

siRNA-27

Application Guide

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Package Contents and Storage Conditions

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

Storage Conditions

The dried duplexes can be stored at 4°C. However, once reconstituted with dH₂O, the plasmids must be stored at -20°C.

Related products

- Cy3-labeled fluorescent transfection control siRNA duplex
- HPRT1 Positive Control siRNA duplex
- TrueORF™ Tagged ORF clones www.origene.com/orf/
- HUSH™ shRNA plasmids www.origene.com/rnai/
- siTRAN siRNA Transfection Reagent www.origene.com/cdna/transfection.mspix

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.mspix
- 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/anti-body.

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).

In cells, small interfering RNAs (siRNAs) are produced by enzymatic cleavage of long dsRNAs 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 are chemically synthesized RNA duplexes that mimic Dicer products and bypass the need for Dicer processing. Dicer-Substrate RNAs are chemically synthesized 27-mer RNA duplexes that are optimized for Dicer processing and show increased potency when compared with 21-mer duplexes [1, 2].

The siRNA-27 kit contains three Dicer-Substrate 27-mer duplexes targeting a specific gene that are selected from a pre-designed set of duplexes from the RefSeq collection of human Genbank. OriGene's siRNA collection of 27-mer sites were chosen by a rational design algorithm that integrates 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 (DS Scrambled Neg) that is absent in human, mouse, and rat genomes. OriGene guarantees that at two of the three Dicer-Substrate duplexes in the siRNA-27 kit will give at least 70% knockdown of the target mRNA when used at 10 nM concentration and assayed by quantitative RT-PCR when the fluorescent transfection control duplex indicates that >90% of the cells have been transfected and the HPRT positive control works with the expected efficiency.

Positive control Dicer-Substrate RNA duplex (HPRT1 Positive Control) which targets a site in the HPRT (hypoxanthine guanine phosphoribosyltransferase 1) and a Cy3TM - 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 and not in the cap before opening for the first time. Dried oligo often dislodges during shipping and can be lost.
3. Resuspend duplexes in the provided 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 duplex is provided (including the control duplex). 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.
 - c. Each 1 nmole tube contains enough duplex for the number of transfections shown in Table 1. Target-specific duplexes are provided with 2 nmole yield and will accordingly yield more transfections.

Table 1. Transfections per1 nmole duplex RNA.

Plate Format	Media Volume (µL)	# of Transfections at 1 nM	# of Transfections at 10 nM
6 well	2500	400	40
12 well	1200	820	82
24 well	600	1660	166
48 well	250	4000	400
96 well	150	6660	666

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.
5. Refills of gene-specific or control duplexes can be ordered on www.origene.com. Note that additional control duplexes are available. These are not included in the kit but may be useful for certain applications. Sequences can be ordered in larger scale as needed.

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. Duplexes targeting reporter genes such as EGFP or Firefly Luciferase can also be employed as controls, however these duplexes have not been optimized for this use.

Choice of Transfection Method

A successful RNAi experiment starts with good transfection. Unfortunately, the same methods that work well for plasmid DNA transfection may not work well when using short duplex RNAs; reagents and protocols must be optimized for each type of nucleic acid transfected and for each different cell type employed. The Cy3-labeled Transfection Control and HPRT1 Positive Control duplexes are available and can be purchased to assist with this phase of the RNAi project.

Cationic lipids are commonly employed to transfect duplex RNAs into tissue culture cells. It may be necessary to test a variety of lipid agents with each cell type used to identify the optimal reagent that introduces duplex RNA into cells with high efficiency yet results in minimal toxicity. After successful transfection of a fluorescent duplex, the cells will show both punctuate and diffuse cytoplasmic staining and also remain “healthy appearing” when examined using fluorescence microscopy. A Cy3-labeled transfection control duplex

(excitation max 556 nm, emission max 570 nm) is available for purchase. OriGene recommends use of the fluorescent transfection control oligos at 10 nM concentration; optimal amounts may vary with the cell types and equipment employed. Transfection efficiency should be assessed within a 4-24 hour window of transfection.

Some cell types are resistant to transfection using cation lipids. Introduction of RNAi duplexes into such cells can often be achieved using electroporation. Liposomal and peptide-based transfection reagents are also available and may be of benefit. Like cationic lipid transfection, electroporation requires optimization. Important variables include voltage, pulse length, pulse number, buffer composition and RNA concentration. A recently published report suggest that the off-target effects (alteration in gene expression profiles not specifically targeted by the siRNA) can be reduced using electroporation mediated transfections as compared to those performed with lipid reagents [3].

