TriFECTa® RNAi Kit

Overview—Dicer-substrate siRNAs (DsiRNAs) and the TriFECTa RNAi Kits

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 21mer siRNAs are chemically synthesized RNA duplexes that mimic Dicer products and bypass the need for Dicer processing. In contrast, Dicer-substrate siRNAs (DsiRNAs) are chemically synthesized 27mer RNA duplexes that are processed by Dicer and can show increased potency when compared with 21mer duplexes [1,2].

The TriFECTa kit contains three DsiRNAs that target a select gene. The DsiRNAs are selected from over 322,000 predesigned duplexes that cover the complete human, mouse, or rat transcriptomes in the RefSeq Genbank collection (www.ncbi.nlm.nih.gov/RefSeq). The IDT predesigned DsiRNAs were chosen using a rational design algorithm that integrates traditional 21mer siRNA design rules and updated 27mer design criteria. Additional analysis is performed to be sure that the chosen sites do not target alternatively spliced exons nor include any known single-nucleotide polymorphisms. Sequences are then tested to minimize the potential for cross-hybridization and off-target effects (Smith-Waterman analysis).

In addition to the three targeted duplexes, the TriFECTa kit contains three control sequences that are needed to perform RNAi experiments:

1. A TYE™ 563-labeled transfection control
2. A nontargeting universal negative control RNA duplex that does not interact with sequences in human, mouse, and rat genomes
3. A positive control DsiRNA that targets a site in the *HPRT1* (*hypoxanthine guanine phosphoribosyltransferase*), a gene common among human, mouse, and rat*

*This control is shown to give >90% knockdown of HPRT when transfected with cationic lipid at a 10 nM concentration in cell culture.

TriFECTa RNAi Kit Guarantee

IDT guarantees that at least 2 of the 3 DsiRNAs in the TriFECTa kit will provide ≥70% knockdown of the target mRNA, when used at 10 nM concentration and tested by quantitative RT-PCR. The fluorescent transfection control DsiRNA must also indicate that >90% of the cells have been transfected, and the HPRT positive control must demonstrate 90% knockdown efficiency.

TriFECTa RNAi—Kit contents

To select your predesigned DsiRNA and order a TriFECTa Kit, go to www.idtdna.com/DsiRNA.

All DsiRNA duplexes are provided dry, in separate tubes, and have been QC tested by electrospray ionization mass spectrometry (ESI MS).

TriFECTa RNAi Kit		
Component	**Description**	**Amount**
3 predesigned DsiRNAs	For one target in the human, mouse, or rat transcriptome	2 nmol each
3 control DsiRNAs • TYE™ 563 Transfection Control DsiRNA • HPRT-S1 Positive Control DsiRNA • Negative Control DsiRNA	 • Fluorescently labeled transfection control • Targeting human, mouse, and rat *HPRT1* • Nontargeting in human, mouse, and rat	1 nmol each
Nuclease-Free Duplex Buffer		2 mL

Important: Handle all materials with gloves under RNase-free conditions. Minimize light exposure for dye-labeled DsiRNAs.

Each tube contains enough DsiRNA for the number of transfections shown in Table 1. Note that additional Control DsiRNAs are available (see Related products) which may be useful for certain applications. DsiRNAs can be ordered in larger scale at www.idtdna.com/DsiRNA.

Table 1. Transfections per DsiRNA.

		Control DsiRNA (1 nmol)		Predesigned DsiRNA (2 nmol)	
Plate format	**Media volume (µL)**	**# of transfections at 1 nM**	**# of transfections at 10 nM**	**# of transfections at 1 nM**	**# of transfections at 10 nM**
6-well	2500	400	40	800	80
12-well	1200	820	82	1640	164
24-well	600	1660	166	3320	332
48-well	250	4000	400	8000	800
96-well	150	6660	666	13,320	1332

Resuspension and storage of DsiRNAs

Salts will be present following the annealing and dry-down processes of manufacturing. To maintain suitable salt concentrations for the duplex structure of your product, we recommend using the following resuspension protocol.

