Trilencer-27

Application Guide

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Revision 03.18TS

Package Contents and Storage Conditions

Materials	Format	Quantity
Gene-specific siRNA duplexes	Purified and sequence-verified	2nmol per vial. Three unique duplexes per gene.
SR30004-Universal scrambled negative control siRNA duplex	Purified and sequence-verified	1nmol per vial.
SR30005-Rnase free siRNA duplex re-suspension buffer	100mM KAc/30 mM HEPES pH 7.5	2ml per vial

Storage Conditions

The controls are stable for one year from date of shipping when stored at -20ºC.

Related products

- Trilencer-27 TYE-563 labeled fluorescent transfection control siRNA duplex
- Trilencer-27 HPRT1 Positive Control siRNA duplex
- HUSH™ shRNA plasmids www.origene.com/rnai/
- TrueORF™ Tagged ORF clones www.origene.com/orf/
- siTran siRNA Transfection Reagent www.origene.com/cdna/transfection.mspx

Additional materials recommended

- Transfection reagent: Transfection reagents must be selected and optimized based on the cell type being used. OriGene offers siTran siRNA transfection reagent ideal for most widely used cell types. For details, visit www.origene.com/cdna/transfection.mspx
- Cell line and cell culture supplies: user preferred
- Reagents for cell lysis: user preferred
- 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

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).

In cells, small interfering RNAs (siRNAs) are produced by cleavage of long double-stranded RNAs by the RNase-III class endoribonuclease Dicer. The siRNAs associate with the RNA Induced Silencing Complex (RISC) in a process that is facilitated by Dicer. Dicer-Substrate RNAi methods take advantage of the link between Dicer and RISC loading that occurs when RNAs are processed by Dicer. Traditional 21-mer siRNAs mimic Dicer products and bypass the need for Dicer processing. Dicer-Substrate RNAs are 27-mer RNA duplexes that are optimized for Dicer processing and show increased potency and specificity when compared with 21-mer duplexes [1, 2].

The Trilencer-27 siRNA kit contains three Dicer-Substrate 27-mer duplexes targeting a specific gene that are selected from a predesigned set of duplexes from the RefSeq collection of human Genbank. OriGene's siRNA collections of 27-mer sites were chosen to integrate both traditional 21-mer siRNA design rules as well as new 27-mer design criteria. In addition, analysis was performed to ensure that the chosen sites do not target alternatively spliced exons and also do not include known SNPs; these sequences are therefore optimized at several levels.
In addition to three target-specific duplexes, the siRNA kit contains a scrambled universal negative control RNA duplex that is absent in human, mouse, and rat genomes. OriGene guarantees that at least two of the three Dicer-Substrate duplexes in the kit will provide at least 70% or more knockdown of the target mRNA when used at 10 nM concentration by quantitative RT-PCR when the fluorescent transfection control duplex indicates that >90% of the cells have been transfected and the HPRT positive control provides 90% knockdown efficiency.

Positive control Dicer-Substrate RNA duplex (HPRT1 Positive Control) which targets a site in the HPRT (hypoxanthine guanine phosphoribosyltransferase 1) and a TYE-563™ labeled transfection control RNA duplex are available for purchase.

Product Application

Reconstitution of siRNA duplexes in duplex buffer

1. Materials should only be handled with gloves under RNase-free conditions.
2. Briefly centrifuge each tube to ensure that all material is in the bottom of the tube for the first time. Dried oligo often dislodges during shipping and can be lost.
3. Resuspend duplexes in the provided Rnase-free duplex buffer. Other sterile, RNase-free buffers are suitable that contain at least 50 mM NaCl or KCl. Oligos

require the presence of cations to form stable duplexes, so resuspension in water is NOT recommended. If your buffer contains Mg salts (OriGene's Duplex Buffer does not contain Mg), do not heat above 75°C as this may result in chemical degradation of the RNA.

   a. 2 nmoles of each target specific duplex and negative control is provided. Addition of 100 µl of RNase-free Duplex Buffer will result in 20 µM final concentration; vortex thoroughly and microfuge prior to use.
   b. Heat to 94°C for 2 minutes, remove from heat and allow tube to cool to room temperature. The oligos were dried in duplex form so heating may not be necessary, however following this protocol ensures that the contents will be fully duplexed.