Transfection Optimization using Cationic Lipids

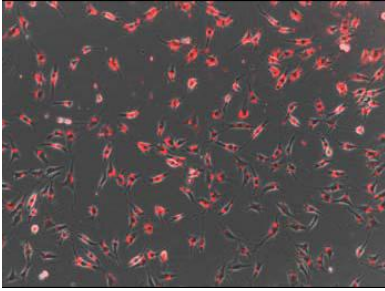
Variables to consider for transfection optimization when using chemical reagents include:

- Reagent choice
- Cell density
- Ratio of reagent to siRNA
- Amount of siRNA
- Length of time delivery reagent is left on cells
- Presence or absence of serum in the medium
- Post transfection incubation period

Reagent selection is important and optimal choice varies with cell type. Some cell lines can be successfully transfected using a wide variety of reagents. Using HeLa cells, we have achieved >90% transfection efficiency using siLentFect™ (BioRad), Oligofectamine™ (Invitrogen), Lipofectamine2000™ (Invitrogen), X-tremeGENE™ (Roche), HiPerFect™ (Qiagen), and TransIT-TKO™ (Mirus). Other cell lines may be refractory to some or most of these reagents. There are dozens of transfection reagents commercially available with varying properties and a survey of a wide range of reagents may be necessary if your cell type is difficult to transfect.

When targeting an endogenous gene, the transfection efficiency must be very high (>90%). A transfection of 80% efficiency will leave 20% of the target RNA intact even if the RNAi duplex is 100% effective! Un-transfected cells will lead to underestimation of the potency of a siRNA duplex and may obscure important biological effects. Transfection efficiencies can be visually estimated using fluorescent transfection control duplexes Cy3-labeled Transfection Control (Figure 1)

NIH3T3 Cells



HeLa S3 Cells

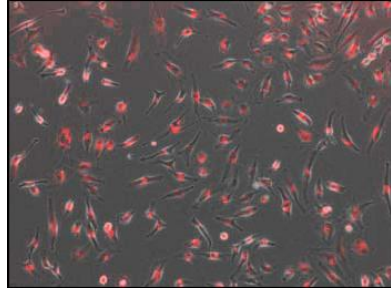


Figure 1. Transfection Efficiency Visualized at 24 hours post-transfection.

Cells were transfected at a final concentration of 10 nM with the Cy3-labeled Transfection Control duplex using X-tremeGENE (Roche) transfection reagent. Cells were imaged 24 hours post transfection.

Each manufacturer provides guidelines for use of their product, but all suggest that the reagent be tested against varying numbers of cells and differing ratios of reagent to siRNA. OriGene recommends testing Dicer-substrate duplexes at 10 nM, 1 nM, and 0.1 nM concentrations.

Another variability to consider is the length of time the cells are exposed to the transfection mixture. With some reagents, the transfection mixture can remain on the cells until harvest or passage with good results. Other reagents need to be washed from the cells or the cell death rate increases. A balance between maximal RNA knockdown and minimal cell death should be experimentally determined.

Commercial kits are available to assess cell viability, such as the CellTiter-GloTM Luminescent Cell Viability Assay (Promega) that monitors ATP levels. Another option is the CellTiter-BlueTM Cell Viability Assay (Promega) that is based on the ability of cells to convert a redox-based dye resazurin into resorufin, a fluorescent molecule.

Traditional transfection protocols for adherent cell lines call for the passage and plating of the cells one day before transfection. A variation that has shown promise for some cells that are difficult to transfect is to mix the reagents with the cells and plate the entire mixture at the same time [4].

The length of incubation time post-transfection before cells are examined for RNAi effect varies with target and the assays employed. Fluorescence to assess transfection efficiency should be examined at around 24 hours (4-24 hour window). RNA levels can usually be studied 24-48 hours after transfection. Protein levels and phenotypes are usually studied 48-72 hours after transfection; however the optimal time can vary depending on protein half-life, cell division rate, and a variety of other factors. Cells may need to be passaged to maintain healthy densities. Duration of gene suppression using

RNAi can vary from ~4 days to two weeks; in general, more potent reagents maintain suppression longer. Repeat transfection may be necessary to study the long-term effects from sustained suppression of a particular gene target.

Positive and Negative Controls

Fluorescent-labeled duplexes permit rapid optimization of transfection protocols. The best control for a good transfection, however, is demonstration of successful suppression of a control gene using a known effective siRNA. OriGene offers a validated HPRT1 Positive control duplex for this purpose. It is a potent Dicer-Substrate duplex that has been shown to suppress HPRT mRNA levels >90% when used at 10 nM concentration. OriGene also provides a validated negative control duplex as part of the kit that is not present in the human, mouse, or rat genomes.

Validated primers for use in a HPRT SYBR Green Q-PCR assay are available from OriGene's qSTAR primer collection.

Measuring RNAi Knockdown of Gene Expression

OriGene recommends using quantitative assessment of mRNA levels as the primary tool to establish efficacy of RNAi methods. Measurements of protein levels, enzyme activity, or phenotype can vary widely depending on protein half-life, turnover rates, and other factors such that it is possible to observe a seemingly negative result even when mRNA levels have been substantially suppressed. Protein levels and phenotype effects can be assessed after it has been established that mRNA levels are reduced.

