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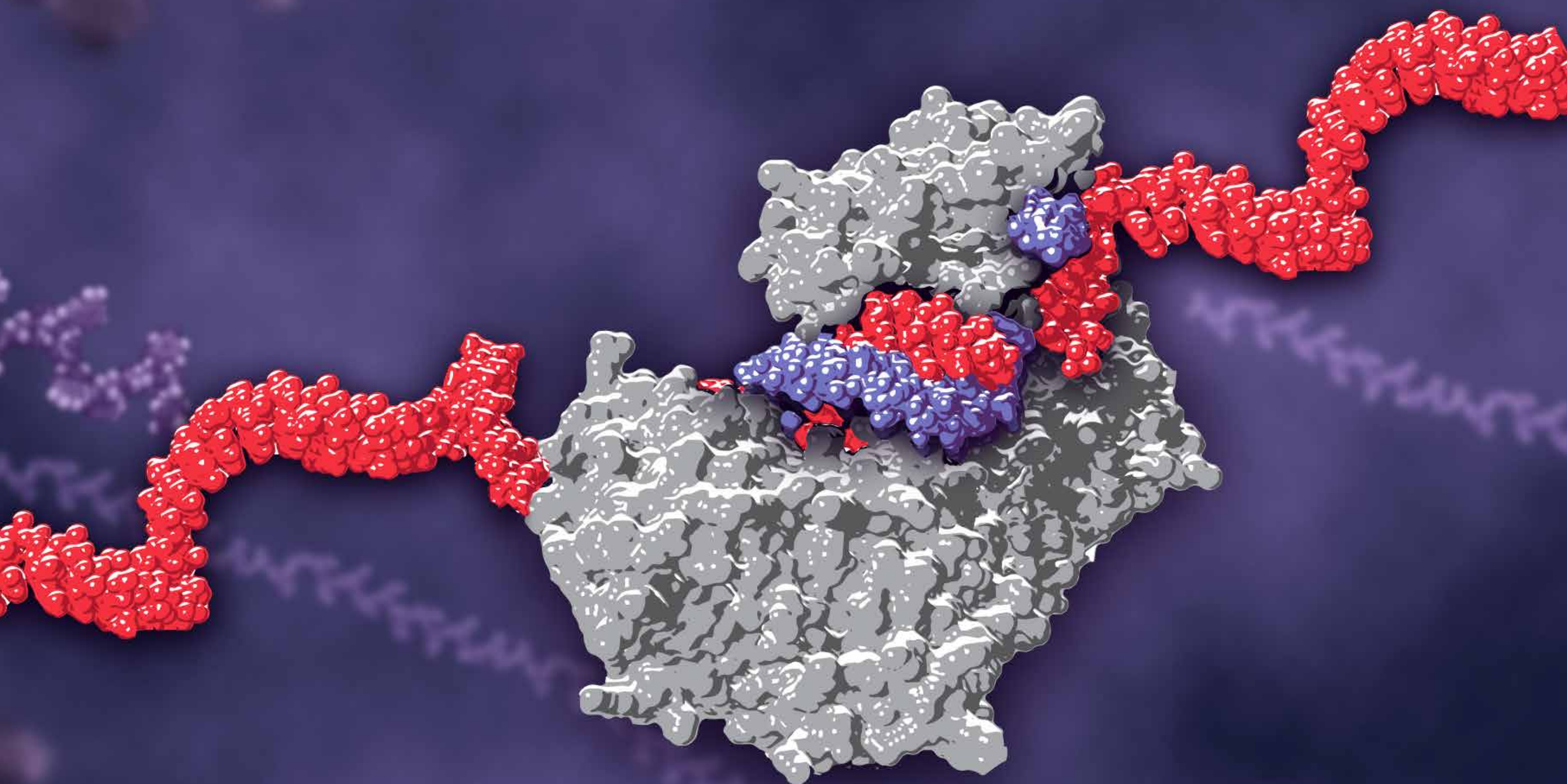


RNAi handbook

A guide to products and applications for efficient gene silencing experiments

ThermoFisher SCIENTIFIC

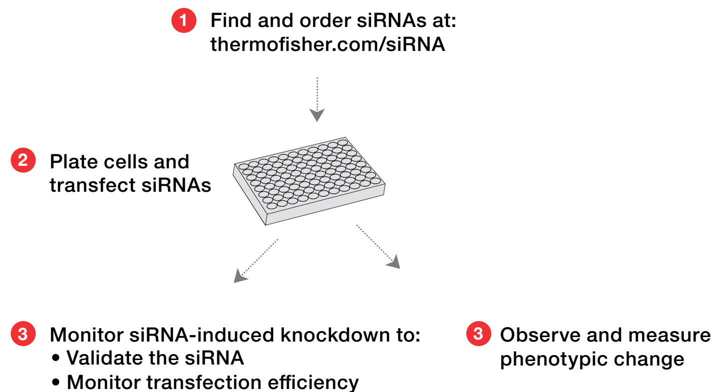
Quick start guide

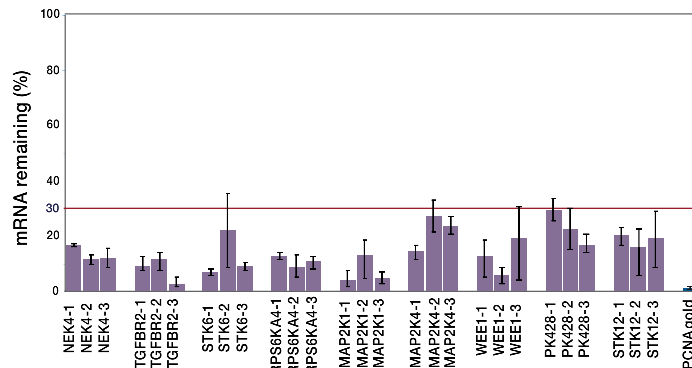
1. Find your gene online: **thermofisher.com/siRNA**
2. Decide on siRNA product type—Chapters 1 and 7
3. Decide on experimental controls—Chapters 2 and 10
4. Choose delivery vehicle: transfection reagent, electroporation, or viral delivery—Chapters 3 and 9
5. Validate to measure loss of function—Chapter 11

siRNA experimental workflow

Have on hand:

- Transfection or electroporation agent and protocol
- Assays to evaluate knockdown and other RNAi effect(s)
- Positive and negative control siRNAs
- Two or more siRNAs targeting your gene of interest





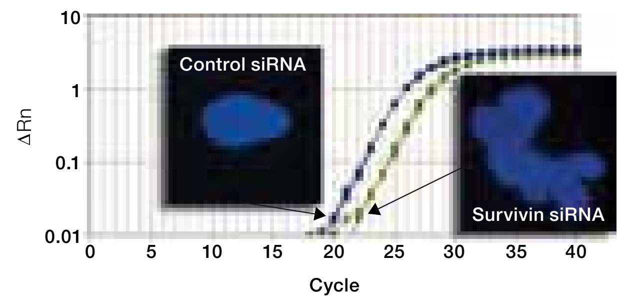


Figure 1. RNAi workflow. RNAi experimental workflow following siRNA design and synthesis.

Glossary of common RNAi terms

RNAi
Ribonucleic acid interference (first used by Fire and Mello et al., 1998).

siRNA
Short, or small, interfering RNA. siRNAs are 21–25 bp double-stranded RNA (dsRNA), typically with dinucleotide 3′ overhangs. Introduction of synthetic siRNAs can induce RNA interference in mammalian cells.

shRNA
Short hairpin RNA. shRNAs are used in vector-based approaches for supplying siRNA to cells to produce stable gene silencing. A strong Pol III type promoter is used to drive transcription of a target sequence designed to form hairpins and loops of variable length, which are processed by cellular machinery into an siRNA.

miRNA
microRNA. miRNAs are 19–23 nt duplexed RNAs, originating from endogenous single-stranded precursor transcripts that are characterized by imperfectly base-paired hairpins. miRNAs function as native gene regulators by utilizing the RNAi pathway. These regulatory RNAs are often studied using miRNA mimics to upregulate the miRNA expression or miRNA inhibitors to prevent miRNA-mediated gene silencing. siRNAs act in the same way as miRNAs but are designed to target a single gene, whereas an endogenous miRNA might have multiple gene targets.

RISC
RNA-induced silencing complex. A nuclease complex, composed of proteins and siRNA or miRNA, that targets and cleaves endogenous mRNAs complementary to the RNA strand within the RISC complex.

Off-target effects
Effects that occur when one or more nontarget genes show loss of gene function following the introduction of an siRNA. The effect may be mediated by the sense strand of an siRNA, which may initiate a loss-of-function response from an unrelated gene. Off-target effects can also occur as a secondary effect of the antisense strand of a specific siRNA if it has sufficient homology to knock down the expression of a nontarget gene.

Your source for RNAi solutions

RNAi is a powerful and well-characterized method for gene silencing. This technology is a proven tool in elucidation of biological pathways, disease gene analysis, and target gene discovery.

At Thermo Fisher Scientific, we have developed a broad suite of tools for RNAi applications, including short interfering RNA (siRNA) for targeted gene knockdown and microRNA (miRNA) to mimic or inhibit endogenous gene regulators. These industry-leading tools are suitable for *in vivo* or *in vitro* research, including high-throughput screening with RNAi libraries. Use this handbook to learn more about how we can help with end-to-end solutions for your entire RNAi workflow.

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Chapter 1
Introduction to RNAi

RNA interference (RNAi) is one of the most important technological breakthroughs in modern biology, allowing us to directly observe the effects of loss of function of specific genes in mammalian systems. Once viewed as a technique used only by select laboratories, RNAi is now considered essential for studying gene function. It has become a prominent tool for protein knockdown studies, phenotype analysis, function recovery, pathway analysis, *in vivo* knockdown, and drug target discovery.

A brief history of RNAi

In the early 1990s, scientists first observed that RNA inhibited protein expression in plants and fungi. In 1998, Andrew Fire and Craig Mello, working with *Caenorhabditis elegans*, discovered that double-stranded RNA (dsRNA) was the source of the inhibition, and they called this phenomenon RNA interference [1]. While studies in *C. elegans* were encouraging, the use of RNAi was limited to lower organisms because delivering long dsRNA for RNAi caused nonspecific inhibition in mammalian cells. In 2001, shorter RNA duplexes called small interfering RNA, or siRNA, were shown to directly trigger RNAi in mammalian cells, without evoking the nonspecific effects observed with longer dsRNAs. In 2006, just 8 years after the discovery of RNAi, the Nobel Prize in Physiology or Medicine was awarded to Fire and Mello for their discovery, underscoring the importance of RNAi as an investigative tool.

How RNAi works

The molecules that mediate RNAi are short dsRNA oligonucleotides, approximately 21 nucleotides in length. When longer dsRNA or short hairpin (shRNA) structures for triggering RNAi are introduced, they must first be cleaved into shorter duplexes by the cellular enzyme Dicer. At this point, RNAi technology takes advantage of the cell's natural machinery to effectively knock down expression of a gene with transfected siRNA. There are several ways to induce RNAi: synthetic molecules, RNAi vectors, and *in vitro* dicing of long dsRNA (Figure 1.1). In mammalian cells, short pieces of dsRNA—short interfering RNA—initiate the specific degradation of a targeted cellular mRNA. In this process, the antisense strand of siRNA becomes part of a multiprotein complex, or RNA-induced silencing complex (RISC), which then identifies the corresponding mRNA and cleaves it at a specific site. Next, this cleaved sequence is targeted for degradation, which ultimately results in the loss of protein expression.

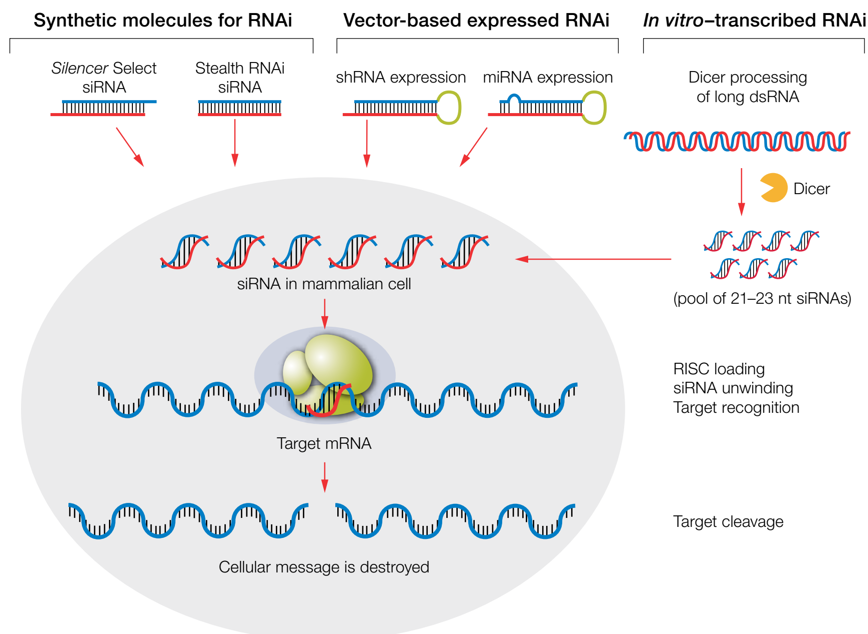


Figure 1.1. Methods of RNA knockdown in mammalian cells.