1. Briefly centrifuge each tube before opening to make sure that all material remains at the bottom of the tube in case the product was dislodged during shipping.
2. Resuspend duplexed oligos in Nuclease-Free Water (Cat # 11-04-02-01) to make a 100 µM stock solution. For example:

Duplexed oligo (nmol)	Nuclease-Free Water
1	10 µL
2	20 µL
10	100 µL

Notes:

- Use these instructions to resuspend the control DsiRNA sequences (such as Negative Control DsiRNA or HPRT-S1 Positive Control DsiRNA). Follow the instructions above (Resuspension and storage of DsiRNAs) using Nuclease Free Duplex Buffer (30 mM HEPES, pH 7.5, 100 mM potassium acetate) to resuspend. Vortex briefly, followed by heating the solution to 94°C for 2 minutes. Remove the tube from the heat source and allow to cool to room temperature.
- The resulting product will be fully resuspended in a stable, double-stranded form and should be stored at –20°C.

3. Make a 20 µM working solution. For example:

Reagent	Amount*
Buffer containing 100 mM Na+ or K+	40 µL
100 µM DsiRNA (Step 2)	10 µL

* Scale volume as needed for your experiment. See next step for storage conditions.

4. Store resuspended DsiRNAs at –20°C or –80°C.

Notes:

- While generally stable to freeze-thaw cycles, we recommend making aliquots for routine use to minimize the frequency of freeze-thaw events for primary stock solutions.
- Minimize light exposure for dye-labeled DsiRNAs.

Outline of an RNAi experiment

1. **Establish the right transfection method for your cell type and culture media.**

   We recommend using the fluorescent-labeled transfection control DsiRNA and fluorescence microscopy. More than 80% of cells should show dye uptake when examined 4–24 hours after transfection.

2. **Demonstrate that RNAi is working using a positive control.**

   We recommend using the HPRT-S1 Positive Control DsiRNA and quantitative RT-PCR. At 10 nM DsiRNA, *HPRT* should show >90% knockdown 24 hours after transfection.

3. **Test predesigned DsiRNAs with a dose response curve.**

   We recommend testing duplexes at 10 nM, 1 nM, and 0.1 nM concentrations. Knockdown of RNA levels should be analyzed 24–48 hours after 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 RNA.

4. **Perform RNAi studies using DsiRNAs identified as "effective by >70% reduction in RNA levels."**

   We recommend that the results of two DsiRNAs against the same target be compared to controls for potential off-target effects and other artifacts.

   a. mRNA levels can generally be measured 24–48 hours after transfection.

   b. Protein levels can generally be measured 48–72 hours after 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, we recommend that control cultures transfected with control DsiRNAs be used for target level normalization. A nontargeting DsiRNA (Negative Control DsiRNA) that has no known targets in human, mouse, or rat is provided for this purpose. DsiRNAs targeting reporter genes such as EGFP, or firefly luciferase, can also be used as controls; however, these DsiRNAs have not been evaluated 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. Therefore, reagents and protocols must be refined for both the type of nucleic acid transfected and each cell type used. The TYE™ 563 Transfection Control DsiRNAs and HPRT-S1 Positive Control DsiRNAs are included in TriFECTa Kits to assist with this phase of the RNAi project.

Cationic lipids are commonly used to transfect DsiRNAs into tissue culture cells. It may be necessary to test a variety of lipid agents with each cell type used to identify the best performing reagent that introduces DsiRNAs into cells with high efficiency but results in minimal toxicity. After successful transfection of a fluorescent DsiRNA, the cells will show both punctate and diffuse cytoplasmic staining and also remain "healthy appearing" when examined using fluorescence microscopy. A TYE™ 563 labeled transfection control duplex (excitation max 556 nm, emission max 570 nm) is included in the TriFECTa Kit. We recommend use of the fluorescent transfection control oligos at 10 nM concentration; required amounts may vary with the cell types and equipment used. Transfection efficiency should be assessed 4–24 hours after transfection.

