Optimizing knockdown of gene expression using the TriFECTa™ Dicer-substrate RNAi reagent system

Integrated DNA Technologies, Inc. (IDT) has developed a library of predesigned Dicer-substrate RNA duplexes. These potent RNA interference (RNAi) reagents are available in a kit (TriFECTa) that includes three specific RNA duplexes for target-gene knockdown plus three optimized control duplexes, which can be used to optimize transfection efficiency and other aspects of RNAi experiments.

In mammalian cells, small interfering RNAs (siRNAs) are produced by the enzymatic processing of long dsRNAs by the RNase III class endoribonuclease Dicer. Newly formed siRNAs associate with Dicer, TRBP and Ago2 to form the RNA-induced silencing complex (RISC). Once in RISC, one strand of the siRNA (the 'passenger' strand) is degraded or discarded, whereas the other strand (the 'guide' strand) is retained and directs the sequence specificity of gene silencing. This natural pathway can be exploited as an experimental tool to selectively reduce expression of targeted genes.

As research reagents, siRNAs have traditionally been chemically synthesized as 21-mer RNA duplexes that mimic natural Dicer products and bypass Dicer processing. Alternative approaches to trigger RNAi exist that may have certain advantages. Dicer-substrate RNAs are chemically synthesized 27-mer RNA duplexes that are optimized for Dicer processing. These duplexes are cleaved by Dicer into well-defined 21-mers, which are the final active species. Dicer-substrate RNAi methods take advantage of the link between Dicer and RISC loading that occurs when RNAs are processed by Dicer, and can boost potency by tenfold or more compared with 21-mer siRNAs at the same site¹. Notably, the direction of Dicer processing can favor loading of the antisense strand into RISC, which can be exploited when designing these reagents². Correct design of the 27-mers is therefore crucial to realize the full benefits of this approach.

The TriFECTa kit

Dicer-substrate RNAi methods were developed as a collaborative project between IDT and John Rossi and Dongho Kim at the Beckman Research Institute, City of Hope National Medical Center^{1,2}. IDT has commercialized Dicer-substrate methods and reagents for research applications, and offers synthesis of custom Dicer-substrate RNA

duplexes, pre-made libraries of Dicer-substrate RNA duplexes (including a new 550-target, 2,200-duplex human kinome library) and predesigned sets of Dicer-substrate RNA duplexes for individual genes in kit form.

Figure 1 | Transfection controls and dose optimization. **(a)** NIH3T3 cells were transfected with the TriFECTa Cy3 transfection control duplex. Cells were washed and examined at 24 h after transfection. Fluorescence and phase-contrast images are overlaid. Scale bar, 100 μ m. **(b)** HeLa cells were transfected using TriFECTa duplexes specific for *HPRT1*, *SSB*, *STAT1* and *HNRPH1* at the concentrations indicated. Relative mRNA levels were measured using qRT-PCR at 24 h post-transfection; data were normalized against an internal *RPLP0* control using the 'DS scrambled neg' duplex as baseline (100%). Con, control.

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APPLICATION NOTES

Figure 2 | HPRT mRNA and protein knockdown. HeLa cells were transfected with HPRT-S1 duplex at 10 nM and collected at the indicated time points. **(a)** HPRT mRNA amounts were measured by qRT-PCR. **(b)** HPRT protein levels were assessed by western blot; β-actin loading standard is shown. Each lane represents a separate transfection. **(c)** HPRT protein levels were averaged and relative knockdown at the indicated times after transfection was quantified.

IDT's TriFECTa kit contains three Dicer-substrate 27-mer RNA duplexes specific for a single target gene. Duplexes are provided in individual tubes and can be used singly or pooled, if desired. Sequences are selected from a predesigned library of optimized 27-mers based upon the latest release of the RefSeq database in GenBank (<http://www.ncbi.nlm.nih.gov/RefSeq/>). The TriFECTa sequence library currently includes seven of the genomes in the RefSeq collection, including human, mouse and rat. TriFECTa duplexes are selected using a rational design algorithm that integrates both traditional 21-mer siRNA design rules as well as new

27-mer-specific criteria. Additionally, analysis is performed to ensure that the chosen sites do not target alternatively spliced exons, do not include known single-nucleotide polymorphisms and share minimal homology to other genes in that species' transcriptome (Smith-Waterman analysis). The TriFECTa library can be accessed online (<http://www.idtdna.com/Scitools/Applications/Predesign/default.aspx>).

In addition to the target-specific duplexes, each TriFECTa kit contains three controls including a fluorescent dye-labeled duplex (the Cy3TM DS transfection control), a 'universal' negative control duplex ('DS scrambled neg', which targets a site that is absent from human, mouse and rat genomes) and a positive control duplex ('HPRT-S1 DS positive control'), which targets a site in the hypoxanthine phosphoribosyltransferase 1 gene that is common between human, mouse and rat). These control reagents can be used to optimize the RNAi experimental system before undertaking studies on new targets. It is good practice to optimize transfection conditions for each different cell line studied as well as for each different form of nucleic acid used (for example, large DNA plasmids often require different transfection conditions than short dsRNA oligonucleotides). Dicer-substrate RNA duplexes can be used with all commonly used transfection methods, such as cationic lipids, liposomes and electroporation.

