

TriFECTa™ Dicer-Substrate RNAi Manual

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Introduction

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 TriFECTa kit contains three Dicer-Substrate 27-mer duplexes targeting a specific gene that are selected from a predesigned set of duplexes from the human, mouse, rat, cow, dog, chicken, and chimp transcriptomes in The RefSeq Genbank collection. The IDT TriFECTa collection of 27-mer sites was 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 TriFECTa kit contains three control sequences which are needed to perform RNAi experiments including a TYE™ 563-labeled transfection control (TYE™ 563 DS Transfection Control RNA duplex), a scrambled universal negative control RNA duplex (NC1, negative control duplex) which is absent in human, mouse, and rat genomes, and a positive control Dicer-Substrate RNA duplex (HPRT-S1 DS Positive Control) which targets a

site in the HPRT (hypoxanthine guanine phosphoribosyltransferase 1) gene that is common between human, mouse, and rat and is prevalidated to give >90% knockdown of HPRT when transfected at 10 nM concentration. IDT guarantees that at least two of the three Dicer-Substrate duplexes in the TriFECTa 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 demonstrates 90% knockdown efficiency.

Receiving a TriFECTa Dicer-Substrate RNAi Kit

Kit Contents:

The TriFECTa Kit includes 6 tubes that contain RNA duplexes (dry) 1 tube that contains nuclease free water. All duplexes have been HPLC purified and QC tested by ESI-MS.

- Three target-specific Dicer-Substrate siRNA duplexes (2 nmoles each)
- TYE™ 563 DS Transfection Control, fluorescent-labeled transfection control duplex (1 nmole)
- HPRT-S1 DS Positive Control duplex (1 nmole)
- NC1, negative control duplex (1 nmole)
- RNase-free Duplex Buffer (100mM KAc/30 mM HEPES pH 7.5) (2 mL)

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) DsiRNA duplexes are already annealed, and water is recommended for their resuspension. Be sure to use only nuclease-free water (HPLC-grade is preferable). DEPC treated water is NOT recommended for use with oligonucleotides, and water coming straight from a Millipore or other type of deionizing system can be acidic with pH's as low as 5.0.

- a. 2 nmoles of each target specific duplex is provided. Addition of 100 µl of nuclease free water will result in 20 µM final concentration; vortex thoroughly and microfuge prior to use.
- b. 1 nmole of each control duplex is provided. Addition of 50 µl of nuclease free water will result in 20 µM final concentration; vortex thoroughly and microfuge prior to use.
- 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 per 1 nmole duplex RNA.

Plate Format	Media Volume (μ L)	# 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, IDT 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 through IDT's *TriFECTa* Catalog page on the website.

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.

The *TriFECTa* Library

Almost 1,200,000 Dicer-Substrate RNAi duplexes have been designed against the approximately 25,000 genes from each of the human, mouse, and rat cow, dog, chicken, and chimp transcriptomes in The RefSeq Genbank collection: <http://www.ncbi.nlm.nih.gov/RefSeq/>.

Site selection was first performed using a novel algorithm that incorporates published 21mer siRNA criteria with new 27mer specific design rules. Sequences that passed this stage were next screened to minimize the potential for cross-hybridization and off-target effects; sites were also eliminated that included known SNPs. Finally, the analysis was extended to include the presence of alternatively spliced exons (if any are present in RefSeq). Two *TriFECTa* sequence collections are therefore available, including 1) sequences that target all forms of a given gene, and 2) sequences that are splice-form specific.

Splice common: targets all known variants of a gene in RefSeq; duplexes lie within common exons. This constitutes the bulk of the *TriFECTa* Dicer-Substrate RNAi collection and is appropriate for most users.

Splice specific: targets only exons present in specific splice forms. In some cases, the unique exon may be very small or comprise sequence unfavorable for selection of RNAi duplexes (knock-down is not guaranteed for these sites). This collection is intended for those researchers who have a need to selectively target specific splice variants.