4. Once hydrated, duplexes should be stored at -20°C or -80°C. While generally stable to freeze/thaw cycles, OriGene recommends that daughter aliquots be made for routine use to minimize the frequency of freeze/thaw events for primary stock tubes. Minimize light exposure for dye-labeled duplexes such as the TYE-563 transfection control.

Outline of an RNAi Experiment

1. Establish optimal transfection method for your cell type and culture media (use fluorescent-labeled transfection control duplex); favored approach is fluorescence microscopy. Greater than 90% of cells should show dye uptake when examined 4-24 hours after transfection.
2. Demonstrate that RNAi is working using positive control (HPRT1 Positive Control duplex); favored approach is quantitative real-time RT-PCR. HPRT should show >90% knockdown 24 hours post-transfection at 10 nM dose.
3. Test target specific duplexes and perform dose response curve. OriGene recommends testing duplexes at 10 nM, 1 nM and 0.1 nM concentrations. Knockdown of mRNA levels should be assayed at 24-48 hours post transfection. To limit off-target effects, routine studies should subsequently be performed using the lowest concentration of RNA duplex that achieves the desired level of suppression of the target mRNA.
4. Perform RNAi studies using duplexes identified as "effective by >70% reduction in RNA levels". OriGene recommends that the results of two duplexes against the same target be compared to control for potential off-target effects and other artifacts.
   a. mRNA levels can generally be measured 24-48 hours post transfection.
   b. Protein levels can generally be measured at 48-72 hours post transfection, however this may vary depending on the half-life of the protein studied and cell growth rate.
   c. Phenotype studies should parallel protein evaluation.
5. Controls: While examination of non-transfection and mock-transfection cultures (lipid or electroporation alone) are useful, OriGene recommends that control cultures transfected using control RNA duplexes be used for target level normalization. A randomized sequence (Universal Scrambled Negative Control) duplex is provided for this purpose, which is not present in human, mouse, or rat.

Transfection Optimization

Although the transfection protocol below has been shown to result in highly efficiency transfection using OriGene's siTran transfection reagent, it is recommended to optimize the reaction conditions for each individual cell type and reagents from other manufacturers. The following variables should be considered:

A. Cell density (% confluence at transfection): The recommended confluence for most adherent cell types at transfection is 50-70%. We recommend that customers determine the optimal cell density for the particular cell type used in their experiments.
B. Transfection reagent to siRNA ratio: We recommend that you optimize the ratio by adjusting the volume of transfection reagent within the guidelines of recommended usage by the manufacturer.

Table 1. Recommended starting transfection conditions for siRNA using siTran 1.0

Plate Format	Final Volume (µL)	Vol. siRNA (5.0 uM), (10 nM final)	siTran 1.0 reagent	Opti-MEM
6 well	2800	5.0 ul	20.0 ul	140 ul
12 well	1200	2.0 ul	10.0 ul	60 ul
24 well	680	1.20 ul	6.0 ul	34 ul
48 well	340	0.6 ul	3.0 ul	17 ul
96 well	120	0.3 ul	1.5 ul	8.5 ul

Table 2. Recommended starting transfection conditions for siRNA and plasmid DNA in a co-transfection using siTran 1.0

Plate Format	Final Volume (µL)	Vol. siRNA (5.0 uM), (10 nM final)	Plasmid DNA	siTran 1.0 reagent	Opti-MEM
6 well	2800	5.0 ul	360 ng	20.0 ul	140 ul
12 well	1200	2.0 ul	180 ng	10.0 ul	60 ul
24 well	680	1.20 ul	120 ng	6.0 ul	34 ul
48 well	340	0.6 ul	60 ng	3.0 ul	17 ul
96 well	120	0.3 ul	30 ng	1.5 ul	8.5 ul

Protocol for transient transfection in a 96-well plate

1. Plate cells one day prior to transfection. Monitor the cell number so that upon performing transfection, the cells are at 50-70% confluence.
2. Dilute siRNA to a concentration of 5 uM using sterilized duplex buffer supplied by siRNA manufacturer.
3. Set up a series of sterilized 0.2 ml PCR tubes. Add 10 ul of Opti-MEM media and 0.3 ul of 5 uM siRNA to each well.
4. Prepare adequate volume of diluted siTran 1.0 (10 ul per well) in Opti-MEM using the recommended ratio siTran (1.5ul siTran/8.5 ul Opti-MEM) and/or ratio suitable for your cell line. Add 10ul of the siTran dilution to each PCR tube with siRNA solution.