Eight tips for a successful siRNA experiment

1. Go to **thermofisher.com/siRNA** and utilize our search tool for predesigned siRNA targeting human, mouse, or rat genes with guaranteed silencing. Multiple siRNAs are recommended to verify phenotypes are due to target gene silencing and not an off-target effect.

2. Avoid RNases. Trace amounts of ribonucleases can sabotage siRNA experiments. Since RNases are present throughout the laboratory environment on your skin, in the air, on anything touched by bare hands or on anything left open to the air, it is important to take steps to prevent and eliminate RNase contamination. Thermo Fisher Scientific offers a complete line of products designed to detect and eliminate RNases.

3. Maintain healthy cell cultures and strict protocols for good transfection reproducibility. In general, healthy cells are transfected at higher efficiency than poorly maintained cells. Routinely subculturing cells at a low passage number ensures that there will be minimal instability in continuous cell lines from one experiment to the next. When performing optimization experiments we recommend transfecting cells within 50 passages, since transfection efficiency drops over time.

4. Avoid antibiotic use. Avoid the use of antibiotics during plating and up to 72 hours after transfection. Antibiotics have been shown to accumulate to toxic levels in permeabilized cells. Additionally, some cells and transfection reagents require serum-free conditions for optimal siRNA delivery. We suggest you perform a pilot transfection experiment in both normal growth medium and serum-free medium to determine the best condition for each transfection.

5. Transfect siRNAs using optimized reagents. Use an optimized siRNA transfection reagent and protocol for your cell type. The choice of transfection reagent is critical for success in siRNA experiments. It is essential to use transfection reagents formulated to deliver small RNAs (most commercially available transfection reagents were designed for large plasmid DNA, not small RNA molecules). Also, some reagents have been developed for the transfection of specific cell lines, while others have broader specificity.

   Note: Invitrogen™ Lipofectamine™ RNAiMAX Transfection Reagent (Cat. No. 137780-75) is our best-performing transfection reagent for siRNA. See page 75 for more information.

6. Use an appropriate positive control to optimize transfection and assay conditions. Housekeeping genes are suitable positive controls for most cell types. To optimize conditions, transfect target cells with several concentrations of an siRNA specific to your chosen positive control. Measure the reduction in the control protein or mRNA level compared to untransfected cells 48 hours after transfection. Too much siRNA can lead to cell toxicity. For maximum convenience, we offer validated positive control siRNAs for this purpose.

7. Use a negative control siRNA to distinguish nonspecific effects. Negative controls, most commonly nontargeting siRNAs, should have no significant homology to any known gene. They are useful for characterizing any nonspecific cellular responses to siRNA delivery that are independent of gene silencing. We offer multiple nontargeting controls that have been verified to have no significant similarity to any human, mouse, or rat gene.

8. Use labeled siRNAs for protocol optimization. Fluorescently labeled siRNA can be used to analyze transfection efficiency. The cells with the greatest concentration of labeled siRNA are likely using the best transfection conditions.

siRNA vs. vector approaches

Both siRNA and vector-based RNAi can be extremely effective at producing loss-of-function phenotypes. In general, most researchers choose siRNA because they can start quickly and there are no special preparations needed other than basic cell culture techniques. However, there are a number of reasons why a researcher might choose either siRNA or a vector-based RNAi. Table 1.1 contains criteria that will help you make the decision.

Table 1.1. Synthetic siRNA vs. vector-expressed siRNA.

	siRNA	RNAi vectors (miR RNAi and shRNA)
Long-term stable knockdown		•
Inducible knockdown		•
Delivery to hard-to-transfect cells		•
Least hands-on time	•	
Most immediate effect	•	
Higher potency likely	•	
Makes use of chemical modifications for specificity and/or stability	•	

Chapter reference

1. Fire A, Xu S, Montgomery MK et al. (1998) *Nature* 391, 806–811.

Chapter 2

Controls for RNAi experiments

Proper controls are essential to ensure success in every RNAi experiment. The number and types of controls chosen depends upon the ultimate research goal (Table 2.1). Our RNAi control reagents allow you to:

- Achieve greater knockdown by optimizing transfection protocols
- Save time by confirming cell viability early in an experiment
- Proceed with confidence by comparing targeted RNAi reagents to a negative control designed to be nontargeting in any human, mouse, or rat system

Transfection controls

With overexpression experiments, even low transfection efficiencies can often yield desired results. In contrast, for gene knockdown to be measurable in a cell population, it is important to have the highest transfection efficiency possible. Even small reductions in transfection efficiency can limit your ability to identify functional differences in your experimental samples or validate knockdown by qRT-PCR or western blot analysis.

To achieve the highest transfection efficiency possible, particularly for gene knockdown experiments, first optimize transfection conditions for your cell lines, so you know you have optimal transfection conditions for effective RNAi experimentation. Keep in mind that it is also important to monitor experiment-to-experiment transfection variation.

We recommend Invitrogen™ Lipofectamine™ RNAiMAX Transfection Reagent for efficient delivery of siRNA in most cell lines. To monitor transfection efficiency, we have a selection of RNAi controls labeled with Applied Biosystems™ FAM™, Invitrogen™ Cy®3, and Invitrogen™ Alexa Fluor™ dyes to choose from. It is important to note that fluorescent controls are a good tool for transfection optimization, but the percentage of transfected cells as indicated by fluorescence will not directly correlate with target gene silencing.

For more information on controls to use for optimizing transfection with siRNA or a table listing the contents for each of the transfection control kits, go to **thermofisher.com/sirnacontrols**

Table 2.1. RNA interference controls.

Type of control	Recommended use	Products
Transfection control	Calculate and monitor transfection efficiency with fluorescence	See pages 85–87
Negative controls	Nontargeting controls used to measure knockdown levels vs. background	See pages 82, 83, and 87
Positive controls	RNAi reagents known to achieve high levels of knockdown used to measure delivery and optimize experimental conditions	See pages 82, 84, and 88
Untransfected control	Measure normal gene expression level and phenotype	–
Multiple RNAi reagents to the same target	Verify phenotypic change; control for off-target effects for generating publication-quality results	–
Titration of RNAi reagent	Use the lowest effective level to avoid altering the cells' normal processes	–
Rescue experiments	Turn off inducible RNAi or introduce a plasmid expressing the target mRNA that will not be affected by the RNAi reagent	Invitrogen™ BLOCK-iT™ Pol II miR RNA with inducible promoter (CMV/TO) (see page 62)

Negative controls

Negative control siRNAs, or nontargeting controls—siRNAs with sequences that do not target any gene product—are essential for determining the effects of siRNA delivery and for providing a baseline to compare siRNA-treated samples. There are two Invitrogen™ *Silencer*™ Select Negative Control siRNAs. These siRNAs include the same modifications for reducing off-target effects as found in other *Silencer* Select siRNAs and have no significant similarity to mouse, rat, or human gene sequences. These negative control siRNAs have been tested by microarray analysis and shown to have minimal effects on gene expression. In addition, these two controls have been tested in multiparametric cell-based assays and are proven to have no significant effect on cell proliferation, viability, or morphology in the cell lines tested.

We also provide a negative control in our RNAi vector cloning kits. The negative control should be the same chemical structure as the target RNAi molecules. For example, if you are using shRNA vectors, the negative control should have the same vector backbone but a different RNAi sequence.

For experiments using Invitrogen™ Stealth RNAi™ siRNA, we have three predesigned negative controls with the following features:

- Three levels of GC content to match that of the experimental Stealth RNAi siRNA; the medium GC content is most widely applicable and commonly used when analyzing multiple siRNAs
- No homology to any known vertebrate gene
- Tested sequences that do not induce a stress response

We recommend using one or more nontargeting control in every RNAi experiment.

Positive controls

Positive controls provide more confidence in your RNAi experiments by ensuring that the experimental conditions were met to achieve robust data. Positive controls are siRNAs designed to target constitutively expressed genes and are known to achieve high levels of knockdown (>70%). A positive control should be used to optimize RNAi delivery conditions and to reconfirm high levels of delivery in each RNAi experiment. When a positive control fails to produce knockdown, carefully evaluate your experimental conditions and determine if some factors need to be adjusted. Examples of positive control targets are genes expressed at easily detectable levels, such as p53, lamin, or GAPDH. However, if you are looking at a particular phenotype such as apoptosis, you will most likely want to choose a positive control known to elicit apoptosis, such as Eg5, a kinesin spindle protein.

Downstream controls

Before transfecting cells and performing qRT-PCR and western blots to measure mRNA and protein levels, we recommend validating your downstream assay reagents. Validating qRT-PCR primers or antibodies for your positive control and target genes before performing knockdown experiments in your cell line ensures that your reagents are sensitive enough to detect changes in expression of your target gene due to knockdown. Without sufficient sensitivity, it can be difficult to interpret knockdown results from genes or proteins with low expression levels.

Interferon controls

The introduction of RNAi reagents to cells can induce cellular stress response pathways, such as the interferon response. Activation of these stress response pathways can lead to translational arrest, growth inhibition, and cellular toxicity. These events make it difficult to assess whether observed cellular phenotypes are due to nonspecific stress responses or the loss of function of the targeted gene. Validated qRT-PCR primers for *PKR* and *IFIT-1* stress response genes provide a specific and sensitive way to monitor whether toxic cellular effects are complicating the interpretation of your RNAi experimental data.

Use multiple siRNA sequences per target to verify results

siRNA sequences with partial homology to other targets may contribute to off-target activity. Gene profiling experiments have shown that duplexes with partial homology to other transcripts can cleave the target or act like a microRNA (miRNA), inhibiting translation of the target mRNA. Specificity studies have revealed that siRNA duplexes can have varying activities, depending on the number, position, and base pair composition of mismatches with respect to the target RNA. To ensure that knockdown of the intended gene causes a particular siRNA phenotype, the phenotypic results should be confirmed by at least two siRNA molecules that target nonoverlapping regions of the target mRNA. Thus, if one siRNA sequence produces a particular phenotype, but the second siRNA sequence (designed to target the same gene) produces a different phenotype, yet the target gene shows depletion by qRT-PCR, then you cannot conclude that the phenotype of interest is due to knockdown of the intended target.

Titrate siRNA

Silencer Select siRNA and Stealth RNAi siRNA can be very effective even at low concentrations. Titrating down the dose of the siRNA enables you to reduce any off-target or nonspecific effects while achieving robust knockdown.

Rescue experiments

RNAi rescue experiments are performed to ensure that the observed effect is due to knocking down the target gene of interest. If you are using an inducible RNAi vector system, turn off the RNAi vector expression by removing tetracycline from the medium. If you are using *Silencer* Select siRNA or Stealth RNAi siRNA, there are two main methods used to rescue the phenotype. The first method involves designing RNAi sequences to target the 3´ UTR and then transfecting the cells after knockdown with a vector expressing the open reading frame (ORF) of the gene of interest. If the RNAi sequences were designed to target the ORF, you can use a mutagenesis kit to create one or more silent third-codon point mutations within the region targeted by the RNAi sequence, preferably the seed and cleavage regions on the antisense strand (bases 2–12).

Chapter 3
Delivery of RNAi reagents to cells

The two common approaches for delivering RNAi agents into cells are lipid-mediated transfection and virus-mediated transduction. Determining which one of these approaches to use depends on the cell type being studied and whether transient or stable knockdown is desired (Table 3.1). The most popular application, transient transfection of synthetic siRNA, uses cationic lipid–based reagents because they are suitable for delivering molecules across a diverse range of commonly used cell lines.

For cell types that are not amenable to lipid-mediated transfection, viral vectors are often employed. Adenoviral vectors work well for transient delivery in many cell types. However, when stable RNAi expression is desired, or for difficult cell lines, such as nondividing cells, lentiviral vectors are the best delivery method. A third possibility is the application of electroporation—an instrument-based approach—which uses an electrical current to temporarily create pores in the cell membrane, enabling entry of siRNA, microRNA, or plasmids.

Methods to achieve high transfection efficiency

Transfection efficiency describes the percentage of cells that have received the siRNA or RNAi expression construct. High-transfection efficiency is particularly important for RNAi applications because untransfected cells will continue to express the gene targeted for knockdown, thus contributing to background expression levels.

Once you have determined the delivery technique that is most suitable for your cell type and duration of silencing, the next step is the selection of the optimal delivery reagent to achieve high-efficiency transfection. We recommend testing multiple transfection reagents and using those that are specifically intended for delivery of your nucleic acid payload—a reagent intended for small RNA delivery to transfect siRNA, for instance, rather than one designed for plasmid DNA.

Table 3.1. Recommended RNAi delivery methods.*

Cell type	Transient expression (<7 days)	Transient expression (>7 days)	Stable expression
Fast-growing adherent cells (A549, HeLa)	Lipid transfection of synthetic siRNA	Lipid transfection of RNAi vectors or adenoviral delivery	Lipid transfection of RNAi vectors or lentiviral delivery
Fast-growing suspension cells (THP-1)	Lipid transfection or electroporation of synthetic siRNA	Lipid transfection of RNAi vectors or adenoviral delivery	Lipid transfection or electroporation of RNAi vectors or lentiviral delivery
Primary cells	–	–	Lentiviral delivery
Nondividing cells	–	–	Lentiviral delivery

* For lipid transfection we recommend Lipofectamine RNAiMAX reagent (**thermofisher.com/rnaimax**), and for electroporation we recommend the Invitrogen™ Neon™ Transfection System (**thermofisher.com/neon**). See Chapter 9 for details.