Some cell types are resistant to transfection using cationic lipids. Introduction of RNAi duplexes into such cells can often be achieved using electroporation. Liposomal and peptide-based transfection reagents may also be of benefit. Like cationic lipid transfection, electroporation requires refined. Important variables include voltage, pulse length, pulse number, buffer composition, and RNA concentration. One report suggested that off-target effects (alteration in gene expression profiles not 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 lipid reagents include:

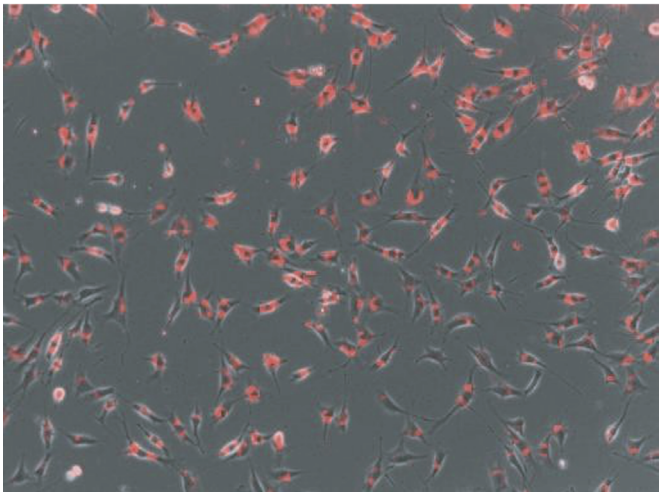
- reagent choice
- cell density
- ratio of reagent to DsiRNA
- amount of DsiRNA
- length of time delivery reagent is left on cells
- presence or absence of serum in the media
- post-transfection incubation period

Reagent selection is important, and the 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 Lipofectamine® RNAiMAX™ reagent (Thermo Fisher Scientific), siLentFect™ (BioRad), Oligofectamine™ (Thermo Fisher Scientific), Lipofectamine 2000 (Thermo Fisher Scientific), X-tremeGENE™ (Roche), HiPerFect™ (Qiagen), and TransIT- TKO™ (Mirus) reagents. Other cell lines may be refractory to some or most of these lipid reagents. There are a wide variety of transfection reagents commercially available with varying properties and surveying multiple 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%) for effective RNAi knockdown. For example, a transfection with 80% efficiency will leave 20% of the target RNA intact, even if the RNAi duplex is 100% effective. Untransfected cells will lead to an underestimation of the potency of an RNAi duplex and may obscure important biological effects. Transfection efficiencies can be visually estimated using fluorescent transfection control duplexes: TYE 563 Transfection Control DsiRNA (Figure 1).

A. NIH3T3 Cells



B. HeLa S3 Cells

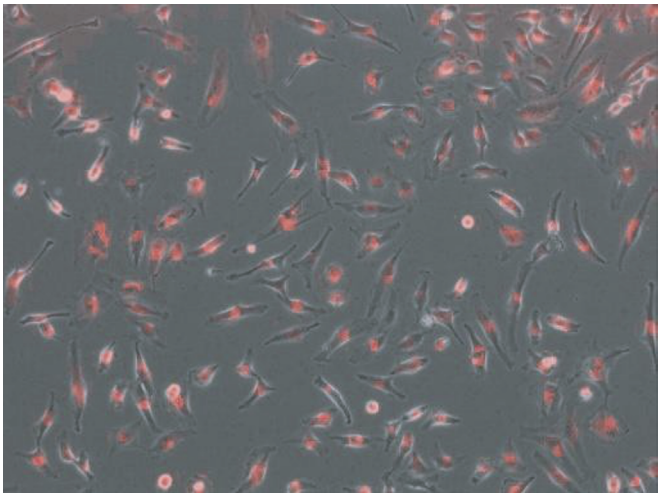


Figure 1. Transfection efficiency visualized at 24 hours after transfection. Cells were transfected at a final concentration of 10 nM TYE 563 Transfection Control DsiRNA using X-tremeGENE transfection reagent (Roche). Cells were imaged 24 hours after transfection.

Each transfection reagent 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 RNAi duplex. As a starting point, IDT recommends testing DsiRNAs at 10 nM, 1 nM, and 0.1 nM concentrations.