RNA \longleftrightarrow **Protein** \longleftrightarrow **Phenotype**

The method used to isolate RNA is an important consideration. It is difficult to obtain useful data with degraded RNA. A wide variety of reagents and kits are available to assist with RNA isolation. For small sample sets, a simple reagent like RNA STAT-60™ (Tel-Test Inc Friendswood, Texas) works well and can yield consistent high quality RNA inexpensively. Isolation of RNA from a large numbers of samples is better accomplished using one of many commercial kits available such as SV96 Total RNA Isolation Kit (Promega, Madison, WI) or Aurum Total RNA Kit (Bio-Rad, Hercules, CA). Some kits like the Aurum series are available in different formats, vacuum filtration or column spin filtration). Verifying RNA quality is important and can be assessed by either direct visualization using denaturing gel electrophoresis or through the use of a specialized microfluidics based platform like the Agilent 2100 Bioanalyzer (Agilent Technologies, GmbH) or the Experion (BioRad, Hercules, CA). The advantage of the Bioanalyzer or the Experion is that both RNA quality and RNA concentration can be determined consuming a minimal amount of sample.

Northern blots, RNase Protection Assays (RPAs), or quantitative real-time RT-PCR can be employed to measure relative gene expression levels in the RNA sample. OriGene recommends use of qRT-PCR as the method of choice; this approach is robust and is quantitative over a wide range of RNA levels. It is essential to employ a reliable internal control for standardization and normalization. We have employed an assay for acidic ribosomal protein P0 as internal control with good results (RPLP0) [2, 5].

Assessing the degree of target knockdown can also be done at the protein level. Protein levels can be determined via Western blots or a functional activity assay (when available). Commercial expression plasmids that allow for cloning of a target gene sequence into the 3' UTR of a gene with an easily assayable activity like Luciferase (psiCHECKTM-2, Promega, Madison, WI) allow for rapid analysis of numerous samples. The psiCHECKTM-2 vector contains both the Firefly and Renilla Luciferase genes; Renilla Luciferase is used as the target and the Firefly Luciferase is used for normalization.

FAQ: siRNA-27 kit

What are Dicer-Substrate RNAs?

Dicer-Substrate RNAs are chemically synthesized RNA duplexes that are optimized for Dicer processing and show increased potency when compared with 21-mer duplexes.

What is the difference between DsiRNAs (27 mers) and traditional 21mer siRNAs?

Many researchers today employ synthetic 21-mer RNA duplexes as their RNAi reagents, which mimic the natural siRNAs that are the result of Dicer processing of long substrate RNAs. An alternative approach is to use synthetic RNA duplexes (DsiRNAs) that are greater than 21-mer in length, which are substrates for Dicer. DsiRNAs are processed by Dicer into 21-mer siRNAs and designed so that cleavage results in a single, desired product. This DsiRNA design provides Dicer with a single favorable PAZ binding site that helps direct the cleavage reaction. Functional polarity is introduced by this processing event, which favors AS strand loading into RISC, and the increased potency of these reagents is thought to relate to increased DsiRNAs being loaded into and processed through Dicer. The Dicer-substrate approach can result in reagents having as much as 10-fold higher potency than traditional 21-mer siRNAs at the same site.

Is the designed RNA duplex specific?

Our Bioinformatics department did a full screening of the recommended sequences before adding them to the database. This included blasting the sequences to ensure they only line up with the gene in questions, performing secondary structure checks, and making sure the end stabilities were favorable for incorporation into the RISC complex.

How do I order GFP controls to go along with the siRNA kit?

We do not offer any GFP controls along with the siRNA kit. The controls we do offer are listed below

Cy3-labeled transfection control RNA duplex (SR30002)

HPRT1 Positive Control duplex (SR30003)

Universal scrambled negative control duplex (SR30004)

These duplexes can be ordered separately at www.origene.com/siRNAcontrols

Does the siRNA kit come with primers and RT-PCR materials?

The siRNA kit includes:

3 Dicer-Substrate RNAi duplexes, 2 nmoles each- to test knockdown you will need to

order primers separately.
2 nmole negative control duplex
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 concentration of DsiRNAs do I need to use in my experiment to see knockdown?

For an RNAi or antisense experiment, 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 test DsiRNA ranging from 0.1 to 50 nM can be used to determine which DsiRNA shows maximum knockdown

Do you provide any non-silencing controls for Dicer Substrate RNAi duplexes?

We currently include a scrambled negative control for our siRNA kit. Other controls are available for purchase separately. The normal turn around time for the siRNA kit is 7-9 business days. The number of assays that can be done will vary widely with the transfection reagent used and the cell line you are trying to transfect. Optimization will be necessary. 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 give at least 70% or more knockdown of the target mRNA when used at 10 nM concentration and assayed by quantitative RT-PCR when the fluorescent transfection control duplex indicates that >90% of the cells have been transfected and the HPRT positive control works with the expected efficiency.

References

1. Kim, D.H., et al., Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol*, 2005. **23**(2): p. 222-6.
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