Minimizing transfection-mediated cytotoxicity

The delivery of RNAi reagents, or the delivery method itself, can give rise to cytotoxicity in gene silencing experiments. Minimizing transfection-mediated cytotoxicity is essential for proper interpretation of the outcome of any RNAi experiment, as cytotoxic effects can be difficult to distinguish from a phenotype resulting from target gene knockdown. Cytotoxicity from the delivery method itself is likely when apparent knockdown of the target gene is seen when cells are transfected with a negative control. The easiest way to combat the issue of delivery-related cytotoxicity is to choose a transfection reagent that has been designed for either double-stranded RNA or plasmid-based RNAi transfections. Most often, these reagents have been formulated to maximize efficiency (to achieve high knockdown levels) while minimizing cytotoxicity. Optimization experiments using supplier or published protocols as guidelines can help to determine which concentration of transfection reagent works best for the cell line of interest. We recommend using the lowest amount of transfection reagent necessary to achieve the highest level of knockdown.

Reducing off-target effects

As well as being a source of cytotoxicity, a suboptimal delivery reagent or excess reagent can result in apparent off-target effects. One cause of off-target effects is the up- or downregulation of genes due to response to the transfection reagent itself. However, with appropriate controls, these effects can be identified and diminished. Again, use the lowest amount of transfection reagent that provides the best gene silencing activity.

The potential also exists for off-target effects due to knockdown from the siRNA itself. To determine the most favorable conditions, vary the concentration of siRNA while holding the concentration of transfection reagent constant at the lowest concentration previously identified. Utilize the lowest siRNA concentration that gives the desired level of knockdown in RNAi experiments. Keep in mind that the specificity of the siRNA will have an impact on potential off-target effects. The use of exceptionally specific reagents, such as *Silencer* Select siRNA can help alleviate these concerns.

Transfecting *Silencer* Select siRNA into A549 cells using Lipofectamine RNAiMAX Transfection Reagent

Introduction

Invitrogen™ Lipofectamine™ RNAiMAX Transfection Reagent is a proprietary formulation specifically developed for highly efficient delivery of *Silencer* Select siRNA to mammalian cells for RNAi analysis. This reference provides a recommended procedure to transfect Invitrogen™ *Silencer* Select siRNA into human A549 lung carcinoma cells (ATCC, Cat. No. CCL-185) using Lipofectamine RNAiMAX reagent (Cat. Nos. 13778-075, 13778-150). Lipofectamine RNAiMAX reagent has a broad range of activity, enabling maximal knockdown levels with a minimum of optimization required.

Important guidelines for transfection

Follow these important guidelines when transfecting *Silencer*™ Select siRNA into A549 cells using Lipofectamine RNAiMAX reagent:

- Both reverse transfection and forward transfection protocols (page 16) can be used for transfecting A549 cells.
- We recommend using 10 nM of the siRNA and indicated procedures. However, the efficacy of the siRNA chosen, the transcription rate of the target gene, and the stability of the resulting protein influence the degree of target gene knockdown observed. You may need to adjust the siRNA concentration (1–50 nM can be used) and assay time (up to 72 hours) to establish optimal knockdown of your target gene.
- We recommend Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985-062) to dilute siRNA and Lipofectamine RNAiMAX reagent before complexing.
- Do not add antibiotics to media during transfection, as this causes cell death.
- Test serum-free media for compatibility with Lipofectamine RNAiMAX reagent.
- Lipofectamine RNAiMAX reagent has a broad peak of activity; for a range of cell densities and volumes of transfection reagent suitable for use, see "Acceptable range for maximal activity" (page 17).

Materials needed

Have the following reagents on hand before beginning:

- A549 cells maintained in Gibco™ DMEM (Cat. No. 11965-092) supplemented with 10% fetal bovine serum (Cat. No. 26140-079), 2 mM glutamine (Cat. No. 25030-149), and penicillin–streptomycin (Cat. No. 15070-063)

  Note: Use low-passage cells (<50 passages); make sure that cells are healthy and greater than 90% viable before transfection.
- *Silencer* Select siRNAs targeting the gene of interest
- Lipofectamine RNAiMAX reagent (store at +2–8°C until use)
- Opti-MEM I Reduced Serum Medium
- Appropriate tissue culture plates and supplies

Reverse transfection

Use this procedure to reverse-transfect siRNA into A549 cells in a 24-well format (for other formats, see "Scaling up or down transfections", page 17). In reverse transfections, the complexes are prepared inside the wells, after which cells and medium are added. Reverse transfections are faster to perform than forward transfections, and are the method of choice for high-throughput transfection. All amounts and volumes are given on a per well basis.

1. For each well to be transfected, prepare siRNA–Lipofectamine RNAiMAX reagent complexes as follows:
   - Dilute 6 pmol siRNA in 100 µL Gibco Opti-MEM I Reduced Serum Medium without serum in the well of the tissue culture plate. Mix gently.
     Note: If the volume of your siRNA solution is too small to dispense accurately (less than 1 µL) and you cannot pool dilutions, predilute your siRNA 10-fold in RNase-free water (or the dilution buffer recommended by your siRNA manufacturer), and dispense a 10-fold higher amount (should be at least 1 µL per well). For example, to get 6 pmol of siRNA from a 20 µM siRNA stock solution, dilute your siRNA 10-fold to a concentration of 2 µM, and dispense 3 µL.
   - Mix Lipofectamine RNAiMAX reagent gently before use, then add 1 µL Lipofectamine RNAiMAX reagent to each well containing the diluted siRNA. Mix gently and incubate for 10–20 minutes at room temperature.
2. Dilute A549 cells in complete growth medium without antibiotics so that 500 µL of the medium contains 30,000 cells (cell density should be 30–50% confluent 24 hours after plating).
3. To each well with siRNA–Lipofectamine RNAiMAX reagent complexes, add 500 µL of the diluted cells. This gives a final volume of 600 µL and a final RNA concentration of 10 nM. Mix gently by rocking the plate back and forth.
4. Incubate the cells for 24–72 hours at 37°C in a CO_2 incubator until you are ready to assay for gene knockdown.

Forward transfection

Use this procedure to forward-transfect siRNA into A549 cells in a 24-well format (for other formats, see "Scaling up or down transfections", page 17). In forward transfections, cells are plated in the wells, and the transfection mix is generally prepared and added the next day. All amounts and volumes are given on a per-well basis.

1. One day before transfection, plate 30,000 cells in 500 µL of growth medium without antibiotics. The cell density should be 30–50% confluent at the time of transfection.
2. For each well to be transfected, prepare siRNA–Lipofectamine RNAiMAX reagent complexes as follows:
   - Dilute 6 pmol siRNA in 50 µL Opti-MEM I Reduced Serum Medium without serum. Mix gently.
     Note: If the volume of your siRNA solution is too small to dispense accurately (less than 1 µL), and you cannot pool dilutions, predilute your siRNA 10-fold in 1X RNA Annealing/Dilution Buffer (or dilution buffer recommended by your siRNA manufacturer), and dispense the proper higher amount (should be at least 1 µL per well). For example, to get 6 pmol of siRNA from a 20 µM siRNA stock solution, dilute your siRNA 10-fold to a concentration of 2 µM, and dispense 3 µL.
   - Mix Lipofectamine RNAiMAX reagent gently before use, then dilute 1 µL in 50 µL Opti-MEM I Reduced Serum Medium. Mix gently.
   - Combine the diluted siRNA with the diluted Lipofectamine RNAiMAX reagent. Mix gently and incubate for 10–20 minutes at room temperature.
3. Add the siRNA–Lipofectamine RNAiMAX reagent complexes to each well containing cells. This gives a final volume of 600 µL and a final RNA concentration of 10 nM. Mix gently by rocking the plate back and forth.
4. Incubate the cells for 24–48 hours at 37°C in a CO_2 incubator until you are ready to assay for gene knockdown. Medium may be changed after 4–6 hours, but this is not required.

Note: The Invitrogen™ BLOCK-iT™ Fluorescent Oligo (Cat. No. 2013) is optimized for use with Invitrogen™ Lipofectamine™ 2000 Transfection Reagent, and is not recommended for use with Lipofectamine RNAiMAX reagent.

Acceptable range for maximal activity

Due to the broad range of maximal activity exhibited by Lipofectamine RNAiMAX reagent, a range of cell densities and volumes of Lipofectamine RNAiMAX reagent can be used for transfection. For transfecting A549 cells in 24-well format, 0.5–1.25 µL Lipofectamine RNAiMAX reagent and 20,000–50,000 cells per well is suitable. For extended time course experiments (72 hours), consider using the lower cell number; for short-term experiments (24 hours), consider the higher cell number. The final concentration of siRNA can be varied between 1 and 50 nM.
A concentration of 10 nM siRNA is suitable to knock down many target genes. However, the optimal concentration of siRNA will vary depending on the efficacy of the duplex, and should be determined empirically.

Scaling up or down transfections

To transfect A549 cells in different tissue culture formats, vary the amounts of siRNA, Lipofectamine RNAiMAX reagent, cells, and medium used in proportion to the relative surface area, as shown below.

Note: 20 µM siRNA = 20 pmol/µL.

Recommended reagent amounts and volumes.

Culture vessel	Relative surface area*	Volume of plating medium	Cells plated per well		Dilution medium		siRNA amount		Final siRNA concentration		Lipofectamine RNAiMAX reagent**	
			Start point	Acceptable range	Reverse transfection (µL)	Forward transfection (µL)	Start point (pmol)	Acceptable range (pmol)	Start point (nM)	Acceptable range (nM)	Start point (µL)	Acceptable range (µL)
96-well	0.2	100 µL	7,500	5,000–10,000	20	2 x 10	1.2	0.12–6	10	1–50	0.2	0.1–0.25
48-well	0.4	200 µL	15,000	10,000–20,000	40	2 x 20	2.4	0.24–12	10	1–50	0.4	0.25–0.5
24-well	1	500 µL	30,000	20,000–50,000	100	2 x 50	6	0.6–30	10	1–50	1	0.5–1.25
6-well	5	2.5 mL	150,000	100,000–250,000	500	2 x 250	30	3–150	10	1–50	5	2.5–6.25

* Surface areas may vary depending on the manufacturer.
** If the volume of Lipofectamine RNAiMAX reagent is too small to dispense accurately, and you cannot pool dilutions, predilute Lipofectamine RNAiMAX reagent 10-fold in Opti-MEM I Reduced Serum Medium, and dispense a 10-fold higher amount (should be at least 1.0 µL per well). Discard any unused diluted Lipofectamine RNAiMAX reagent.

Want additional transfection protocols?
Go to **thermofisher.com/rnaitransfectionprotocol**

Cell health

In general, healthy cells take up nucleic acids better than poorly maintained cells. When they are stressed by culture conditions, many cells undergo expression profile changes that can adversely affect your experiments. Routinely subculturing cells before they become overcrowded or unhealthy will minimize instability in continuous cell lines. Information on basic cell culture technique can be found in "Culture of Animal Cells: A Manual of Basic Technique" [1].

Culture conditions

Overly crowded or sparse cultures are not conducive to healthy cells. As a rule, cells should be replated before the medium becomes depleted. As cell cultures approach confluence, they typically contain some unhealthy or dead cells, which make cell counts inaccurate. In addition, cells that have grown in depleted medium between subculturing events have been deprived of nutrients and may have experienced pH shifts that are detrimental to health and viability. Therefore, avoid overgrowing cells and subjecting them to frequent pH and temperature shifts.

Some adherent cell lines are sensitive to trypsin exposure and to shear forces from vigorous pipetting or high-speed centrifugation. (For most cell lines, trypsinization should be kept shorter than 10 minutes.) In addition to treating cells gently, maintaining strict protocols, including harvesting cells for experiments at similar confluencies and maintaining consistent time intervals between plating and transfecting cells, will improve experimental reproducibility.

Mycoplasma contamination is another common stress factor for cells in culture that can deleteriously affect experimental results. Mycloplasmas are small, free-living prokaryotes that are not observable by light microscopy. Because they grow as filamentous or coccal forms without cell walls, they are not sensitive to antibiotics that interfere with cell wall production. Mycoplasmas can alter cell growth characteristics, inhibit cell metabolism, and disrupt nucleic acid synthesis, causing chromosomal abnormalities, and altering transfection or infection rates. In most situations, mycoplasmas from an infected cell line spread to other cultures in a laboratory via aerosolization during routine pipetting and handling or from shared reagents (e.g., medium, serum) that become contaminated. The best prevention and control requires good aseptic technique (including working with cultures in order of clean to untested to infected during the work day or week) and routine testing. Many commercial kits (PCR-, ELISA-, fluorescence-, luminescence-, and culture-based assays) are available to test cultures for mycoplasma contamination. Cultures infected with mycoplasmas are usually discarded and replaced, but for irreplaceable cultures, treatment options are available to inhibit or eliminate mycoplasmas.

Tips

- Let freshly thawed cells recover for at least 48 hours. Do not perform analyses on freshly thawed cells within 48 hours of plating.
- Optimize siRNA delivery for different phenotypic assays. Similar to balancing siRNA-induced knockdown and cell viability, there may also be a balance between siRNA delivery and downstream assay conditions. It may be necessary to reoptimize siRNA delivery conditions for different downstream assays that are used in siRNA screening passes.