5. Mix the solution and let it stay at room temperature for 10 mins.
6. Transfer the siTran and siRNA mixtures to each well of the microtiter plate prepared one day earlier.
7. Incubate the plate at 37oC in a CO2 incubator.
8. Observe cells under a fluorescent microscope or harvest cells 24 to 48 hrs after transfection

Determining siRNA 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 –80oC 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 PMSF[2], 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 buffer 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^{o}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 manufacture'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^{o}C for several weeks. However, once the membrane is dried, it needs to be wetted by methanol followed by PBS.

*2X 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 TBST 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^{o}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.

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

FAQ: Trilencer-27 siRNA kit

How are 27-mer Dicer-Substrate siRNA different from traditional 21mer siRNAs?
Traditional 21-mer RNA duplexes mimic the natural siRNAs that are the result of Dicer processing of long substrate RNAs. In contrast, 27-mer siRNAs go through natural mechanism of Dicer processing to produce 21-mer siRNAs. The Dicer-substrate approach can result in 10-fold higher potency than traditional 21-mer siRNAs at the same site.

What are the benefits of the Trilencer-27 siRNA duplex design?
A full screening was performed which included blasting the sequences to ensure they only line up with the target gene, performing secondary structure checks, and making duplex stability check for incorporation into the RISC complex.

How should I use the products?
Customers can use the 3 siRNA duplexes 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 siRNA duplex, or RNA can be harvested from transfected cells and used in quantitative RT-PCR

to determine the loss of gene expression. If desired, the validated siRNA target can be integrated into OriGene's retroviral plasmid vector using exact-shRNA service and these shRNA plasmids can be re-transformed for unlimited supply.

What controls are offered with the Trilencer-27 kit?
The control we offer with the kit is Universal scrambled negative control duplex (SR30004)

Other controls are available for purchase and are listed below:
TYE 563-labeled transfection control RNA duplex (SR30002)
HPRT1 Positive Control duplex (SR30003)
These duplexes can be ordered separately at www.origene.com/siRNA/Controls.aspx

What use does the scrambled non-effective siRNA duplex serve?
To specifically rule out the potential non-specific effect induced by expression of the siRNA, OriGene provides customers with a negative control (SR30004). The duplex should serve as a negative control for gene-specific knockdown experiments and exclude any potential interferon response.

Does the siRNA kit come with RT-PCR reagents?
The siRNA kit includes:
3 Dicer-Substrate RNAi duplexes, 2 nmol each
1 negative control duplex, 2 nmol
Rnase-free duplex re-suspension buffer

qSTAR primer pairs and SYBR Green master mix are available for purchase. For more information, visit www.origene.com/geneexpression/

What is the concentration of Trilencer-27 siRNA needed to conduct the experiment and see knockdown?
The actual level of target gene knockdown relates to the transfection efficiency. A positive control such as HPRT siRNA should always be used in each experiment to assess transfection efficiency.
In addition, varying amounts of Trilencer-27 duplexes ranging from 0.1 to 50 nM can be used to determine which siRNA shows maximum knockdown. Normal optimization concentrations vary between 1-50nM siRNA, with 10nM being most common.

What is OriGene's guarantee on siRNA duplexes?
OriGene guarantees that at least two of the three Dicer-Substrate duplexes in the kit will provide at least 70% or more knockdown of the target mRNA when used at 10 nM concentration by quantitative RT-PCR when the fluorescent transfection control duplex indicates that >90% of the cells have been transfected and the HPRT positive control provides 90% knockdown efficiency.

I am writing a paper for publication and need to describe this product. How should I cite?
We recommend that you refer to the product by its specific catalog number and refer to us as OriGene Technologies (Rockville, MD). Furthermore, we'd love to hear from you when your paper is published. Inform us and we will send a gift.

References

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4. Amarzguioui, M., Improved siRNA-mediated silencing in refractory adherent cell lines by detachment and transfection in suspension. Biotechniques, 2004. **36**(5): p. 766-8, 770.
5. Bieche, I., et al., Quantitation of hTERT gene expression in sporadic breast tumors with a real-time reverse transcription-polymerase chain reaction assay. Clin Cancer Res, 2000. **6**(2): p. 452-9.