' end of the forward primer and the sequence for the 3' restriction site at the 5' end of the reverse primer.
- Include additional bases at the 5' end of each primer to allow for efficient digestion by the restriction enzymes. Some of these extra bases can form a G/C clamp.
- Avoid too many Gs or Cs at the 3' ends of either primer.
- Avoid long runs of any single nucleotide.
- Make sure the forward and reverse primers don't form primer dimers (especially avoid complementary sequences at the 3' ends) or significant secondary structure.

- 94°C for 1 minute
- 65°C for 1 minute
- 72°C for 2 minutes
- Repeat 30-33 times

- 72°C for 10 minutes

Purify the PCR product and digest with the restriction enzymes whose sites were added to the ends (Not I and Sma I in the above example). Purify the digestion product, and continue with the ligation and transformation protocol, below.

Ligation and transformation

Prepare a ligation according to the following protocol

- 1 µL 10x ligation buffer
- 10 ng purified digested pCMV-LUC (V) vector
- 10 ng* purified digested insert from donor plasmid
- 0.25 U ligase
- up to 10 µL H₂O

*The optimal insert:vector molar ratio is about 2:1. Adjust the insert concentration accordingly, making sure to account for vector/insert sizes in the calculations.

Incubate the tube at room temperature for two hours or 16°C overnight (according to the manufacturer's recommendation).

Transform the mixture according to the directions from the manufacturer of the competent cells. A sample protocol for chemically competent cells is listed here.

1. Add 3 to 5 µL of the ligation mixture to 50 µL of competent cells (efficiency rated > 10⁸ cfu/µg DNA) on ice.
2. Mix the tube gently and incubate on ice for 20 minutes.
3. Heat shock the tube for 45 seconds at 42°C.
4. Incubate the tube on ice for 2 minutes, then add 500 µL SOC (recovery medium).
5. Incubate the tube at 37°C with agitation for 1 hour.
6. Spread 50 µl of the cells on an LB ampicillin- agar plate. You may also wish to plate larger amounts of this transformation mixture on separate LB-amp plates after pelleting the cells and resuspending in a smaller volume.
7. Incubate the plates at 37°C for 16 hours to allow colony formation.
8. Pick single colonies and inoculate liquid LB-amp cultures; grow at 37°C for 16 hours with agitation.
9. Perform DNA purification, and estimate the DNA quantity by A₂₆₀ spectrophotometric analysis or by running 1 µL of the purified eluate on an agarose gel and comparing the band intensity to a reference such as DNA Quanti-Ladder (cat #QLD200).
10. If the ligation was not directional (using a single restriction site such as Not I to insert the cDNA), sequence several independent clones with the LucVP3

primer to confirm that the cDNA was inserted in the sense orientation with respect to the CMV promoter. If none of the clones contain cDNA in the sense orientation, screen a larger number of clones until you identify one that has been inserted correctly.

Validating the RNAi effect

Introduction of the validation construct and gene specific shRNA into mammalian cells via transfection

1. Plate HEK293 cells at 3×10^3 cells/ well / 100 μL in a 96-well plate. Grow the cells overnight in a 37°C , 5% CO_2 incubator to achieve 50% confluence.
2. In a small sterile tube, combine the following reagents in the prescribed order. The amounts given below are for triplicate wells.

Note: Add TurboFectin 8.0 directly into the serum-free media. Do NOT let TurboFectin 8.0 touch any plastic other than the pipette tip.

Serum-free medium (OptiMEM I)	30 μL
TurboFectin 8.0 transfection reagent	1.8 μL
Validation construct (pCMV-LUC (V)+cDNA)	45 ng in 1-3 μL
HuSH shRNA plasmid against the target gene	0.3 μg in 1-3 μL

3. Mix the tube contents gently. Do NOT vortex.
4. Incubate at room temperature for 15-45 minutes.
5. Add the DNA-TurboFectin mix to the triplicate wells of the tissue culture plate (10 μL in each of three wells) directly without removal of the culture media. Mix by gently rocking the plate.
6. Twenty-four to forty-eight hours post-transfection, add 15 μL of luciferase substrate to each well. The luminescent signal can be determined by a plate reader immediately and for up to 15 minutes with virtually no loss in sensitivity.

Introduction of the validation construct and gene specific siRNA oligonucleotides into mammalian cells via transfection

Refer to protocols available from your siRNA oligonucleotide provider. Remember to include non-effective siRNA oligonucleotides in your co-transfection experiment with the pCMV-LUC (V) + cDNA construct as the background control.

Frequently Asked Questions

Can I use the validation vector with other RNAi products like siRNA oligos?

