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# Dicer-Substrate siRNA Technology

## Advances in siRNA Designs Improve Gene-Specific Silencing

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RNA interference (RNAi) is an intrinsic cellular mechanism, conserved in most eukaryotes, that helps to regulate the expression of genes critical to cell fate determination, differentiation, survival, and defense from viral infection. Researchers have exploited this natural mechanism by designing synthetic double-stranded RNAs (dsRNAs) for sequence-specific gene silencing to elucidate gene function (Hannon 2002, Hutvagner and Zamore 2002, Sharp 1999). Such research has helped forge a rapid transition from discovery and research to potential therapeutic application (Xia et al. 2004).

Since it was first demonstrated that 19–23 nt small interfering RNAs (siRNAs) are mediators of gene-specific silencing (Elbashir et al. 2001a), design of siRNAs has sought to improve specificity and potency, which can reduce off-target effects (Birmingham et al. 2006, Jagla et al. 2005, Naito et al. 2004). While traditional synthetic siRNAs based on the 21- to 23-mer designs have been effective, increased understanding of the discrete events and enzymes involved in the RNAi pathway have recently led to significant improvements in the design of siRNAs, making them even more efficient tools for the induction, control, and interpretation of gene silencing events in everyday research (Khvorova et al. 2003, Schwarz et al. 2003). This article describes studies by researchers at City of Hope and Integrated DNA Technologies (IDT) that led to the development of one such tool, Dicer-substrate siRNA, a highly potent mediator of RNAi.

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### RNAi Overview

The RNAi pathway, part of a larger network that uses small RNA molecules as regulators of cellular signaling, relies on dsRNA as a trigger for sequence-specific gene silencing (Figure 1). In this pathway, longer dsRNAs associate with Dicer endonuclease, a member of the RNase III family, which precisely cleaves the dsRNA into smaller functional siRNAs (MacRae et al. 2006). These siRNAs then associate with an RNA-induced silencing complex (RISC), which targets any homologous mRNA for degradation. It has recently been suggested that in addition to cleaving longer dsRNAs, Dicer endonuclease plays roles in loading processed dsRNA into RISC and in RISC assembly (Lee et al. 2004, Rose et al. 2005, Sontheimer 2005). This hypothesis has helped drive the development of a new class of siRNAs, termed Dicer-substrate siRNAs, that are highly potent mediators of gene-specific silencing.

**25- to 30-mers are up to 100-fold more potent than 21-mer siRNAs targeting the same sequence.**

### Dicer-Substrate siRNA

While long (>30 nt) dsRNAs have been used successfully to regulate gene expression in a number of eukaryotic organisms, including fungi, plants, and *C. elegans* (Napoli et al. 1990, Romano and Macino 1992, Fire et al. 1998), they often activate intrinsic cellular immune responses that result in broad, nonspecific silencing when applied to mammalian systems (Minks et al. 1979, Stark et al. 1998). To prevent activation of these immune responses during RNAi experiments, researchers have generally used shorter (19–23 nt) dsRNAs (Elbashir et al. 2001b).

More recent studies have demonstrated that dsRNAs 25–30 nt in length are even more powerful effectors of gene-specific silencing than 21-mers. Specifically, 25- to 30-mers are up to 100-fold more potent than 21-mer siRNAs targeting the same sequence (Kim et al. 2005). This greater potency appears to depend on processing of the longer dsRNAs by Dicer, which cleaves the longer dsRNAs to produce 21-mers. When 27-mer siRNAs were selectively labeled with 6-carboxyfluorescein (6-FAM) to reduce cleavage by Dicer, a corresponding decrease in potency of siRNA was observed (Kim et al. 2005).

Dicer cleavage of 27-mers into specific 21-mers does not, by itself, explain the greater potency of the 27-mers. Different 21-mer siRNAs (with 2-base 3' overhangs) were synthesized to correspond to all possible Dicer products that could be derived from a blunt 27-mer duplex. None of these 21-mers, acting individually or pooled, produced the same level of silencing observed with 27-mers at low concentrations of siRNA (Kim et al. 2005). Since specific cleavage by Dicer is not sufficient to explain the increased potency, it has been hypothesized that providing Dicer with a substrate for cleavage (i.e., a 27-mer) improves the efficiency of the secondary role of Dicer — that of introducing siRNAs into RISC — and that this is responsible for the enhanced silencing by 27-mers (Rose et al. 2005).