Another variable 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 to prevent an increase in cell toxicity. The 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-Glo™ Luminescent Cell Viability Assay (Promega) for monitoring ATP levels. Another option is the CellTiter-Blue™ Cell Viability Assay (Promega), which relies 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, often called reverse transfection, has shown promise for some cells that are difficult to transfect. In reverse transfection experiments, reagents are mixed with the cells and the entire mixture is plated at the same time [4].

The duration of post-transfection, before cells are analyzed for an RNAi effect, varies with the target used. Fluorescence measurements to assess transfection efficiency should be taken at approximately 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 2 weeks. In general, more potent reagents maintain suppression for longer. Repeat transfection may be necessary to study the long-term effects of sustained suppression of a particular gene target.

Positive and negative controls

Fluorescently labeled duplexes permit rapid optimization of transfection protocols. However, the best control for a good transfection is demonstration of successful suppression of a control gene using a known effective RNAi duplex. IDT provides an HPRT-S1 Positive Control DsiRNA for this purpose. The HPRT-S1 DsiRNA has been shown to suppress HPRT mRNA levels >90% when used at 10 nM concentration with cationic lipid in cell culture. IDT also provides a negative control DsiRNA (NC1) that is nontargeting in the human, mouse, or rat transcriptomes.

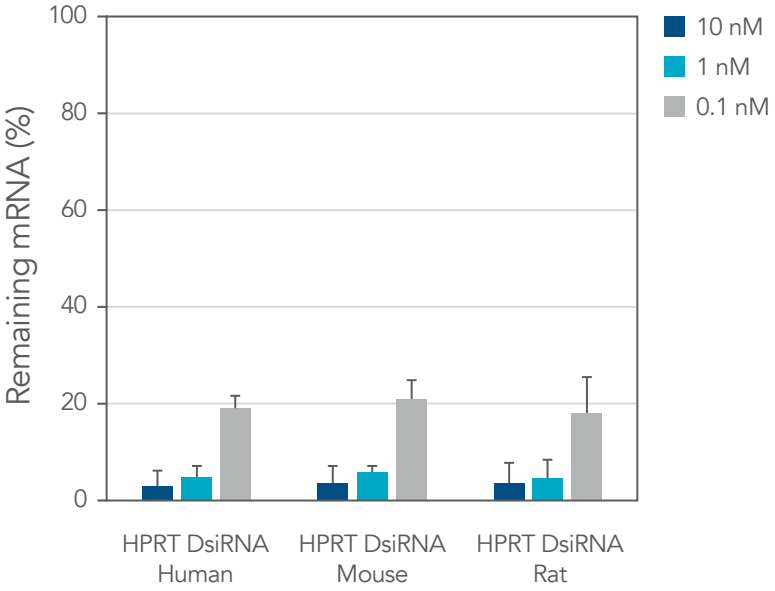


Figure 2. HPRT mRNA knockdown using HPRT-S1 Positive Control DsiRNA. The HPRT-S1 Positive Control DsiRNA was transfected into A549 (human), Neuro 2A (mouse) or RG2 (rat) cell lines in triplicate with Lipofectamine® RNAiMAX. RNA was isolated 24 hours later using the SV96 Total RNA Isolation Kit (Promega) and RNA levels were measured by RT-qPCR on a QuantStudio™ 7 Flex Real-time PCR System (Thermo Fisher Scientific). Relative HPRT expression was normalized to the internal reference gene SFRS9 using two DsiRNA negative controls as baseline (100%). Data shown is a representative transfection from 3 independent experiments. Error bars represent SEM, *n* = 3.

Dicer-substrate control duplexes are also available for EGFP and firefly luciferase. Primers for quantifying HPRT using a SYBR® Green-based qPCR test are also available from IDT (see Related products).

Measuring RNAi knockdown of gene expression

IDT recommends using quantitative assessment of RNA 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, making it 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 RNA levels are reduced.