Passage number

Because some cell lines may gradually change in culture, we recommend using normal or primary cell types within 10 passages of determining optimal siRNA delivery conditions. If transfection or electroporation efficiency begins to drop, thaw fresh cells for subsequent experiments. If frozen stocks are not available for reculturing, it may be necessary to reoptimize the transfection conditions using existing stocks. Passage number is usually not as critical for immortalized or transformed cell lines.

siRNA quality

The quality of siRNA can significantly influence RNAi experiments. siRNAs must be free of reagents carried over from synthesis, such as ethanol, salts, and proteins. Also, dsRNA contaminants longer than 30 bp are known to alter gene expression by activating the nonspecific interferon response and causing cytotoxicity [2]. Therefore, we recommend using siRNAs that are greater than 80% full length (standard purity siRNAs).

siRNA quantity

The optimal amount of siRNA and its capacity for gene silencing are influenced in part by properties of the target gene products, including the following: mRNA localization, stability, abundance, as well as target protein stability and abundance. Although many siRNA experiments are still performed by transfecting cells with 100 nM siRNA, published results indicate that transfecting lower siRNA concentrations can reduce off-target effects exhibited by siRNAs [4,5]. For lipid-mediated reverse transfections, 10 nM of siRNA (range 1–30 nM) is usually sufficient. For siRNA delivery using electroporation, siRNA quantity has a less pronounced effect, but typically 1 µg of siRNA per 50 µL of cells (range: 0.5–2.5 µg per 50 µL of cells, or 0.75–3.75 µM) is sufficient.

Keep in mind that while too much siRNA may lead to off-target or cytotoxic effects, too little siRNA may not reduce target gene expression effectively. Because there are so many variables involved, it is important to optimize the siRNA amount for every cell line used. In addition, the amount of nontargeting negative control siRNA should be the same as the experimental siRNAs.

Choice of transfection agent

It is important to select the appropriate transfection agent for the cell line being used. Different cell types vary in their response to different transfection agents; thus, the best transfection agent for a particular cell type must be determined experimentally. Lipofectamine RNAiMAX reagent is known to be best performing siRNA transfection reagent on the market. For more information, see Chapter 9.

Volume of transfection agent

The volume of transfection agent is a critical parameter to optimize because too little can limit transfection, and too much can be toxic. The overall transfection efficiency is influenced by the amount of transfection agent complexed to the siRNA. To optimize, titrate the transfection agent over a broad dilution range, and choose the most dilute concentration that still gives good gene knockdown. This critical volume should be determined empirically for each cell line.

While cell density is important for traditional, preplated transfection experiments, cell density is less critical and requires little to no optimization, when siRNAs are delivered by reverse transfection. However, if too many cells are used, and the amount of siRNA is not increased proportionally, the concentration of siRNA in the sample may be too low to effectively elicit gene silencing. When cell density is too low, cultures can become unstable. Instability can vary from well to well because culture conditions (e.g., pH, temperature) may not be uniform across a multiwell plate and can differentially influence unstable cultures.

Exposure to transfection agent:siRNA complexes

Although most transfection agents are designed to induce minimal cytotoxicity, exposing cells to excessive amounts of transfection agent or for an extended time can be detrimental to the overall health of the cell culture. Sensitive cells may begin to die from exposure to the transfection agent after a few hours. If transfection causes excessive cell death with your cells, remove the transfection mixture and replenish with fresh growth medium after 8–24 hours.

Presence of serum in the medium during transfection

Complex formation between transfection agents and siRNA should be performed in reduced-serum or serum-free medium, so that serum components will not interfere with the reaction. However, once the complex is formed, some transfection agents will permit transfection in serum-containing, normal growth medium (follow manufacturer's instructions). No culture medium addition or replacement is usually required following transfection, but changing the media can be beneficial in some cases, even when serum compatible reagents are used. Be sure to check for serum compatibility before using a particular agent. Some transfection agents require serum-free medium during the transfection and a change to complete growth media after an initial incubation with transfection complexes.

Optimizing transfection experiments

Maximizing transfection efficiency while minimizing cytotoxicity are crucial for optimal gene silencing. The best transfection efficiencies are achieved for each cell type by identifying (in order of importance):

1. Choice of transfection reagent
2. Volume of transfection agent
3. Amount of siRNA
4. Cell density at the time of transfection
5. Length of exposure of cells to transfection agent:siRNA complexes
6. Transfection method: traditional transfection where cells are preplated or reverse transfection where cells are transfected as they adhere to the plate
7. Presence or absence of serum

Once the conditions for maximal gene silencing are determined, keep them constant among experiments with a given cell type.

Tips

- **siRNA storage:** Store siRNAs at –20°C or –80°C, but do not use a frost-free freezer. Our data indicate that up to 50 freeze/thaw cycles are not detrimental to siRNAs in solution at 100 μM (as assessed by mass spectrometry and analytical HPLC). However, we recommend that siRNAs that have been resuspended in RNase-free water or buffer be stored in small aliquots to avoid potential contamination.
- **Nuclease resistance of siRNAs:** Annealed, double-stranded siRNAs are much more nuclease resistant than single-stranded RNA. However, stringent RNase-free techniques should be used during all RNAi experiments.
- **Checking siRNA for degradation:** If you suspect that a preparation of siRNA may be degraded, check the integrity of the siRNA by running ~2.5 μg on a nondenaturing 15–20% acrylamide gel. Visualize the RNA by staining with ethidium bromide, and verify that it is the expected size and intensity. The siRNA should migrate as a tight band; smearing indicates degradation.

Chapter references

1. Freshney RI (2000) 4th ed. New York (NY): Wiley-Liss.
2. Stark GR et al. (1998) *Ann Rev Biochem* 67:227–264.

Chapter 4

Vector-based RNAi technologies

Introduction to adenoviral and lentiviral RNAi expression vectors

Having evolved to proficiently deliver nucleic acids to cells, viruses offer a means to reach hard-to-transfect cell types for protein overexpression or knockdown. Adenoviral, oncoretroviral, and lentiviral vectors have been used extensively for delivery in cell culture and *in vivo* (Table 4.1). Adenoviruses are DNA viruses that can transiently transduce nearly any mammalian cell type, including nondividing primary and growth-arrested cells. Adenoviruses enter target cells by binding to the coxsackievirus and adenovirus receptor (CAR). After binding to the CAR, the adenovirus is internalized via integrin-mediated endocytosis, followed by active transport to the nucleus, where its DNA is expressed episomally. Oncoretroviruses and lentiviruses are positive-strand RNA viruses that stably integrate their genomes into host cell chromosomes. When pseudotyped with an envelope that has a broad tropism, such as vesicular stomatitis virus glycoprotein (VSV-G), these viruses can enter virtually any mammalian cell type. However, the oncoretroviruses depend upon nuclear membrane breakdown during cell division to transduce cells. In contrast, lentiviruses are more versatile tools, as they use an active nuclear import pathway to transduce nondividing cells (Figure 4.1).

Table 4.1. Viral delivery strategies for RNAi.

	Transient expression		Stable expression			
	Dividing cells	Nondividing cells	Dividing cells	Neuronal cells	Drug- or growth-arrested cells	Contact-inhibited cells
Adenovirus	•	•				
Lentivirus	•	•	•	•	•	•
Oncoretrovirus	•		•			

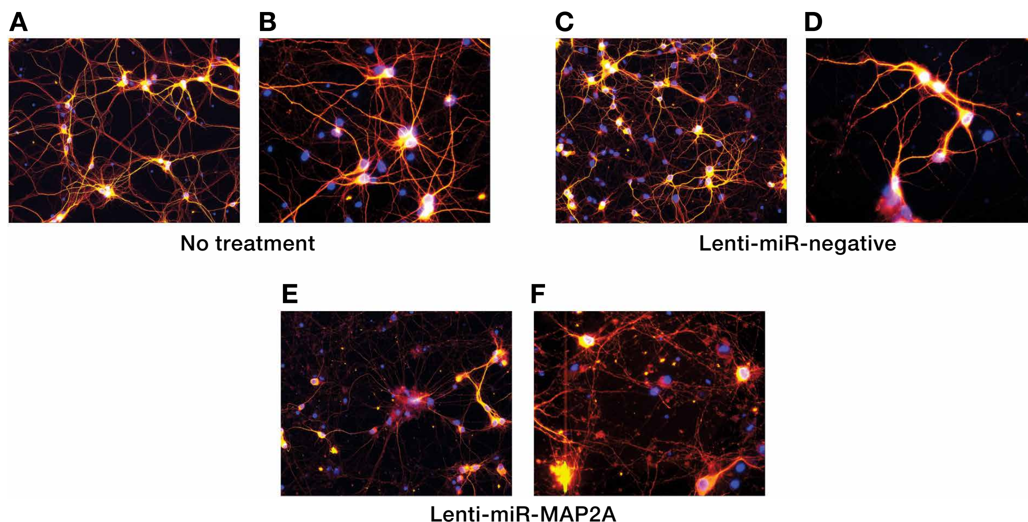


Figure 4.1. Lentiviral transduction of miR RNAi. Untreated **(A, B)** and transduced **(C–F)** samples were stained with MAP2 antibodies (orange) and DAPI (blue). **(E)** and **(F)** clearly show less expression of target protein compared to the untreated neurons and those transduced with the Lenti-miR-negative control.

How vector-mediated RNAi works

For utilization of vector-based RNAi, there are two primary types of expression constructs in use. Many systems use a standard stem-loop, short hairpin RNA (shRNA). It has been found that improvements in hairpin processing, and therefore efficiency of silencing, can be achieved with a microRNA (miR)-based scaffold. Both miR RNAi and shRNA vector systems take advantage of the endogenous RNAi pathway found in all animal cells. Compared to shRNA vectors, the miR RNAi vector systems, by using artificial microRNAs (miRNAs), utilize more of the components of the endogenous machinery, resulting in more efficient processing of expressed RNA hairpins (Figure 4.2).

Artificial miRNAs expressed from an expression vector are transcribed by RNA polymerase II. The primary miRNA (pri-miRNA) transcript contains one or more precursor miRNAs (pre-miRNAs). The RNase type III enzyme Drosha recognizes the flanking sequences of the pre-miRNAs and excises them from the pri-miRNA transcript. Each precursor miRNA is then actively transported out of the nucleus by Xpo-5.

Once in the cytoplasm, the pre-miRNA hairpins are processed further by Dicer, which converts them into miRNAs. Finally, the miRNAs unwind, load into the RNA-induced silencing complex (RISC), and hybridize with their mRNA target. While most endogenous mammalian miRNAs do not perfectly complement the target mRNA sequence and thus result in translational inhibition, the artificially designed miRNAs used in a vector-based RNAi system are 100% homologous to the target mRNA sequence and result in target cleavage.

The short hairpin RNA (shRNA) vectors contain an RNA polymerase III (Pol III) promoter (H1 or U6) for nuclear expression of shRNAs [1-5]. Xpo-5 actively exports shRNA to the cytoplasm [6,7], where it is recognized and cleaved by the RNase III enzyme Dicer to produce short interfering RNA (siRNA) [3].

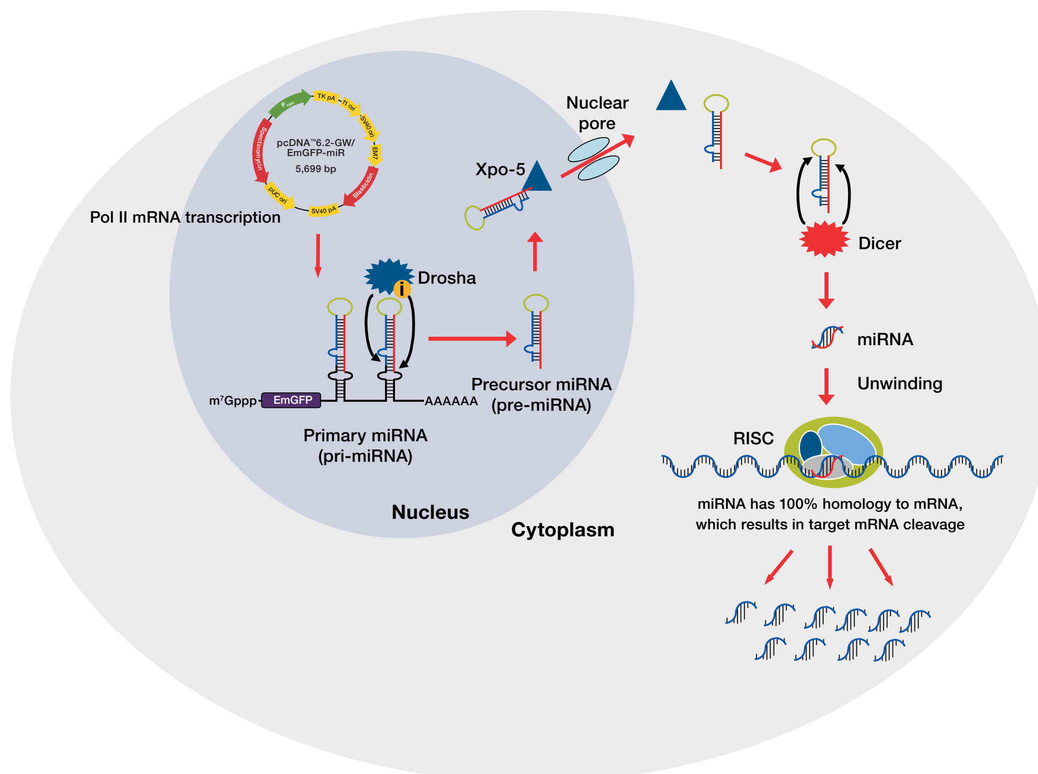


Figure 4.2. Expression of miRNA sequences using the BLOCK-iT™ Pol II miR RNAi expression vectors.