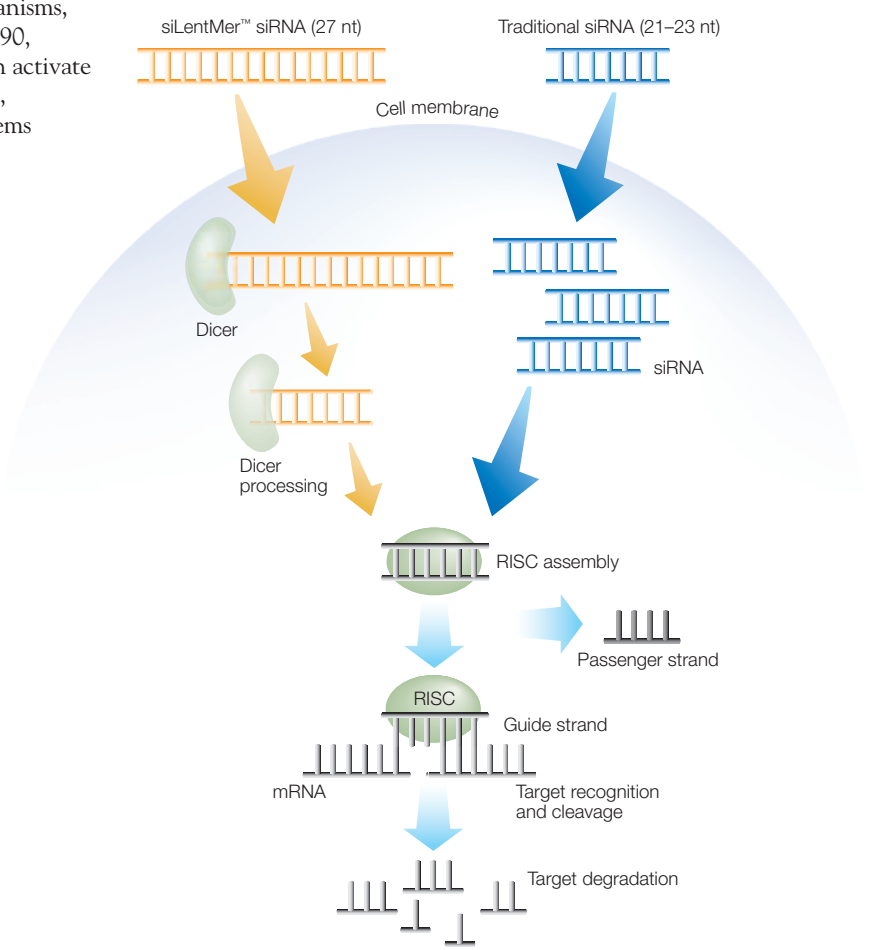


**Fig. 1. Activation of the RNAi pathway by dsRNAs.** Long dsRNAs are cleaved by Dicer endonuclease to form 21–23 nt duplexes. After cleavage, siRNA duplexes are incorporated into RISC. Unwinding of the siRNA duplex results in retention of the guide strand. The guide strand then pairs with complementary mRNA sequences, which are cleaved by RISC and degraded. This allows silencing of a specific gene. Dicer, in addition to cleaving dsRNAs longer than 21 nt, may facilitate the loading of siRNAs into RISC. This may explain why synthetic 21-mer dsRNAs, which are not cleaved by Dicer, are less effective than 27-mers containing the same sequence. Because Dicer may influence loading, and because siRNA structure influences the orientation of Dicer binding, Dicer-substrate siRNAs can be designed to promote specific cleavage by Dicer and preferential retention of the guide strand complementary to the target mRNA.