Answer: The pCMV-LUC (V) vector is appropriate for screening siRNA as well as shRNA constructs. You must be certain to subclone into the validation vector the region of the cDNA targeted by your RNAi constructs; for example, if your siRNA targets the 5' UTR, you must clone the cDNA including 5' UTR into pCMV-LUC (V).

What advantages does this system have over other similar reporter systems?

Answer: Our vector is particularly suitable for the subcloning of OriGene's TrueClone cDNA clones, as Not I sites exist on both vectors. Moreover, our vector utilizes firefly luciferase for measurement of the effect; the substrate of firefly luciferase is readily and commercially available, unlike the substrate for other forms of luciferase.

Does this replace the use of antibodies for Western blot analysis or qRT-PCR knockdown detection?

Answer: No. This vector is recommended as a prescreening method to choose the most effective construct with which to do your experiments. It is still advisable to perform Western blot analysis, qPCR, or functional assays to monitor the level of gene knockdown after you find the best knockdown construct using this screening method.

What advantages does this vector offer in the design of large screening assays?

Answer: If OriGene TrueClones are used in the design, one can subclone many genes into this vector in parallel using Not I. Bulk clone pricing is available; contact OriGene's Customer Service (custsupport@origene.com, 888-267-4436 or 301-340-3188) for more information.

Does this vector produce a luciferase – gene fusion protein?

Answer: No. Since the luciferase cDNA cloned into this vector contains its own stop codon, there will be no fusion protein of luciferase and your target gene produced. However, an intact luciferase protein will be translated if the chimeric transcript is not degraded due to RNAi against the target gene.

How does a gene specific shRNA or siRNA end up degrading the luciferase reporter in this vector?

Answer: The shRNA/siRNA forms a complex with DICER and the corresponding target mRNA in the RISC in order to degrade the mRNA sequence. This RNAi effect extends in the 5' direction, into the luciferase mRNA, leading to a decrease in luciferase enzymatic activity.

Is the coexpressed gene really similar to the gene I'm trying to silence?

Answer: In principal, the chimeric mRNA containing luciferase and your targeting gene should be similar to the endogenous version of your target mRNA. However, since the amounts of overexpressed mRNA may be much higher than endogenous mRNA, it may be more difficult to quantitatively knockdown your target gene as part of the chimeric mRNA compared to the endogenous target.

Has the validation vector been shown by other means that it provides specific quantifiable knockdown?

Answer: OriGene is currently testing this and we would also appreciate if you would share with us your own experimental results of this comparison. Please contact OriGene's Technical Support (techsupport@origene.com, 888-267-4436, 301-340-3188) for details about setting up such collaborations.

Can I subclone genes that are not from the OriGene TrueClone collection into the validation vector?

Answer: Yes, you can subclone any cDNA into this vector in order to determine the potential knockdown effect of shRNA/siRNA constructs. Please see the Protocols section in this manual for advice on subcloning cDNA into pCMV-LUC (V).

Other than Not I, what other MCS restriction sites are available for subcloning?

Answer: Sac II and Sma I are also available at the MCS. Please confirm that these restriction sites do not exist in the internal region of the cDNA if you are using this subcloning strategy.

Which cloning methods work best with the pCMV-LUC (V) vector?

Answer: Non-directional Not I subcloning or restriction and PCR-based directional subcloning are equally compatible with this vector. It is the customer's preference which to choose. Please note that non-directional cloning requires a screening step to confirm that the cDNA is cloned in the sense orientation with respect to the CMV promoter and luciferase cDNA.

How can I subclone a Gateway compatible clone into this vector?

Answer: Determine if there are any cloning sites in the Gateway vector compatible with pCMV-LUC (V). If not, use the PCR directional cloning strategy (see the Protocols section in this manual) to clone the Gateway cDNA into pCMV-LUC (V).

Why must the cDNA be cloned in the sense orientation to validate my shRNA or siRNA?

The mechanism of RNA-induced transcript degradation depends on an enzyme complex called the RNA-induced silencing complex (RISC). The RISC complex contains an endoribonuclease that utilizes only the anti-sense strand of the trigger shRNAs (or siRNAs) to target the complementary mRNA sequence for degradation. A cDNA cloned in the anti-sense orientation will not produce a transcript that is complementary to the anti-sense sequence used by RISC and thus will not be degraded.

What is the sequence of the primer LucVP3?

Answer: The nucleotide sequence of our sequencing primer is 5' GGAAACTCGACGCAAGAAA 3'. This primer anneals near the end of the firefly luciferase coding region, and will read through the MCS and into your subcloned cDNA so that you can confirm the orientation of the subcloned cDNA.