The *TriFECTa* library is available at:

<http://www.idtdna.com/scitools/Applications/trifecta/default.aspx>

Results for a given gene are displayed showing “splice common” choices first; “splice specific” choices are shown below as an alternative. If only one splice form is identified for that gene in RefSeq, then only one set of duplexes is shown to choose from.

TriFECTa gene-specific duplexes are available in sets of three with stock controls in kit form (small scale) or can be ordered individually (small, medium, or large scale). The “top three candidates” are displayed for easy ordering. To access additional pre-designed duplexes to a given target, contact IDT Technical Support Services at 800-321-2661.

Custom Dicer-Substrate RNAi duplexes can be designed using the free online design tool at: <http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx>

IDT Bioinformatics support personnel can assist with special design needs. Please contact IDT Technical Support Services at 800-321-2661.

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 (HPRT-S1 DS 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. IDT 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”. IDT recommends that the results of two duplexes 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 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, IDT recommends that control cultures transfected using control RNA duplexes be used for target level normalization. A randomized

sequence (NC1- Scrambled Neg) 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 TYE™ 563 DS Transfection Control and HPRT-S1 DS Positive Control duplexes are included 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 TYE™ 563 labeled transfection control duplex (excitation max 556 nm, emission max 570 nm) is included in the TriFecta kit. IDT 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) are 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. IDT offers two types of siRNA delivery reagents, Transductin and Trifectin.

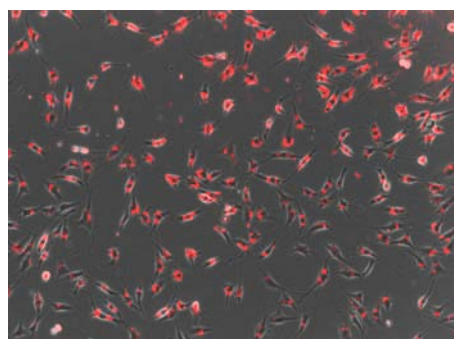
Transductin is a peptide-based transduction delivery reagent specific for delivery of siRNAs. Transductin works through a mechanism distinct from cationic lipids and can be used to deliver dsRNAs to most cell lines, including primary cells and ES cells. It produces fewer off-target effects than cationic lipids and can minimize the risk of triggering an immune response <http://www.idtdna.com/Catalog/transductin/transductin.aspx>

TriFECTin, a proprietary cationic lipid formulation, that has been optimized for deliver of IDT's Dicer-Substrate siRNAs into a wide variety of cell types with minimal toxicity. It is equally potent for delivery of traditional 21-mer siRNAs and other kinds of nucleic acids. For a list of cells lines tested, please read the TriFECTin tech manual. <http://www.idtdna.com/catalog/Trifectin/Page1.aspx>

Using HeLa cells, in addition to Trifectin 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 an siRNA duplex and may obscure important biological effects. Transfection efficiencies can be visually estimated using fluorescent transfection control duplexes TYE™ 563 DS Transfection Control (Figure 1)

NIH3T3 Cells



HeLa S3 Cells

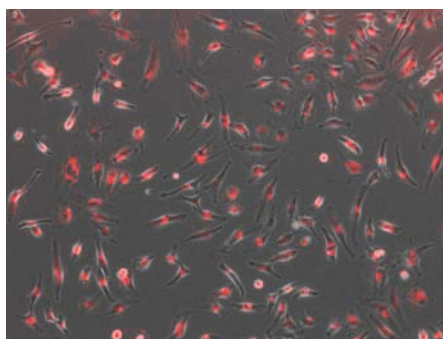


Figure 1. Transfection Efficiency Visualized at 24 hours post-transfection.

Cells were transfected at a final concentration of 10 nM with the TYE™ 563 DS 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. IDT 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-Glo™ Luminescent Cell Viability Assay (Promega) which monitors ATP levels. Another option is the CellTiter-Blue™ Cell Viability Assay (Promega) which 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. IDT provides a validated HPRT-S1 DS Positive control duplex for this purpose. HPRT-S1 is a potent Dicer-Substrate duplex that has been shown to suppress HPRT mRNA levels >90% when used at 10 nM concentration. IDT also provides a validated negative control duplex (NC1) that is not present in the human, mouse, or rat genomes.