### Asymmetric Design of 27-mers Confers Functional Polarity

Subsequent work on Dicer-substrate siRNAs has sought to improve their design to further increase the efficacy of silencing (Rose et al. 2005). General rules of siRNA duplex design, such as length, sequence preference, and target accessibility, play a role in the relative potency of any given siRNA (Reynolds et al. 2004, Brown et al. 2005, Overhoff et al. 2005). In addition to these rules, Dicer-substrate siRNAs can be designed to promote Dicer cleavage at a specific position to produce the most potent 21-mer product. Additional subtle design features can influence the dynamics of strand incorporation into RISC, promoting selective retention of the guide strand (the antisense strand, which is complementary to the target message) and leading to a significant impact on the performance of siRNAs in vitro.

**Dicer-substrate siRNAs are potent at concentrations as low as 100 pM, which minimizes the potential for off-target effects.**

By using electrospray ionization mass spectrometry (ESI-MS) to analyze the Dicer products derived from a variety of 27-mers, researchers at IDT and City of Hope were able to identify structural features that encourage the production of a single, predictable, maximally active product (Rose et al. 2005). Specifically, an asymmetric design that includes a 2-base 3' overhang on one strand and the addition of 2 DNA residues to the 3' end of the other strand (Figure 2) severely limits heterogeneity of the cleaved siRNA product — the blunt end is unfavorable for Dicer binding, and cleavage preferentially occurs 21–22 bases from the overhang. Maximum potency is obtained when the 2-base overhang is present on the antisense strand while the DNA bases are added to the sense strand. In this case, Dicer binds to the 5' end of the antisense strand, leading to preferential retention of this strand in RISC. These design features, which are incorporated into Bio-Rad's siLentMer Dicer-substrate siRNAs (see sidebar, next page), ensure maximum potency in RNAi.

### Benefits of Dicer-Substrate siRNAs

Effective use of RNAi for research and therapeutics requires that nonspecific effects be minimized. Nonspecific effects may be related to sequence homology of an untargeted mRNA or activation of cellular responses — particularly induction of the interferon response, which can result in global translational arrest. In addition, some data suggest that the RNAi machinery can be saturated, inhibiting the proper processing of precursors of microRNA (miRNA; Bitko et al. 2005), leading to toxicity. Nonspecific effects and toxicity can be limited by using low concentrations of siRNA. For example, while full activation of the interferon pathway can be avoided by the modest use of siRNAs <30 nt in length (Elbashir et al. 2001a), proinflammatory responses can be activated by higher concentrations of siRNA (Persengiev et al. 2004). Dicer-substrate siRNAs, when used with an effective transfection reagent and protocol, are potent at concentrations as low as 100 pM (see figure in sidebar), which minimizes the potential for off-target effects. To further reduce the potential for activating immune responses, Dicer-substrate siRNAs can be specifically designed to prevent the activation of proinflammatory cytokines (IFN-α and IFN-β) and the protein kinase R (PKR) pathway (Kim et al. 2004). To demonstrate this, cells were transfected with a 27-mer dsRNA, a 21-mer siRNA, or a triphosphate-containing single-stranded RNA (ssRNA). The latter was used as a positive control, because it is highly effective in activating IFN-α and IFN-β when introduced into cells. Compared to cells transfected with ssRNA, assays of cell lysates from cells transfected with 27-mer dsRNA or 21-mer siRNA showed no detectable levels of IFN-α and IFN-β and no evidence of PKR activation. While the risk of other off-target effects remains, this risk can be tempered by using reagents that permit the use of low nanomolar concentrations of siRNA (Persengiev et al. 2004).

An additional benefit of Dicer-substrate siRNAs is longevity of silencing. When NIH 3T3 cells stably expressing enhanced Green Fluorescent Protein (eGFP) were transfected with 21-mers or 27-mers targeting the same site, eGFP suppression by the 21-mer lasted for about 4 days, while suppression by the 27-mer lasted up to 10 days (Kim et al. 2005). While these results match observations made in other studies (Persengiev et al. 2004), some 21-mer siRNAs, described as hyperfunctional siRNAs, can produce comparable long-term silencing (Reynolds et al. 2004). Still, controlling the processing of 27-mers by Dicer to ensure the production of a single specific siRNA can allow lasting and consistently potent silencing at low concentrations and reduce the chance of off-target silencing events.


**Fig. 2. 27-mer Dicer-substrate siRNA.** The functional siRNA is designed with a 25-base sense strand and a 27-base antisense strand. A 2-base DNA pair is added at the 3' end to create a blunt end, directing Dicer-mediated processing to yield a predicted, functional siRNA.