Do I have to cotransfect the shRNA and the validation vector or should they be introduced to the cells separately? Which would be best?

Answer: We recommend that you cotransfect both the pCMV-LUC (V) + cDNA construct and shRNA plasmids simultaneously. Commercially available transfection reagents, such as OriGene's Turbofectin 8.0 (catalog # [TF81001](#)), work very well for the cotransfection of cDNA and shRNA plasmids.

What luciferase substrate would you recommend for use with the RNAi Target Validation Vector?

Answer: We typically use Perkin Elmer's Ultra-High Sensitivity Luminescence Reporter Gene Assay System (No. 6016977).

Once I have determined which knockdown construct works best, should I keep or discard the others?

Answer: One construct may work better in one cell line but be less efficient in another. If you change the cell type used in your experiment, it may be necessary to rescreen to find the best performer. The other constructs may also be useful for partial knockdown studies.

Can the validation vector itself interfere with gene expression in my cells?

Answer: We do not have data to exclude this possibility. Thus, it is very critical to include suitable negative controls in the cotransfection experiments.

What can I conclude from seeing positive results (reduction of luciferase activity) using this validation vector?

Answer: A reduction in the activity of luciferase indicates successful targeting of the chimeric mRNA by your shRNA or siRNA construct(s). When screening a number of constructs, choose the one producing the greatest decrease in luciferase activity for use in future experiments.

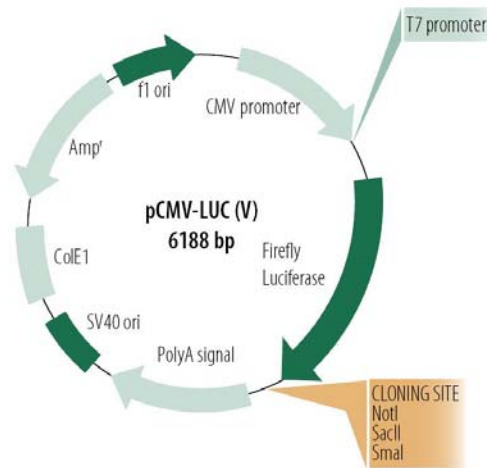
What should I do if none of my constructs result in a reduction in luciferase activity?

Answer: Please use the provided controls to exclude any problems with your experimental setup or readout. Transfect pCMV-LUC(V)-CASP1 together with the HuSH-29 construct targeting CASP1 into your target cells using the protocol provided in this manual, *Validating the RNAi effect*. Measure the resulting luciferase activity and compare with the luciferase activity obtained from cells transfected with pCMV-LUC(V)-CASP1 and empty pRS vector (TR20003). You should see at least a 50-60% decrease in luciferase activity with the HuSH construct; this result will indicate that your experimental conditions and this system are working well.

Does OriGene provide any guarantee of knockdown in subsequent experiments when an RNAi construct has been validated using this method?

Answer: No. The degree of knockdown of your target gene will be dependent on a number of factors, including transfection or infection method and efficiency, target cells, and transcript or protein half-life. For this reason, OriGene cannot guarantee knockdown using a pCMV-LUC (V) validated construct.

Appendix Vector map



Vector features

The OriGene pCMV6-XL5 vector has been adapted for this validation assay by the insertion of firefly luciferase using Xba I and destruction of one of the NotI sites. The remaining Not I site provides a simple, single digestion cloning strategy for any OriGene TrueClone into the vector. This pCMV-based vector allows for maximal expression of luciferase in most mammalian cells transfected.

- Vector size: 6.2kb
- Selection marker in *E. coli*: Ampicillin
- Selection marker in mammalian cells: None. For transient transfection only
- Promoter for *in vitro* expression in mammalian cells: CMV promoter
- Promoter for *in vitro* expression in a cell free system: T7
- Cloning sites: Not I, Sac II, Sma I

Transcription termination and polyadenylation signals for firefly luciferase are from human growth hormone (hGH) gene.