When analyzing RNAi efficacy, 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 for RNA isolation. For small sample sets, a simple reagent like RNA STAT-60™ (Tel-Test, Inc) works well and can yield consistently high-quality RNA inexpensively. Isolation of RNA from large numbers of samples is better accomplished using one of many commercial kits available such as SV 96 Total RNA Isolation Kit (Promega) or Aurum™ Total RNA Kit (Bio-Rad).

Some kits like the Aurum series are available in different formats (e.g., vacuum filtration or spin-column filtration). 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 2100 Bioanalyzer (Agilent Technologies) or the Experion™ System (BioRad). The advantage of the Bioanalyzer or Experion systems is that both RNA quality and RNA concentration can be determined with a minimal amount of sample.

Northern blots, RNase protection assays (RPAs), or quantitative real-time RT-PCR can be used to measure relative gene expression levels in the RNA sample. We recommend using qRT-PCR because it is efficient and quantitative over a wide range of RNA levels. It is essential to use a reliable internal control for standardization and normalization. We have used acidic ribosomal protein P0 and SFRS9 as internal controls with good results (RPLP0) [2,5-6].

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 facilitate cloning of a target gene sequence into the 3′ UTR of a gene with an easily measurable activity like luciferase (psiCHECK™-2, Promega) enable rapid analysis of numerous samples. The psiCHECK-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.

Related products

To order positive and negative controls, go to www.idtdna.com/DsiRNA.

DsiRNA controls	Amount (nmol)	Catalog #
Fluorescent-labeled transfection efficiency controls		
• TYE™ 563 Transfection Control DsiRNA	1	51-01-20-19
	5	51-01-20-20
• TEX 615 Transfection Control DsiRNA	1	51-01-20-21
	5	51-01-20-22
• Cy® 3 Transfection Control DsiRNA	1	51-01-03-06
	5	51-01-03-08
Endogenous gene positive controls		
• HPRT-S1 Positive Control DsiRNA (human, mouse, and rat)	1	51-01-08-02
	5	51-01-08-03
Exogenous reporter gene positive controls		
• EGFP-S1 Positive Control DsiRNA	1	51-01-05-06
	5	51-01-05-07
• FLuc-S1 Positive Control DsiRNA (Firefly luciferase)	5	51-01-12-10
• RLuc Positive Control DsiRNA (*Renilla* luciferase)	1	51-01-08-21
	5	51-01-03-22
Negative controls*		
• Negative Control DsiRNA (nontargeting)	1	51-01-14-03
	5	51-01-14-04
• Scrambled Negative Control DsiRNA	1	51-01-19-08
	5	51-01-19-09
Companion intercalating dye-based qPCR controls		
• Human HPRT qPCR Assay	1	51-01-08-04
	5	51-01-08-05
• Mouse HPRT qPCR Assay	1	51-01-13-07

* The recommended negative control is the nontargeting Negative Control DsiRNA.

References

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2. Rose SD, Kim DH, Amarzguioui M, *et al*. **Functional polarity is introduced by Dicer processing of short substrate RNAs**. *Nucleic Acids Res*. 2005;33(13):4140-4156. Published 2005 Jul 26.
3. Fedorov Y, King A, Anderson E, *et al*. **Different delivery methods-different expression profiles** [published correction appears in Nat Methods. 2005 Jul;2(7):559]. *Nat Methods*. 2005;2(4):241.
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6. Lennox KA, Behlke MA. **Cellular localization of long non-coding RNAs affects silencing by RNAi more than by antisense oligonucleotides**. *Nucleic Acids Res*. 2016;44(2):863-877.

Revision history

Version	Release date	Description of changes
4	2023	Update template, Figure 2, and editorial changes
3	2018	• Updated instructions for resuspension of DsiRNAs • Updated product names, email addresses, and URLs • Removed discontinued products • Reorganized and simplified procedure • Updated template
2	2011	• Updated names of target species, transfection controls, and negative controls to reflect updated kit contents • Updated TriFECTa guarantee and recommendations for transfection reagents • Deleted instructions for duplexing oligos, since DsiRNAs will now be supplied in duplex form • Updated template
1	2005	Original document

TriFECTa® RNAi Kit

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