## Summary

Synthetic 27-mer Dicer-substrate dsRNAs can be designed to be processed by Dicer in a predictable way, to ensure appropriately oriented loading into RISC and thus maximum efficiency in RNAi. Use of an efficient transfection method allows these siRNAs to be used at low concentrations, minimizing the potential for off-target effects.


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## Bio-Rad Partners With Integrated DNA Technologies (IDT) to Provide siLentMer Products

Bio-Rad Laboratories offers a selection of 27-mer Dicer-substrate siRNAs, including siRNAs validated to achieve ≥85% knockdown of mRNA in specific human genes. Bio-Rad has combined its effective transfection and analysis capabilities with IDT's expertise in high-quality oligonucleotide manufacturing and quality control to produce these siLentMer 27-mer Dicer-substrate siRNAs. The siRNAs are designed using an advanced algorithm that incorporates the design features discussed in the accompanying article. Final siRNA designs undergo a comprehensive bioinformatic analysis to avoid homology to other sequences and to ensure specific targeting. Any siRNAs targeting alternatively spliced exons or known single nucleotide polymorphisms (SNPs) are eliminated. siLentMer siRNAs are effective at low concentrations (as low as 100 pM), and this helps minimize the potential for off-target silencing that is typically associated with siRNAs that are effective only at higher concentrations.

## siLentMer Dicer-Substrate siRNA Duplexes

Validated and predesigned siLentMer siRNAs offer convenience by saving the time and expense of developing and screening your own effective siRNA libraries. Multiple siRNA duplexes are available for a variety of targets. This allows you to confirm knockdown results in a specific gene of interest by duplicating the biological effects using additional siRNAs. Both validated and predesigned siLentMer Dicer-substrate siRNA duplexes are purified by HPLC and identified by ESI-MS.

### Validated siRNAs

- Functionally tested by RT-qPCR to guarantee a reduction in mRNA levels by ≥85%
- Validated at siRNA concentrations as low as 5 nM to reduce the chance of off-target effects and toxicity
- Appropriate for targeting a gene of interest or as an experimental control
- Available with up to 2 different siRNA duplexes per target to better confirm that any biological effects observed in experiments are specifically due to loss of the targeted gene

In addition to individual duplexes, selections of validated siRNA duplexes are combined with controls and siLentFect™ lipid reagent for RNAi to create transfection kits. These easy-to-use kits are ideal for optimization.

### Predesigned siRNAs

- Ready-to-order duplexes for various gene targets
- Useful when a validated siRNA is not yet available
- Generally more efficient at lower concentrations (≥5 nM) than 21-mer siRNAs
- Available with up to 4 different siRNA duplexes per target to investigate effectiveness of target gene knockdown

## siLentMer siRNA Transfection Kits

When performing RNAi experiments, it is important to establish effective silencing conditions and a set of positive and negative controls for your cell line. To simplify optimization of transfection conditions, selection of appropriate controls, and assessment of the efficiency of siRNA delivery, siLentMer transfection kits combine siLentFect lipid reagent with both validated and fluorescently labeled siLentMer siRNA duplexes.

### Delivery Optimization Kit

- Contains all the components for establishing optimal delivery conditions for most cell lines
- Includes a fluorescently labeled nonsilencing siRNA and siLentFect lipid reagent
- Contains sufficient reagents for approximately 150 transfections in 24-well plates

### Starter Kits

- Help optimize delivery conditions and establish reliable positive and negative controls for cell lines
- Include a validated Dicer-substrate siRNA, nonsilencing negative control siRNA, and siLentFect lipid reagent
- Contain sufficient reagents for approximately 150 transfections in 24-well plates

### Total Control Kits

- Contain all the appropriate positive and negative controls to optimize delivery and fully evaluate target silencing
- Include a validated Dicer-substrate siRNA, nonsilencing negative control siRNA, fluorescently labeled nonsilencing siRNA, and siLentFect lipid reagent
- Are available with GFP or luciferase validated siRNAs for cotransfection experiments involving plasmid-based reporter genes
- Contain sufficient reagents for approximately 300 transfections in 24-well plates