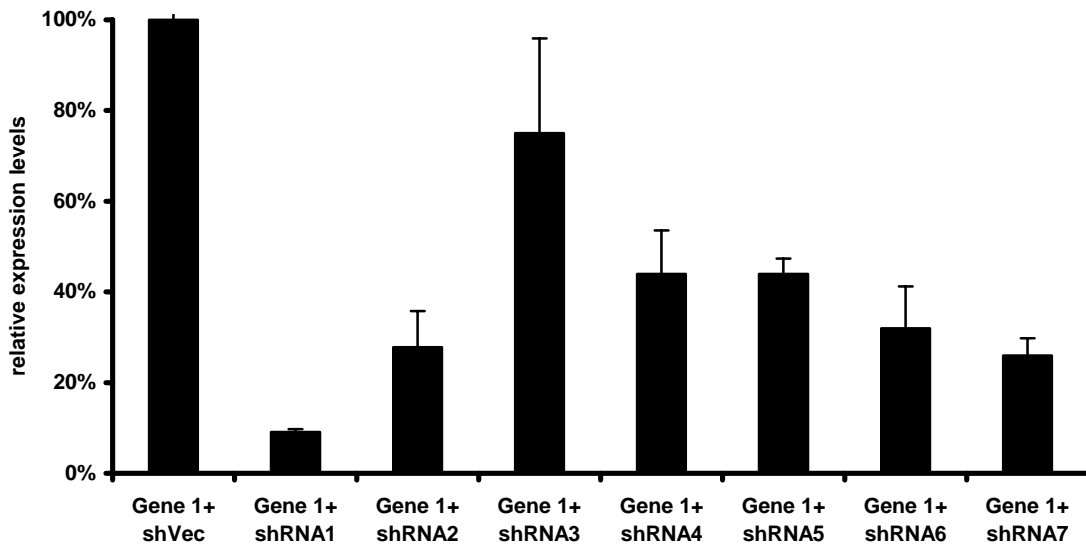
Vector sequence

The nucleotide sequence of pCMV-LUC (V) is available electronically at the following URL:

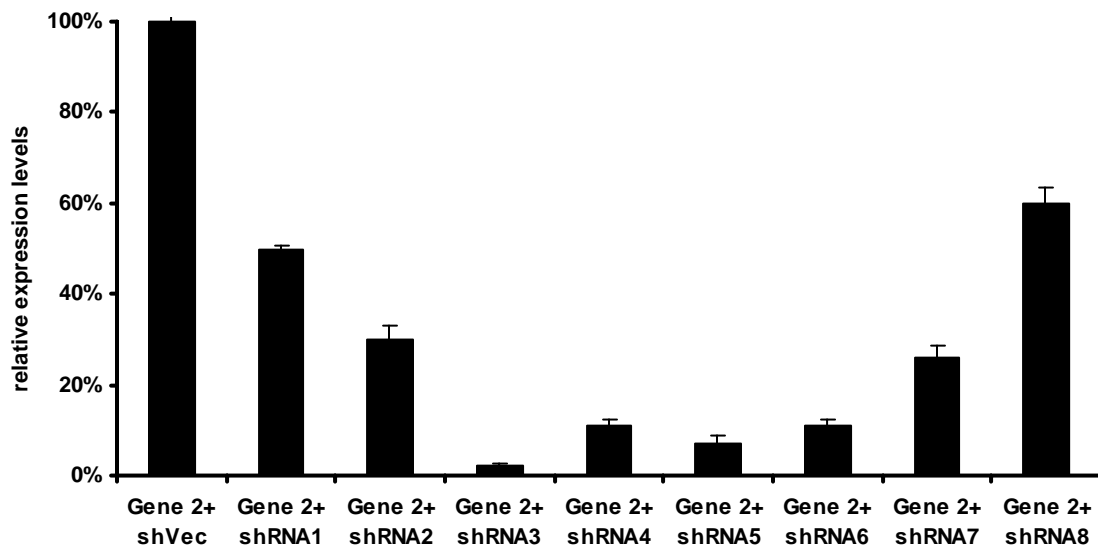
<http://www.origene.com/assets/Documents/NucleotidesequenceforpCMVLUCVector.doc>

Sample results using the pCMV-LUC (V) validation vector

A.



B.



Target genes were cloned into the pCMV-LUC (V) vector in the sense orientation. Fifteen ng of these plasmids were cotransfected with 100 ng of a HuSH-29 shRNA construct or control into HEK293 cells using TurboFectin 8.0. Twenty-four hours post-transfection, cells were lysed using Perkin Elmer's luciferase substrate solution and the luciferase activity was measured with a PE Victor3 plate reader. Relative light units for each condition (measured in triplicate wells) were measured, and are shown as percentage of expression when compared with cotransfection of the target gene and the empty pRS vector. A. Target gene 1 is NM_001363, B. Target gene 2 is NM_001797.

Disclaimer

Use of this product is subject to a license from CSHL or Hairpin Technologies, and CSHL. Hairpin Technologies reserves all other rights under its license. For information on licensing rights for Commercial Entities, including use of this product for purposes other than research and trial licenses, please contact Hairpin Technologies, Inc. at licensing@hairpintechnologies.com or call (631) 881-0844.