Virus-mediated delivery

For many disease models, the most desirable cell types, such as immune system or primary cells, are not amenable to transfection. Viral delivery of RNAi vectors is a powerful alternative to transfection for these cell types as well as for *in vivo* applications. To accurately determine the efficacy of knockdown from an shRNA or miR RNAi molecule in a population of cells, it is critical to deliver the shRNA or miR RNAi molecule to as many cells as possible. Otherwise, when knockdown is measured by qRT-PCR or western blot analysis, the background of mRNA or protein in untransfected cells will make the knockdown appear less effective than it actually is. Viral delivery can be the best option in virtually any mammalian cell type, including hard-to-transfect, primary, and even nondividing cells. Conveniently, lentiviral delivery systems are available for both shRNA and miR RNAi vectors, and an adenoviral delivery system is available for shRNA vectors (Table 4.2).

Table 4.2. Choose a lentiviral or adenoviral RNAi system.

Viral system	When to use	Products
Lentiviral RNAi delivery systems	• Stable RNAi in any cell line, even nondividing cells • Inducible or constitutive shRNA or miR RNAi expression • Studies in animal models	• Invitrogen™ BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System—a complete lentiviral system with all of the advantages of miR RNAi: multiple-target knockdown and a higher design success rate than conventional shRNA (contains pLenti6/V5-DEST™ vector) • BLOCK-iT Lentiviral Pol II miR RNAi Expression System with EmGFP—a system with all of the benefits listed above, plus easy expression tracking with cocistronic EmGFP (contains pLenti6/V5-DEST™ vector) • Invitrogen™ BLOCK-iT™ Lentiviral RNAi Expression System—complete lentiviral system for constitutive shRNA expression in any cell type (contains pLenti6/BLOCK-iT™-DEST vector)
Adenoviral RNAi delivery system	• High-level transient shRNA expression • Effective delivery to a wide range of human cell types • Studies in animal models	• Invitrogen™ BLOCK-iT™ Adenoviral RNAi Expression System—complete system for high-level transient expression of shRNA

The procedure for using both RNAi viral systems (Figure 4.3):

1. Clone a double-stranded DNA oligo encoding an shRNA or miR RNAi into one of the BLOCK-iT entry (shRNA) or expression (miR RNAi) vectors.
2. Transfer the RNAi cassette into the adenoviral (shRNA only) or lentiviral destination vector by Invitrogen™ Gateway™ recombination cloning technology.
3. Transfect vectors into the appropriate packaging cells (use Invitrogen™ ViraPower™ Packaging Mix for lentiviral systems only) to produce viral stocks, which can be used immediately or stored at –80°C.
4. Harvest and (for adenovirus only) amplify the viral supernatant and use it for shRNA or miR RNAi delivery to any cell type.

Although employing RNAi vector systems can be slightly more involved than using synthetic RNAi reagents, the flexibility of the vector-based systems is compelling for many RNAi researchers conducting both *in vitro* and *in vivo* experiments. There are two main types of RNAi expression vector technologies on the market: short hairpin (shRNA) expression vectors and artificial microRNA (miRNA) expression vectors. Most RNAi vectors available today employ shRNA vector technology, which typically involves shRNA expression from a Pol III promoter and may or may not employ viral delivery. These vectors express shRNA sequences, typically from a U6 or H1 promoter, and some have inducible promoters (typically H1/TO, a tetracycline-inducible promoter). While these vectors can be used for *in vivo* RNAi experiments, there are some drawbacks, including low design success rate and inability to track shRNA expression or express the shRNA in a specific target tissue.

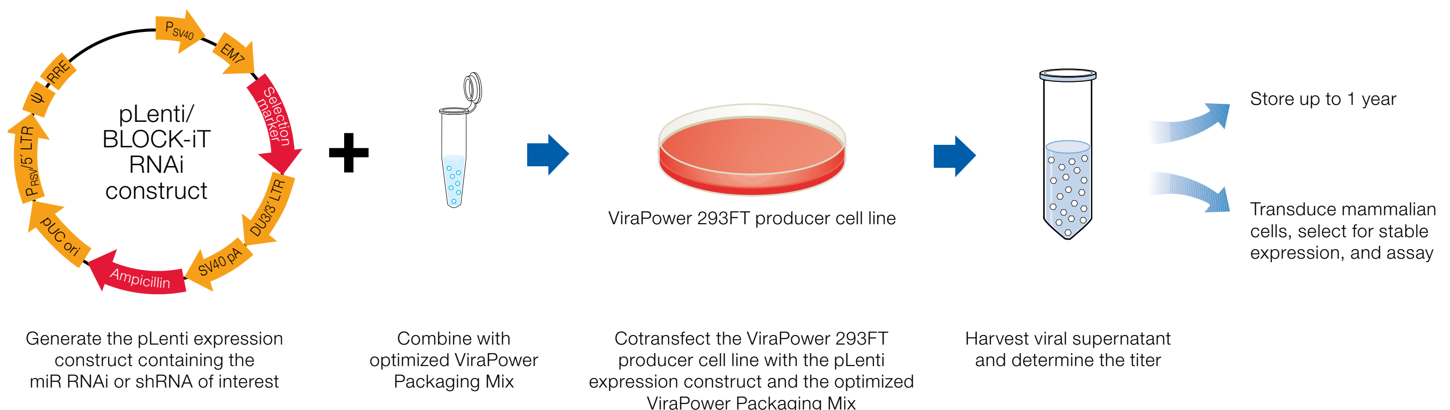


Figure 4.3. How the BLOCK-iT lentiviral RNAi systems work.

Chapter 5
In vivo RNAi

RNAi molecules for *in vivo* applications

The obstacles and challenges for *in vivo* RNAi delivery are very different from those in *in vitro* settings. To achieve successful knockdown, *in vivo* siRNA has to survive opsonization and degradation by nucleases, target particular cells, and traffic into the appropriate cell compartment. This chapter is set up to provide guidelines and protocols enabling successful *in vivo* RNAi experiments.

Choosing RNAi effector molecules

siRNA vs. RNAi vectors

RNAi can be delivered using two different approaches: siRNA synthetic duplexes, or siRNA expressed from plasmids or viral vectors (shRNA, miR RNAi). siRNA is becoming the method of choice for the fast development of therapeutics. It is relatively straightforward to design, synthesize, and screen a number of candidates. RNAi vectors offer steadier expression because the vectors can target nondividing stem cells, lymphocytes, and neurons. The drawbacks of RNAi vectors include danger of oncogenic transformation from insertional mutagenesis, and unanticipated toxicity from long-term silencing of human genes and/or high amounts of expressed siRNA inside the cell [1].

Chemically modified vs. unmodified siRNAs

Delivered *in vivo*, standard siRNA is rapidly degraded and cleared from plasma with a half-life of minutes [2]. Chemical modifications that prolong siRNA half-life, without jeopardizing biological activity, are highly desirable for success *in vivo*. We offer two suitable siRNA product lines for *in vivo* use: Invitrogen™ Stealth RNAi™ siRNA and Invitrogen™ Ambion™ In Vivo siRNA. Both of these product lines offer high stability in serum for longer-lasting knockdown effects in cells (Figure 5.1). See Chapter 7 for more details.

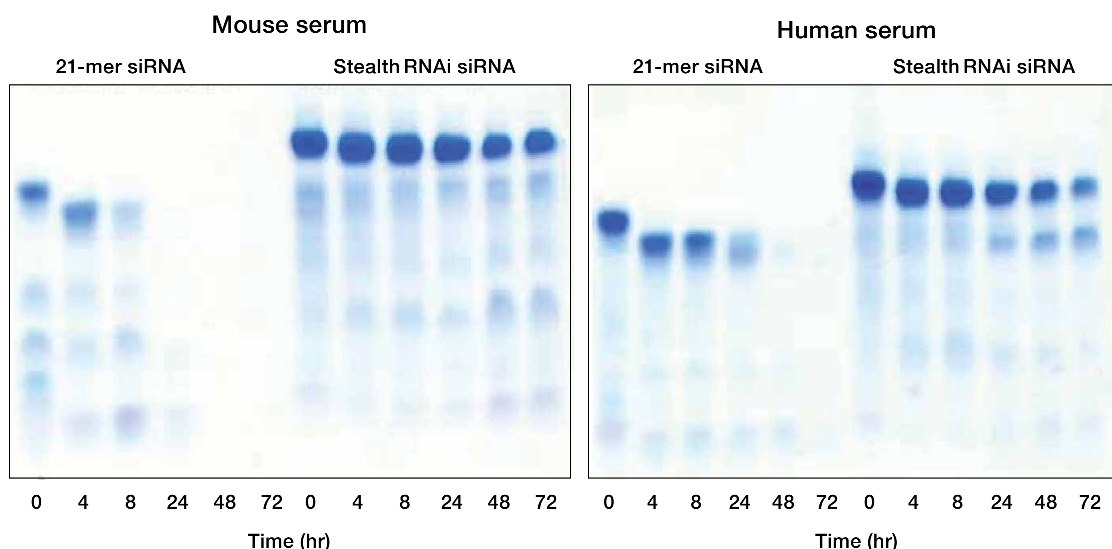


Figure 5.1. Chemical modification makes Stealth RNAi siRNA ideal for *in vivo* applications. Stealth RNAi siRNA duplexes are chemically modified to enhance stability against nucleases in serum. Unmodified 21-mer dsRNA sequences and corresponding Stealth RNAi siRNA sequences were analyzed at 0, 4, 8, 24, 48, and 72 hr following incubation in either 10% mouse or human serum. Samples were separated on a Invitrogen™ Novex™ 15% TBE-Urea polyacrylamide precast gel and stained with methylene blue.

RNAi purification options

The production of *in vivo* RNAi duplexes begins with standard synthesis of RNA oligonucleotides using high-quality starting materials (Figure 5.2). These single-stranded RNA oligos are then duplexed and desalted. At this point the researcher can also request HPLC purification to reduce the amount of non-full-length product. However, this step adds cost to the process and reduces yield. Subsequent *in vivo* purity processing of RNAi duplexes includes a series of dialysis and counterion exchange steps to remove toxic salts and solvents and lower the conductivity to match physiological conditions. The resulting high-quality duplexes are ready for *in vivo* use regardless of whether HPLC purification is requested upstream of this process:

1. **HPLC:** standard RNA oligo synthesis followed by HPLC purification

   Advantage: Improves overall purity and is compatible (and recommended) for siRNAs with any fluorophore or other modifications
   Disadvantage: Does not include extra salt and solvent removal

2. **Desalted, *in vivo* purity:** standard RNA oligo synthesis followed by diafiltration to remove salts and solvents to a level of <200 µS, and sterile filtration

   Advantage: Available in larger amounts (without custom order), offers important salt and solvent removal, most cost-effective
   Disadvantage: Cannot be carried out on siRNAs with fluorophore modifications

3. **HPLC, *in vivo* purity:** standard RNA oligo synthesis followed by HPLC purification, diafiltration to remove salts and solvents to a level of <200 µS, and sterile filtration

   Advantage: Highest purity available
   Disadvantage: Least cost-effective due to lower yields

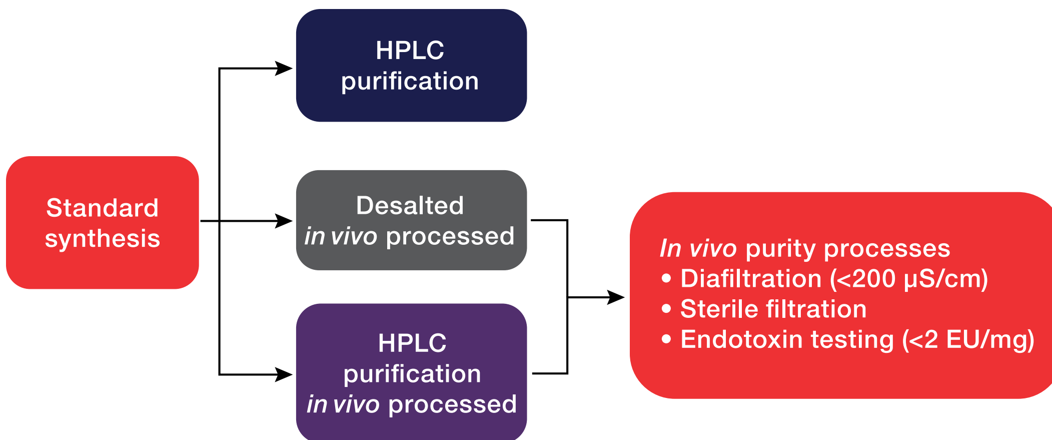


Figure 5.2. Invitrogen products produce high-quality *in vivo* RNAi duplexes at three purity levels.