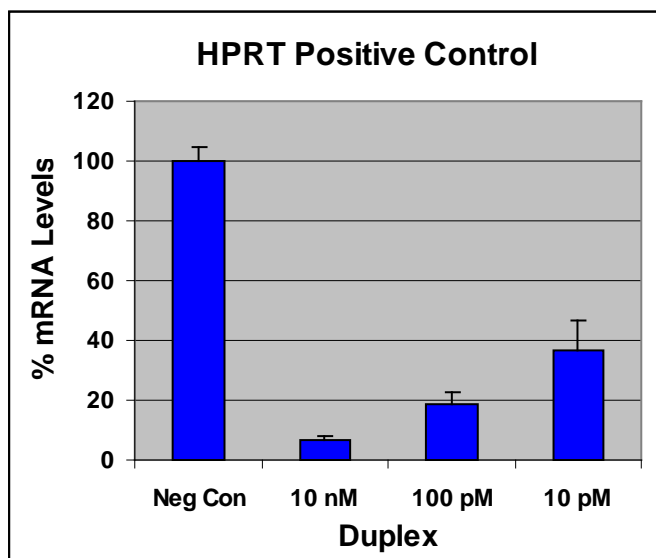


Figure 2 HPRT mRNA Knockdown Using HPRT-1 Positive Control Duplex.

HeLa cells were plated at a density of 2×10^4 cells/well in a 24 well plate. 24 hours later, the cells were transfected using 10 nM, 100 pM or 10 pM of the HPRTS1 DS Positive Control duplex, or 10 nM of the negative control duplex mixed in 1ul of OligofectamineTM. 24 hours post-transfection, RNA was isolated using Promega's SV96 Total RNA Isolation Kit, and HPRT levels were measured using qRT-PCR on an ABI 7000 real-time PCR machine. Relative expression was normalized to RPLP0 internal control mRNA using the negative control as baseline (100%).

Validated Dicer-substrate control duplexes are also available for EGFP and Firefly Luciferase. Validated primers for use in a HPRT SYBRTM Green Q-PCR assay are available from IDT's siRNA readymade primer collection.

Measuring RNAi Knockdown of Gene Expression

IDT 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 → Protein → 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-60TM (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. IDT 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.

TriFECTa Readymade Duplexes (Validated Controls)

Fluorescent Transfection Efficiency Controls

TYETM 563 DS Transfection Control

TEX 613TM DS Transfection Control

Cy3TM DS Transfection Control

Endogenous Gene Positive Control

HPRT-S1 DS Positive Control (human, mouse, and rat)

HPRT-Bt DS Positive Control (Cow)

HPRT-Bt DS Positive Control (Pig)

Exogenous Reporter Gene Positive Controls

EGFP-S1 DS Positive Control

FLuc-S1 DS Positive Control

Negative Control

NC1 negative control

DS Scrambled Neg

SYBR-Green Q-PCR Primer Sets also available.

<http://www.idtdna.com/catalog/Trifecta/Controls.aspx>

References

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4. Amarzguioui M. (2004) Improved siRNA-mediated silencing in refractory adherent cell lines by detachment and transfection in suspension. *Biotechniques*, 36(5): 766–768, 770.
5. Bieche I, Noguès C, et al. (2000) Quantitation of hTERT gene expression in sporadic breast tumors with a real-time reverse transcription-polymerase chain reaction assay. *Clin Cancer Res*, 6(2): 452–459.

THESE PRODUCTS ARE NOT FOR USE IN HUMANS OR NON-HUMAN ANIMALS AND MAY NOT BE USED FOR HUMAN OR VETERINARY DIAGNOSTIC, PROPHYLACTIC OR THERAPEUTIC PURPOSES. Dicer-Substrate RNAi methods were developed in a collaborative project between IDT and Drs. John Rossi and Dongho Kim at the Beckman Research Institute, City of Hope National Medical Center [1, 2], Patent Pending.