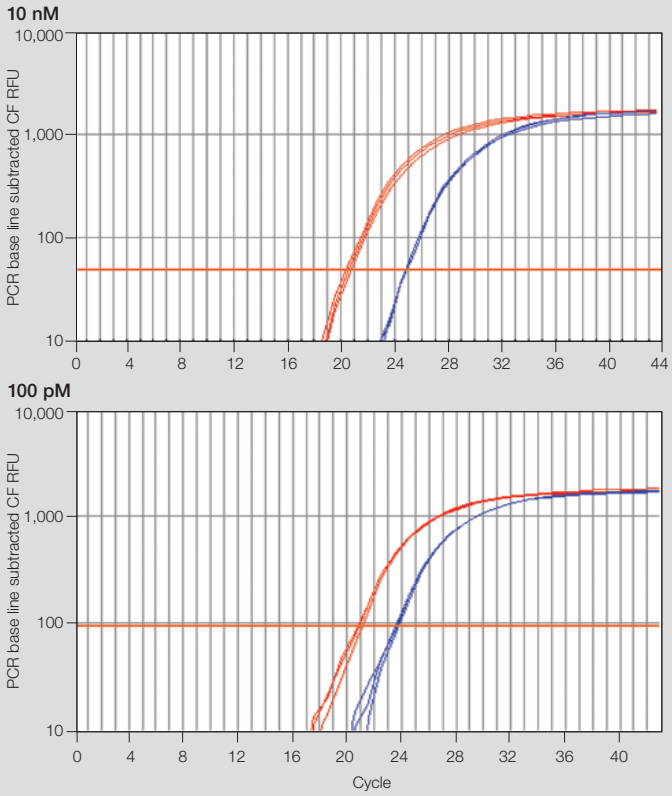
### siLentFect — An Effective Lipid Transfection Reagent

Lipid-mediated transfection is the most popular method for siRNA delivery because it is the most affordable, simple, and consistent delivery method for performing RNAi. Furthermore, it can be broadly applied to a variety of cell lines with effective silencing results.

Bio-Rad's siLentFect lipid transfection reagent was specifically developed to deliver siRNA into cells. siLentFect reagent's high molar efficiency requires only low concentrations of siRNA and small lipid volumes to achieve silencing of up to 90%.

## Custom siRNAs

Bio-Rad's siRNA partner, IDT, specializes in manufacturing custom DNA and RNA oligonucleotides for research applications. IDT has the expertise to deliver custom-synthesized RNA with the yield and purity that researchers demand. Go to **www.idtdna.com** for custom siRNA synthesis inquiries.




**Effective silencing can be achieved with very low siRNA concentrations using siLentMer Dicer-substrate siRNAs and siLentFect lipid reagent.** HeLa cells were grown in 24-well plates to ~70% confluence and transfected with 10 nM or 100 pM of siLentMer Dicer-substrate siRNAs targeting HPRT, or with an anti-EGFP control. 24 hr posttransfection, RNA was purified and RT-qPCR was performed on the iCycler iQ® system. At both concentrations, HPRT (—) was silenced >85% relative to the control (—).

## Research Resources

Bio-Rad offers a variety of resources to assist you with your research. Our support groups (technical support teams, customer service, field application specialists, etc.) are knowledgeable, responsive, and available to help provide necessary information.

The new Gene Expression Gateway (GXG) web site (**www.bio-rad.com/genomics/**) is a valuable application-focused resource for genomic research and Bio-Rad products. The site provides information for the four main application areas consistent with the gene expression workflow — sample preparation, quantification, profiling, and modulation. Another key element of the GXG site is the Citations Library, a searchable database of over 10,000 published research articles citing Bio-Rad products for genomics.

Specific information on RNAi applications is available on the Bio-Rad RNAi web site (**www.bio-rad.com/RNAi/**). From design to detection, Bio-Rad offers an extensive set of tools for effective gene silencing and analysis. Potent Dicer-substrate siRNAs, three delivery technologies, and four detection platforms are supported by high-quality sample preparation kits and quality analysis tools for both RNA and protein methodologies.