RNAi vector *in vivo* delivery methods

Similar to RNAi vectors for *in vitro* applications, you can use either standard transfection techniques or a viral delivery method to deliver RNAi vectors *in vivo*. Currently, the delivery of an RNAi expression vector *in vivo* without using a viral delivery system is similar to delivering synthetic dsRNA *in vivo*. Typically, this would involve complexing the RNAi expression vector with a commonly used lipid-based *in vitro* transfection reagent and injecting directly into the animal. While this may be the easiest approach for delivering RNAi vectors into animals, it has quite a few limitations, including the inability to deliver systemically, and low transfection efficiencies. For these reasons, most researchers choose to use a viral delivery method when employing RNAi vectors for *in vivo* experiments. Regardless of whether one chooses an shRNA or miR RNAi vector system, viral delivery is a huge advantage for many *in vivo* approaches. Most viral delivery approaches involve either adenoviral, retroviral (nonlentiviral), or lentiviral technology:

- Adenovirus can be used for transient RNAi expression in either dividing or nondividing cells
- Retrovirus can be employed for transient or stable expression, but can only be used to transduce dividing cells
- Lentiviral delivery affords the most options, as it can be used for transient or stable expression in dividing or nondividing cells, as well as neuronal cells, drug- or growth-arrested cells, or even primary cells

In vivo RNAi protocols

Refer to the following supplemental protocols for special considerations for *in vivo* experiments. For questions about a protocol, please contact **techsupport@thermofisher.com**

Protocols for successful *in vivo* RNAi experiments:

- Resuspension of siRNA for *in vivo* applications
- Measuring RNA concentration
- Tissue harvest/RNA extraction

For protocols and further recommendations, visit **thermofisher.com/invivornai**

Resuspension of siRNA for *in vivo* applications

In vivo–purity Stealth RNAi siRNA and Ambion *In Vivo* siRNA duplexes are specifically formulated for use in animals. Resuspend the RNA duplex in Invitrogen™ UltraPure™ DNase/RNase-free Distilled Water or appropriate DNase/RNase-free buffer (e.g., PBS, Ringer's solution, 0.9% NaCl). A 5 mg/mL stock solution is recommended for *in vivo* RNAi experiments. Tables 5.1 and 5.2 specify the recommended resuspension volume for *in vivo*–purity Stealth RNAi siRNA and Ambion *In Vivo* siRNA.

Table 5.1. Desalted *in vivo* purity: recommended resuspension volume for 5 mg/mL final concentration.

Desalted *in vivo* quantity	Stealth RNAi siRNA resuspension volume	Ambion *In Vivo* siRNA resuspension volume
25 nmol	80 µL	67 µL
100 nmol	320 µL	260 µL
2 µmol	6.4 mL	5.4 mL

Table 5.2. HPLC purity: recommended resuspension volume for 5 mg/mL final concentration.

HPLC-purified delivered quantity	Stealth RNAi siRNA resuspension volume	Ambion *In Vivo* siRNA resuspension volume
5 nmol	16 µL	13 µL
20 nmol	64 µL	53 µL
500 µmol	1.6 mL	1.3 mL

Measuring RNA concentration

If desired, measure RNA concentration using UV absorbance at 260 nm (A_{260}). Dilute the RNA solution in resuspension buffer or water, and mix well. Measure the A_{260} of the dilution in a spectrophotometer blanked against dilution buffer (using a cuvette with a 1 cm optical path length). Calculate the RNA concentration using the appropriate formula:

Stealth RNAi siRNA formula

RNA concentration (µg/mL) = A_{260} (OD_{260} units) x 44 ((µg/mL)/OD unit) x dilution factor

Ambion *In Vivo* siRNA formula

RNA concentration (µg/mL) = A_{260} (OD_{260} units) x 41 ((µg/mL)/OD unit) x dilution factor

Note: The formulas for Stealth RNAi siRNA and Ambion *In Vivo* siRNA are slightly different due to chemical modifications and length differences.

Harvesting tissue—RNA extraction from tissue

1. Homogenize 50–100 mg of tissue in 1 mL Invitrogen™ TRIzol™ Plus Reagent using lysing matrix D on the FastPrep™-24 Instrument (MP Biomedicals) at 4°C. A tissue homogenizer or rotor stator can also be used.

2. For harder tissues (e.g., tumors, lungs), perform 3 cycles of 60 seconds each at 6 m/sec using a tissue homogenizer or rotor stator. For softer tissues (e.g., brain, liver), 1 cycle of 60 sec at 4.5 m/sec is sufficient to completely dissociate the tissue. Add 0.2 mL of chloroform directly into the tube and process following the protocol described in the Invitrogen™ PureLink™ RNA Mini Kit Purification System manual.

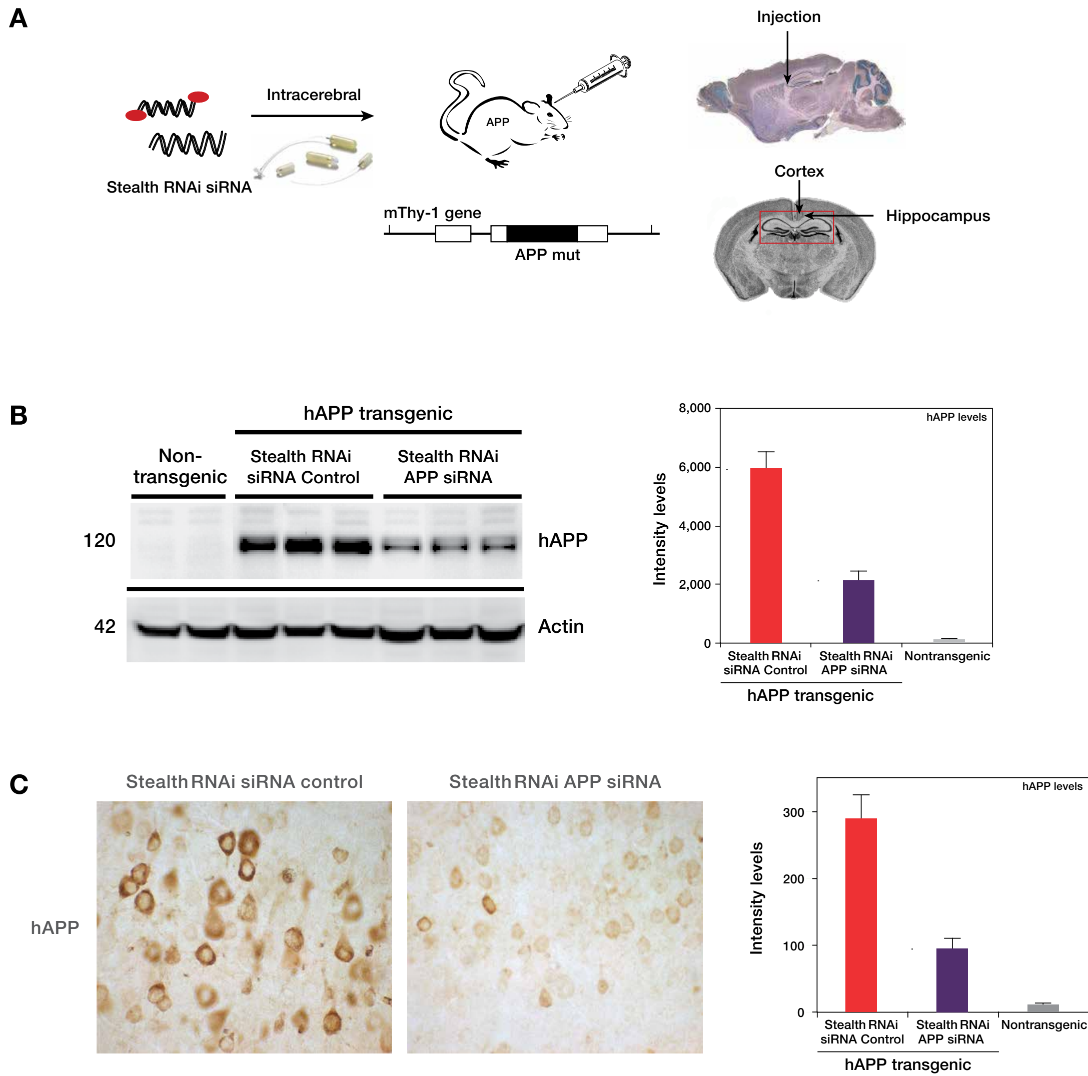
3. Determine the quantity and purity of the purified RNA using UV absorbance at 260 nm or Invitrogen™ Quant-iT™ RNA Assay Kit (Cat. No. Q33140). To determine RNA quality, electrophorese on an Invitrogen™ E-Gel™ agarose gel or analyze on the Agilent 2100 Bioanalyzer™ instrument.

4. After quantification, use 750 ng of total RNA for first-strand synthesis using the Invitrogen™ SuperScript™ VILO™ Kit and measure knockdown by qPCR analysis using Applied Biosystems™ TaqMan® Gene Expression Assays.

Special note and disclaimer: Literature describing *in vivo* delivery of siRNA and modified siRNA has become more abundant. However, the applications and methods described often vary. To the extent Thermo Fisher provides general guidelines for using siRNA in animals, the company makes no guarantees concerning use of these products or guidelines in animal studies.

FEATURED STUDY

Stealth RNAi siRNA *in vivo* delivery to mouse brain as a model of Alzheimer's disease



Effective knockdown achieved in an Alzheimer's disease model using Stealth RNAi siRNA. (A) Experimental design: A transgenic mouse model for Alzheimer's disease (hAPP Tg) overexpressing the *huAPP* gene carrying London and Swedish mutations under the mThy1 promoter was used. A cannula was inserted into the lateral ventricle of the brain. Invitrogen™ Stealth RNAi™ APP siRNA solution (4.75 mg/mL in PBS) was infused via cannula at 6 µL/day for 2 weeks in the lateral ventricle using an osmotic mini pump. **(B)** Brain homogenates from Stealth RNAi siRNA–treated mice were separated into cytosolic and particulate fractions as described in Rockenstein et al. [3]. Western blots were probed with a mouse monoclonal anti-hAPP antibody, screened, and analyzed. **(C)** The fixed brains were serially sectioned at 40 µm, and sections were immunolabeled with anti-hAPP antibody, followed by incubation with a FITC-labeled secondary antibody, and imaged with a Quantimet™ 570C system (Leica) or by laser scanning confocal microscopy (LSCM). APP protein level is reduced in the neocortex treated with the Stealth RNAi APP siRNA but not when treated with the Stealth RNAi™ siRNA Control.

Frequently asked questions about *in vivo* RNAi

For answers to additional questions, please contact **techsupport@thermofisher.com**

Should I use chemically modified duplexes for my *in vivo* RNAi experiments?

Chemically modified siRNA duplexes have a number of advantages over standard RNAi duplexes, including reduction of off-target effects, enhanced stability, and reduced toxicity. For these reasons, Ambion *In Vivo* siRNA and Stealth RNAi chemically modified RNAi duplexes are recommended for *in vivo* RNAi experiments.

I am planning on using siRNA for my *in vivo* experiments. What purity should they be?

The production of Ambion *in vivo* RNAi duplexes begins with standard synthesis of RNAi oligos using high-quality starting materials. The RNA oligos are then duplexed and desalted. At this point, you can also request HPLC purification, prior to further *in vivo* purity processing. However, this step increases cost and reduces yield. Subsequent *in vivo* purity processing includes a series of dialysis and counterion exchange steps to remove toxic salts and solvents from the RNA duplex and to lower the conductivity to physiological conditions. The resulting high-quality duplexes are ready for *in vivo* use regardless of whether HPLC purification is requested upstream of this process.

Should I use siRNA or vectors for *in vivo* RNAi?

RNAi can be delivered using two different approaches: siRNA synthetic duplexes or siRNA expressed from plasmids or viral vectors (shRNA, miRNAi). siRNAs are the method of choice for the rapid development of therapeutics. They are easy to design, easy to synthesize, and easy to use. siRNAs can be rapidly identified and multiple genes can be targeted at the same time. With RNAi vectors, the expression will be steadier as a result of the potential for the plasmid to stably integrate into the genome. Additionally, RNAi vectors are able to target nondividing cells such as stem cells, lymphocytes, and neurons. The drawbacks include the danger of oncogenic transformation from insertional mutagenesis, and unanticipated toxicity from long-term silencing of human genes and/or the expression of high amounts of siRNA inside the cell [1].

How should I deliver my *in vivo* RNAi molecules?

Several different approaches have been used for siRNA delivery, including various local delivery techniques and systemic delivery. Invitrogen™ Invivofectamine™ 3.0 Transfection Reagent is ideally suited for *in vivo* delivery of siRNA and microRNA duplexes. Invivofectamine 3.0 reagent–RNA complexes are delivered using a low-volume, low-pressure method with no toxicity and no stress response in the animal. Invivofectamine 3.0 reagent can also be combined with Invitrogen™ BLOCK-iT™ fluorescent controls to track the biodistribution of the siRNA or microRNA.

How many micrograms or milligrams of the nanomole quantities of Invitrogen siRNA duplexes are delivered for *in vivo* RNAi?

Table 5.3 provides quantity of siRNA in micrograms (µg) and milligrams (mg).

I want to order larger scales than the standard offering. Whom do I contact?

Please contact **RNAiSupport@thermofisher.com** regarding large-scale *in vivo* RNAi.

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Table 5.3. Quantities delivered, in micrograms (µg) and milligrams (mg).