Optimizing RNAi experiments

The following steps offer a general strategy to follow when starting any functional genomics-type project:

- 1) Optimize transfection methods
 - a) Dye-labeled control
 - b) Positive-control knockdown
- 2) Test target-specific duplexes
 - a) Validate that duplexes actually work in your cell line
 - b) Titrate dose response
- 3) Knockdown studies of targeted gene
 - a) Assess changes in mRNA levels
 - b) Assess changes in protein levels
 - c) Assess changes in phenotype

A successful RNAi experiment starts with good transfection. It may be necessary to empirically test several different cationic lipids (or test other approaches) to establish a protocol that performs optimally with each cell line used. Use of a dye-labeled transfection control oligo allows for rapid, easy screening of many reagents in parallel. Ideally the transfection method should result in >90% of the cells transfected with minimal cell death. A transfection with 70% efficiency still leaves 30% of the target RNA intact even if the RNAi duplex is 100% effective. Nontransfected cells will lead to underestimation of the potency of a siRNA duplex and may obscure important biological effects. When optimizing transfection methods, IDT recommends using dye-labeled oligos at 10 nM or less; higher concentrations can increase the amount of nonspecific binding, which can cause high background and falsely elevate the apparent success of transfection. Culture medium should be changed and the

cells should be washed before visualizing fluorescence. The length of incubation time after transfection before cells are examined varies with the target and the assays that are used. Direct visualization of fluorescence is the fastest method to assess transfection efficiency and is usually done less than a day after transfection (6–24 hours is optimal). An example of successful transfection using the TriFECTa Cy3 DS transfection control duplex is shown in **Figure 1a**.

Unfortunately, use of dye-labeled control oligos is not always sufficient to optimize transfection protocols. It is possible to get seemingly good dye-oligo uptake without delivery of the oligos into the correct cytoplasmic location for effective RNAi. Transfection conditions that succeed in the dye-labeled study should next be examined for functional knockdown using a positive control siRNA. The HPRT-S1 DS positive control duplex can be used for this purpose. IDT recommends measuring RNA concentrations for method optimization and duplex validation. Once optimal transfection methods have been established, target-specific duplexes should be tested for relative potency and to establish a dose-response curve. It is usually adequate to test 10 nM, 1 nM and 0.1 nM concentrations. Off-target effects are dose dependent, and the risk of experiencing adverse events can be minimized by using the lowest concentration of RNA duplex that achieves the desired level of knockdown of the target mRNA. To illustrate this point, dose response curves are shown in **Figure 1b** for four TriFECTa duplexes specific for human *HPRT1*, *SSB*, *STAT1* and *HNRPH1* targets. In this particular experimental system, the *HPRT1*, *STAT1* and *HNRPH1* duplexes could be used in the 1–5 nM range while the *SSB* duplex should be used at 10 nM concentration.

RNA assays (quantitative reverse-transcriptase PCR (qRT-PCR), northern blots, RNase protection assays, among others) are commonly performed 24–48 h after transfection. But a decrease in mRNA amounts can often be detected in only a few hours after transfection. For example, the HPRT-S1 DS positive control duplex was transfected into HeLa cells at 10 nM concentration using a cationic lipid, and *HPRT* mRNA levels were measured at different time points using qRT-PCR (**Fig. 2a**). A reduction in mRNA levels was seen as early as 3 h after transfection, and maximum knockdown was reached by 12 h. Protein assays (or assessment of phenotypes) are usually performed

48–72 h after transfection. Measurements of protein concentrations, enzyme activity or phenotype can vary widely depending on protein half-life, rate of cell division (dilution) and other factors, such that it is possible to observe a seemingly negative result even when mRNA concentrations have been substantially suppressed. Protein concentrations and phenotypic effects are best assessed after first establishing that mRNA levels are adequately reduced. The HPRT-S1 duplex was transfected (as before), and protein extracts were examined for HPRT by western blot at 24, 48 and 72 h after transfection (**Fig. 2b**). The average levels of protein knockdown for this series of experiments are shown in **Figure 2c**. *HPRT* mRNA levels were reduced by >90% by 12 h after transfection, whereas protein levels did not reach this level of knockdown until the 72-h time point. In all of the experiments, we used the 'scrambled neg' duplex as negative control and levels of the unknown were normalized against an internal control standard.

RNA duplexes can trigger interferon responses in some cell types and *in vivo*. Stimulation of the innate immune system can occur through a variety of receptor molecules, include members of the TLR family, PKR, OAS and RIG-I^{3,4}. Even minimal modification of the RNA duplex with 2'-O-methyl RNA bases can avoid immune triggering⁵, so it may be wise to use modified duplexes in any *in vivo* studies. Methods for use of Dicer-substrate RNA duplexes *in vivo* are under development⁶, and modified TriFECTa duplexes are available to support these needs.

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