**www.bio-rad.com/RNAi/**

## Ordering Information

| Catalog # | Description |
|---|---|
| **Design** | |
| **siLentMer Dicer-Substrate siRNA Duplexes** | |
| Varies | siLentMer Validated Dicer-Substrate siRNA Duplexes, 2 nmol, designed with proven criteria and functionally tested for ≥85% silencing |
| Varies | siLentMer Predesigned Dicer-Substrate siRNA Duplexes, 2 nmol, designed with proven criteria |
| **siLentMer Delivery Optimization Kit** | |
| 174-9950 | siLentMer Delivery Optimization Kit, includes 1.0 nmol fluorescently labeled siLentMer nonsilencing siRNA, 0.2 ml siLentFect lipid reagent, 1.0 ml siLentMer siRNA resuspension buffer |
| **siLentMer Starter Kits** | |
| 174-9960 | siLentMer Starter Kit for Human GAPDH, includes 0.5 nmol siLentMer validated human GAPDH siRNA (positive control), 0.5 nmol siLentMer nonsilencing siRNA (negative control), 0.2 ml siLentFect lipid reagent, 1.0 ml siLentMer siRNA resuspension buffer |
| 174-9961 | siLentMer Starter Kit for GFP, includes 0.5 nmol siLentMer validated GFP siRNA (positive control), 0.5 nmol siLentMer nonsilencing siRNA (negative control), 0.2 ml siLentFect lipid reagent, 1.0 ml siLentMer siRNA resuspension buffer |
| **siLentMer Total Control Kits*** | |
| 174-9970 | siLentMer Total Control Kit for Human GAPDH |
| 174-9971 | siLentMer Total Control Kit for Human HPRT |
| 174-9972 | siLentMer Total Control Kit for Human Lamin A/C |
| 174-9973 | siLentMer Total Control Kit for Human Cyclophilin |
| 174-9974 | siLentMer Total Control Kit for Human β-Actin |
| 174-9975 | siLentMer Total Control Kit for Human β-Tubulin |
| 174-9976 | siLentMer Total Control Kit for GFP |
| 174-9977 | siLentMer Total Control Kit for Luciferase |

Go to **www.bio-rad.com/RNAi/** for a complete list of catalog numbers for validated and predesigned siRNAs and control kits.

| | |
|---|---|
| **siLentFect Lipid Reagent for RNAi** | |
| 170-3360 | siLentFect Lipid Reagent for RNAi, 0.5 ml |
| 170-3361 | siLentFect Lipid Reagent for RNAi, 1.0 ml |
| 170-3362 | siLentFect Lipid Reagent for RNAi, 5 x 1.0 ml |

* All total control kits include 1.0 nmol siLentMer validated siRNA (positive control), 1.0 nmol fluorescently labeled siLentMer nonsilencing siRNA (control for delivery), 1.0 nmol siLentMer nonsilencing siRNA (negative control for silencing), 0.5 ml siLentFect lipid reagent, 1.0 ml siLentMer siRNA resuspension buffer.

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*Validated, highly potent 27-mer siLentMer™ siRNAs employ Dicer-substrate technology for proven performance.*

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- Are effective at concentrations as low as 5 nM
- Minimize off-target effects associated with siRNAs requiring higher working concentrations

Bio-Rad's quality-controlled HPLC purification of each duplex produces homogeneous samples to ensure specific mRNA targeting and gene silencing.

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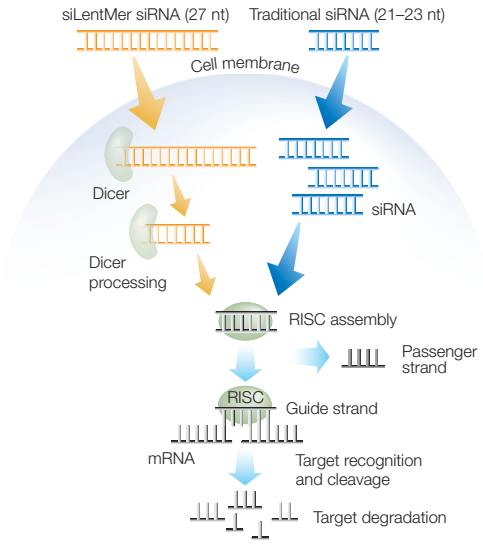


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