			Ambion *In Vivo* siRNA		Stealth RNAi siRNA	
Purity	**Quantity**	**µmol**	**µg**	**mg**	**µg**	**mg**
HPLC	5 nmol	0.005	67	0.067	81	0.081
	20 nmol	0.02	269	0.269	322	0.322
	500 nmol	0.5	6,731	6.731	8,050	8.050
Desalt—*in vivo* purity	25 nmol	0.025	337	0.337	403	0.403
	100 nmol	0.1	1,346	1.346	1,610	1.610
	2 µmol	2	26,922	26.922	32,200	32.200
HPLC—*in vivo* purity	5 nmol	0.005	67	0.067	81	0.081
	20 nmol	0.02	269	0.269	322	0.322
	500 nmol	0.5	6,731	6.731	8,050	8.050

Chapter 6

siRNA screening

Selecting an siRNA library

Customer insights: What is important when selecting an siRNA library?

Data generated from a large-scale siRNA screening experiment are invaluable when searching for gene targets involved in a particular biological process, pathway, or network. However, obtaining a large-scale siRNA library and preparing to run the first screen can involve a significant investment. Many factors should be considered before initial library purchase, including the quality of the reagents, formatting needs, validation strategy, and support. We interviewed scientists from siRNA screening laboratories, and here we provide a summary of the factors they recommend you should consider before purchasing an siRNA library.

Key consideration: siRNA design and performance

Large-scale siRNA screens can generate hundreds of potential "hits"—genes for which one or more siRNAs give a desired phenotype. Unfortunately, in early screens with unmodified siRNA technologies and pooled siRNA approaches, many of these hits actually result from off-target effects. Off-target effects can result from poor siRNA design, using an siRNA technology that is not designed to reduce off-target effects, or using siRNAs with limited potency, which requires delivery of a large amount of siRNA to observe gene target knockdown. "The first concern is the quality of the algorithm that is used to design the siRNAs," says Anand Ganesan, MD, PhD, Assistant Professor, Department of Dermatology and Biological Chemistry at the University of California, Irvine. "Secondly, the number of siRNAs that have been validated either by the company or as a part of other studies should be considered." Invitrogen™ *Silencer*™ Select siRNAs incorporate the latest improvements in siRNA design, off-target effect prediction algorithms, and chemistry. As a result, they enable unrivaled silencing consistency, potency, and specificity, and fewer failed experiments, allowing cleaner, more consistent phenotypic data (Figures 6.1 and 6.2).

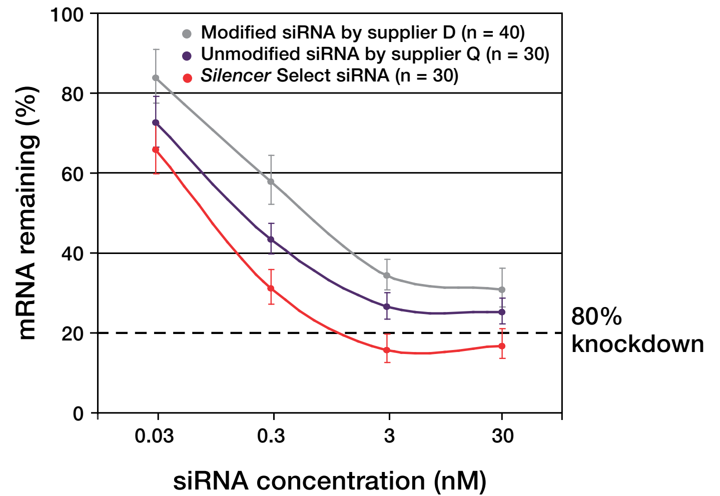

Figure 6.1. *Silencer* Select siRNAs provide up to 100x higher potency compared to other siRNAs. *Silencer* Select siRNAs for 10 different targets and siRNAs from two other suppliers for the same 10 different targets were individually transfected into HeLa cells in triplicate at the indicated siRNA concentrations. mRNA knockdown levels were tested 48 hr later as described in Figure 6.2. Average percent mRNA remaining is shown for each set of siRNAs.

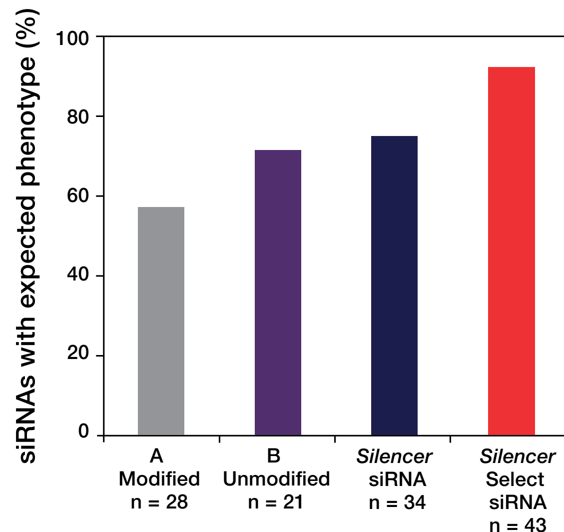

Figure 6.2. *Silencer* Select siRNAs elicit expected phenotype at a higher rate than other siRNAs. siRNAs for 7 gene targets with well-understood RNAi-induced phenotypes were individually transfected at 3 nM, and phenotypes were measured 48 hr later. Each bar represents the percent of siRNAs that gave the expected silenced phenotype. siRNAs to *BUB1B*, *AURKB*, *WEE1*, and *PLK1* were assessed using a multiparametric cell growth/apoptosis assay in U2OS human osteosarcoma cells. siRNAs to *HMGCR*, *LDLR*, and *FDFT1* were assessed using an LDL uptake assay in HUH7 human hepatoma cells.

Silencer Select siRNAs are designed with a newly developed algorithm and include locked nucleic acid (LNA) chemical modifications, making them the best-performing siRNAs commercially available. Experiments designed to assess levels of knockdown and observed phenotypes show that LNA-modified *Silencer* Select siRNAs consistently demonstrate the highest levels of knockdown and the least off-target effects [1]. To date, more than 4,300 *Silencer* Select siRNAs targeted to the most popular gene targets have been bench tested and validated at a concentration of 5 nM to provide ≥80% target mRNA knockdown as measured by qRT-PCR with Applied Biosystems™ TaqMan® Gene Expression Assays. This extensive level of validation provides enhanced confidence in both the *Silencer* Select siRNA design algorithm and the siRNA library's overall performance.

Key consideration: siRNA quality

Design and structure of an siRNA are important; however, siRNA manufacturing quality is equally critical. Cellular viability is a concern in screening experiments, so it is important that any contaminants that result from the production of siRNA be at extremely low levels. *Silencer* Select siRNAs are manufactured under stringent control and are subjected to rigorous quality control procedures to ensure lot-to-lot consistency. Each and every strand is subjected to MALDI-TOF mass spectrometry, and each annealed strand is tested by nondenaturing PAGE to ensure efficient annealing. The average purity of "standard purity" siRNA strands is approximately 90% full-length product.

Key consideration: completeness of the library

All screening scientists agree that a complete siRNA library is crucial to success. The library should contain siRNAs to as many relevant genes as possible, and most researchers prefer to err on the side of screening too many, rather than too few targets. The *Silencer* Select siRNA algorithm has been used to generate siRNAs to the human, rat, and mouse genomes. An siRNA library for 21,585 unique genes within the human genome is available, along with predefined siRNAs sets for a number of important human gene families that include kinases, phosphatases, GPCRs, and several others. Although defined sets are very useful, researchers may prefer to screen their own customized collection of targets. To that end, Thermo Fisher can provide a custom library of *Silencer* Select siRNAs targeting your genes of interest.

Silencer Select siRNA libraries are available at scales of 0.1, 0.25, 1.0, 2.0, and 5.0 nmol per well. Custom aliquotting requests of all types are easily handled, and we can supply siRNAs in your desired plate layouts to match your transfection and cell-based assay protocols.

Key consideration: validation

Once hits from a library screen are identified, validation is a crucial step prior to drawing conclusions. Typically, hits are validated by repeating the experiment with the same siRNA sequences, using a secondary assay or additional siRNA sequences, or using qRT-PCR, often followed by looking at protein levels to assess target knockdown.

Keys to successful validation are having a defined strategy up front and having a streamlined method for obtaining additional siRNAs for validation purposes. We can help you define an appropriate follow-up and hit validation strategy. Custom sets of *Silencer* Select siRNAs are available with as little as 0.1 nmol siRNA per well—useful for focused follow-up studies of primary screen hits. Of course, following up on large numbers of false-positive results can easily drain your hit validation budget. By performing the primary screen with *Silencer* Select siRNAs, which reduce off-target effects by up to 90%, and by purchasing the lowest amount of siRNA you need, you can keep follow-up siRNA costs to a minimum.

Key consideration: communication and support

We realize that purchasing the initial siRNA library, setting up, performing, and validating an siRNA screen can be a large endeavor. To that end, we offer free consultations on your siRNA screening project every step of the way. Our scientists and award-winning technical support staff welcome the opportunity to support you in your siRNA experiments.

For more information about *Silencer* Select siRNA libraries, please go to **thermofisher.com/sirnalibraries**

Screening with siRNA libraries

Advantages

RNAi is currently the easiest and most cost-effective reverse genetics tool for studying gene function. Plate-based and cell-based assays, including higher content multiparameter assays and the availability of libraries of effective siRNAs, allow researchers to quickly evaluate dozens to thousands of genes for their role in cellular processes.

Challenges

While thousands of RNAi screens using siRNA libraries have been successfully carried out, there are still some perceived limitations to adoption of this approach. One is the perception that gene silencing may not be sufficient to identify hits. Keep in mind that RNAi facilitates gene knockdown, not gene knockout; i.e., target gene expression is almost never completely extinguished. Whether the level of knockdown is sufficient to generate a positive outcome in the screening assay (rendering expression rate-limiting) will depend on the endogenous expression level of the gene, protein product's activity and half-life within the cell, and redundancy of specific protein function within that biological pathway. Some proteins may require very little activity to affect a biological process, and are, in general endogenously expressed at much higher levels than biologically necessary. Thus, their residual activity after RNAi knockdown could still be sufficient to fulfill their cellular role. It might therefore be difficult, for example, to achieve sufficient gene knockdown to extinguish the effects of certain highly active proteins.

A second limitation is the possibility of off-target silencing causing false positives or simply confounding the assay results. It is an expensive proposition to follow up on hits that turn out to be irrelevant in the biology of interest. While it is impossible to assure 100% specificity to the target, there are siRNA chemical modification approaches, such as with *Silencer* Select siRNA, that can greatly reduce off-targets to improve the specificity of the siRNA to its intended target. Additional experimental approaches will be discussed in this chapter, including use of multiple, individual siRNAs per gene, that can reduce the role of potential off-targets in siRNA screen analysis.

Definitions

siRNA libraries

Sets of siRNAs, usually provided individually in 96-well or 384-well plates. siRNA libraries may target any number of genes, e.g., an entire genome, a gene family, a biological pathway, or a custom set of genes.

Hits

For this application guide, a "hit" is defined as a gene that triggers the phenotype being assayed in an initial siRNA library screen when it is successfully targeted by an siRNA. In other words, hits (also called candidate or putative hits) are positive results from an siRNA library screen—they may result in a phenotype that is similar to the positive control in the screening assay. Ideally, candidate hits represent genes that directly or indirectly influence the cellular process under study. Confirmed hits are those that have been validated using 2–3 methods to corroborate the initial screening results.

Negatives

Genes for which none of the targeting siRNAs results in a hit.

False positives

siRNAs that give a positive result in the screening assay that is not confirmed by other distinct siRNAs targeting the same gene or by secondary assays. The false positive result may be due to off-target effects of the siRNA or to experimental error. siRNA screens should be designed to rule out all false positives.

False negatives

True hits that are not detected. These siRNAs give a negative signal; however, use of other distinct siRNAs targeting the same gene show that when this target is silenced it gives a positive assay result. False-negative results can cause you to overlook true positives and should be minimized.

Biological false negatives

These occur when the RNAi reagents cannot silence a target sufficiently to render it rate-limiting—thus, no phenotype is observed. For example, the target protein may have a long half-life, or extremely high activity, so that very little protein is needed to fulfill its function. There may also be redundancy of function in the pathway, such that the function of the silenced gene product is replaced by the product of a second gene. These types of false negatives are difficult if not impossible to avoid.

Technical false negatives

Caused by suboptimal screening reagents, conditions, or assay design. They can also result from siRNAs with suboptimal potency or not using enough siRNA to achieve sufficient knockdown for the phenotype to develop. While, of course, one would like to detect as many potential hits as possible, allowing for a higher rate of technical false negatives may be worth the significant decrease in overall costs and time required to perform the screen.

Goals

Design the siRNA screen for comprehensive coverage of the genes involved in the phenomenon being studied while minimizing the rate of technical false negatives (see Definitions on page 35). By careful design of experimental controls and assessment of results, the screen should also strive to eliminate false positives.

These goals are best attained through sequential screening passes, ideally using independent assays for each as false positives. The initial pass is focused on maximizing sensitivity of detection, and therefore should use conditions that favor maximal silencing (e.g., use multiple individual siRNAs per target at relatively high concentrations (30–50 nM)). Depending on the type of assay and the threshold used (usually 2–3 standard deviations from the baseline), a relatively high percentage of targeted genes may show a positive phenotype from at least one siRNA, warranting follow-up evaluation [2]. These candidate hits will, no doubt, include false positives, which will be filtered out using validation experiments.

siRNA library screening workflow (5 steps)

RNAi screening using an siRNA library involves the following five steps; they are briefly introduced here and are described in detail in later chapters.

Step 1. Experimental design

Step 2. Optimize the siRNA delivery method

Step 3. Develop and optimize an effective screening assay

Step 4. Perform screen and analyze data

Step 5. Validate screening results

Step 1. Experimental design

Among the most critical aspects of experimental design for siRNA screening are selection of siRNAs to screen and use as controls, the cell type(s) in which the experiment is to be performed, and the siRNA delivery strategy. These decisions should be based on the biological pathway under study. For instance, to look at oncogenesis, you may wish to screen an siRNA library for differential effects on cell proliferation in a cancer cell line compared to a noncancerous cell line. The choice of siRNA library will depend on the depth of the screen and the overall design of the experiment. Do you want to analyze every possible target, or just potentially druggable targets, such as kinases? siRNA libraries that target complete genomes (e.g., human, mouse, etc.), as well as sets of siRNAs that target specific gene families or biological pathways are available. Libraries can also be custom-designed; the possibilities are almost endless—it is up to each individual researcher to determine how best to approach the experimental problem at hand.

Equally important in choosing an appropriate set of siRNAs for your screen, is to use well-designed, efficacious siRNAs with a high probability of inducing successful knockdown. Effective siRNA design is key to avoid wasting both time and money. We use “intelligent” siRNA design algorithms and validate a selection of our siRNAs. Ultimately, the most important attribute of any algorithm is its performance when tested experimentally. Therefore an algorithm that has been proven using many siRNAs targeting many different endogenous transcripts will produce more effective libraries leading to more useful data. Additionally, algorithms that have been trained to predict the highest potency siRNAs will allow you to use siRNAs in your experiments at lower concentrations, resulting in more effective screening performance.

Even with extensive validation of an siRNA design algorithm, the vast majority of siRNAs in almost all siRNA libraries have not been experimentally proven to knock down their intended target. The use of multiple siRNAs per target is generally accepted to be the best approach [3]. Employing three or more distinct, individual siRNAs, and performing replicate transfections, provides statistical significance and significantly decreases both false positive and false negative rates as compared to screening with pools of siRNAs.

Another important part of the process of selecting siRNAs for screening is to choose appropriate positive and negative control siRNAs. Positive control siRNAs are necessary to prove that the siRNAs in the screen are delivered efficiently and can induce silencing in the experimental conditions used. Additional assay-specific positive control siRNAs are necessary to ensure that the screening assay yields sufficient signal for accurate quantitation of the data. Nontargeting, negative control siRNAs are necessary to control for nonspecific effects due to siRNA delivery and to set an appropriate baseline for assay readout. Finally, in screens that include a treatment to induce the phenotypic effect, untreated controls serve as a baseline for the overall experimental conditions.

Step 2. Optimize the siRNA delivery method

Screening siRNAs that target hundreds to thousands of genes requires conditions for high-throughput, efficient siRNA delivery. Using optimal siRNA delivery conditions eliminates the most common causes of failed gene silencing experiments. Because it is so important, we highly recommend investing the time to select the best siRNA delivery method and conditions for the cells that are to be used for siRNA library screening.

There are two basic methods employed for siRNA delivery: lipid-mediated transfection and electroporation. Lipid-mediated reverse transfection can be used to transfect up to 1,000 wells in less than an hour without the need for robotics [4]. With automated liquid handling, the pace can be even faster. Electroporation can also be used to deliver dozens to hundreds of siRNAs quickly when performed with a 96-well plate electroporation chamber.

The first step in optimizing siRNA delivery conditions for an RNAi screen is to identify a transfection reagent and plating conditions, or electroporation conditions, that maximize uptake of active siRNA while maintaining high cell viability. We find it useful to measure both siRNA-induced target knockdown and cell viability in cells transfected with 2–5 different transfection reagents or 5–10 electroporation conditions (e.g., varying cell concentration, voltage, pulse length, pulse number, siRNA concentration, etc.). Delivery can then be further optimized for the reagent or electroporation condition that worked best among the conditions tested. Choice of delivery method and its optimization will depend on the cell type used.

Step 3. Develop and optimize an effective screening assay

The results of siRNA library screens are typically evaluated using phenotypic assays, such as reporter gene assays, cell-based assays, plate-based assays, etc., rather than through direct monitoring of changes in target mRNA or protein levels. The success of an siRNA library screen depends greatly on the quality of the phenotypic assay used for screening. Thus, it is worthwhile to invest the time and effort required to create an assay with enough precision, signal-to-noise, and linear range to ensure identification of siRNAs that induce the desired phenotype in transfected cells.

The diversity of cell functions that can be characterized using siRNA libraries is limited only by the range of phenotypic assays that can be developed. Assays that are amenable to siRNA screening experiments range from microscopic assays that monitor cell size, cell cycle status, or antibody staining; to enzymatic assays that assess the turnover of a specific substrate in a cell lysate; to direct measurements of biomolecules or small molecules in lysates, on cells, or in medium.

Good quantitative assays will demonstrate a high signal-to-noise ratio—that is, they will exhibit a large difference in the results obtained from assay-specific positive controls and nontargeting negative controls (which may serve as negative controls for both the phenotypic assay and for silencing). Such assays will yield a wide range of results from siRNA screening experiments, and they will maximize the chance of identifying genes with an interesting phenotype when silenced. Maximizing the signal-to-noise ratio involves testing variables like assay time, assay components (e.g., the reporter), cell type, and length of time between transfection and assay.

Step 4. Perform screen and analyze data

With a robust phenotypic assay and optimized siRNA delivery conditions established, a library of individual siRNAs can be introduced into cells. Triplicate transfections for each siRNA provide enough data for reasonable statistical analysis. Use of three siRNAs per target helps to eliminate nonspecific effects caused by any single siRNA sequence (false positives) as well as any false negatives. Positive control and negative control siRNAs on each plate provide quantitative numbers to normalize data from different plates, and to provide the range of phenotypes that can be used to identify siRNAs that yield a positive effect, designated “candidate hits” (see Definitions on page 35). The data are collected and analyzed to identify candidate hits. In many cases, we recommend reserving this designation for target genes that exhibit the expected phenotype when transfected with two of the three individual siRNAs. This should eliminate false positives and false negatives [5]. Another approach is to follow up on all of the siRNAs to a given gene even when one gave a positive result. This approach requires additional follow-up, and few potential hits are confirmed; however, it is a good approach to avoid missing targets.

Candidate hits are essentially a series of genes identified by the initial screen to be potentially interesting and warrant further study. They are typically confirmed using an independent method to eliminate false positives and to ensure that only legitimate genes are evaluated further (Step 5).

Step 5: Validate screening results

Targets identified as candidate hits in the initial screen must be validated. This is done by a second and often, a third screening pass. These experiments will also typically shed light on the roles of the target genes in specific biological pathways. An obvious validation technique used in secondary screens is to show a direct link between the observed phenotype and target gene silencing by documenting a reduction in target mRNA or protein level. However, the specific goals of the library screening and the assay tools available will dictate the techniques used for validation. A major goal of the second screening pass is to retest all candidate genes to identify interesting hits and eliminate false positives. Since fewer genes are analyzed, these validation experiments may also serve to further refine the relevance of candidate hits with respect to the biological process of interest. It would not be uncommon for the second screen to eliminate up to 90% of candidate hits identified in the first pass.

Those genes confirmed as positive in the second screening pass can be subjected to a final third pass wherein, if not shown by previous experiments, the functional phenotypic assay is repeated in parallel with a direct measure of target gene silencing (usually by qRT-PCR or branched DNA assays), thus directly linking the two. Targets that have been validated by two or more screens in addition to the initial screening experiment are excellent candidates for further studies (e.g., *in vivo* studies) to help confirm data independently and expand upon results found in the published literature.

Cellular viability

When siRNAs are introduced into cells via transfection, unexpected and/or toxic effects might be observed in addition to the expected phenotypes involved in the screen.

Off-target effects are gene silencing effects on unintended targets through the RNAi mechanism. They could come from the guide (antisense) or passenger (sense) strand of siRNA. In the case of near-complete complementarity, the strands will induce cleavage of mRNA (act as siRNA), while in the case of limited homology, the strands cause translation inhibition (act as miRNA). The support vendor machine (SVM) algorithm used to design our *Silencer* Select siRNAs selects only the sequences that have little or no homology (<15 nt) to the transcriptome outside the intended mRNA target. Further, the passenger strand of siRNA is inactivated by chemical modifications. However, a high proportion of “off-target” transcripts silenced by siRNAs have been shown to have 3´-UTR sequence complementarity to the seed region of the siRNA, indicating the off-target effect is mediated through an miRNA pathway. Since it is impossible to eliminate all 7–8-base matches of siRNAs to the transcriptome, it is difficult to achieve absolute specificity, even when chemical modifications are introduced. Performing RNAi experiments with hyper-potent siRNAs at low concentrations (<5 nM) minimizes off-target effects.

Another problem can arise from cellular responses to introduction of foreign nucleic acid. Both non-immune and immune cells may be activated by long dsRNA, leading to the activation of cytoplasmic receptors such as the dsRNA-dependent protein kinase R (PKR) and the retinoic acid inducible gene-I (RIG-I). Once PKR is activated, it phosphorylates the eukaryotic translation initiation factor (EIF-2-α), leading to global suppression of protein synthesis and subsequent programmed cell death. PKR can also activate nuclear factor κB (NF-κB) with consequent induction of type-I IFN production. A family of 2959-oligoadenylate synthetases (2959-OAS) can also be activated by dsRNA. This leads to the activation of RNase L, which eventually triggers the nonspecific degradation of mRNA.

RIG-I is an intracellular dsRNA sensor capable of triggering IFN production. The RIG-I helicase recognizes blunt-ended siRNAs, leading to the activation of dsRNA signaling. RIG-I efficiently unwinds siRNAs containing blunt ends, and the efficiency in duplex unwinding is translated into downstream signaling to interferon regulatory factor 3 (IRF-3) and NF-κB activation. Therefore, introduction of long double-stranded RNA (>30 bp), especially blunt-ended siRNA, can induce severe antiviral responses in transfected cells. For these reasons, typical synthetic siRNAs are shorter, 19 bp in length, and contain 2 nt overhangs at 3´ ends to reduce cellular responses. In most cases, cellular responses to such molecules are minimal.

Mammalian immune cells also express a family of toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns including CpG motifs and viral dsRNA. For example, TLR7 and TLR8 were initially shown to mediate the recognition of RNA viruses [6]. TLR7, TLR8, and TLR9 are expressed in endosomes and require endosomal maturation for efficient signaling. siRNA recognition by TLR7, TLR8, and TLR9 results in activation of NF-kB and IRFs, which induce inflammatory cytokines and IFNs, respectively. TLR7 and TLR8 mediate the recognition of siRNAs in a sequence-dependent manner: preferentially U- and G-rich siRNAs are recognized. Recognition of siRNAs by TLRs takes place in the endosome, before the siRNAs enter the cytoplasm. Therefore, if siRNAs can enter the cytoplasm avoiding the endosome, they should bypass the activation of immune systems, but still mediate gene silencing.

Secondary screening: confirmation of candidates

The goal of the secondary screen is to confirm that the phenotype observed is a specific effect of siRNA-induced target knockdown and not an off-target effect dependent on the protocol or a reagent. Because the siRNA delivery conditions were optimized for a positive control siRNA, the siRNAs that target candidate hits should be further tested for silencing efficiency in secondary screens [7], using techniques described in Chapters 10 and 11. If there are a large number of candidate hits, optimization experiments may help prioritize which genes should be followed up first.

Confirming that phenotypes are due to reduced expression of the intended target

Test multiple siRNAs: To minimize false-positive and false-negative results, we recommend using two or more siRNAs that target distinct regions of each gene and delivering each siRNA in triplicate for the initial screening experiment. Although the number of siRNAs targeting the same gene required to confirm a candidate hit can be determined statistically by estimating the probability that any random siRNA from the library will score positively in the assay under the chosen conditions (i.e., the false-positive rate), most researchers consider an siRNA target a confirmed hit if at least two different siRNAs that target the candidate produce the same phenotype. If the initial screen was carried out using multiple individual siRNAs per target, you may want to prioritize your initial follow-up to include only genes for which at least two distinct siRNAs give a phenotype outside the set thresholds of the assay. Note that candidate hits where only one of the siRNAs gives the desired phenotype should not be ignored.

Reproduce the phenotype: For well-characterized target proteins, antibodies or small molecule inhibitors may exist and can be used to mimic or counteract the effect of the siRNA [8]. To confirm that a hit is associated with a specific pathway, additional phenotypes that are known to occur when the pathway of interest is perturbed (e.g., up- or downregulation of other genes, increase or decrease in protein modification), can be assayed after siRNA treatment [9,10]. These additional assays should be based on existing knowledge of the biological process. To further study complex cellular processes, some researchers have begun to develop automated, microscopy-based assays to simultaneously analyze fluorescent markers linked to antibodies or ligands to assess relative intensity and cellular localization over time [7,11].

Perform a rescue experiment: Functional validation of RNAi experiments is supported by successful siRNA-refractory rescue experiments. In these experiments, overexpression from cDNA constructs of target mRNAs that are not recognized by gene-specific siRNAs are used to restore the wild-type phenotype. For example, a cDNA with silent mutations in the siRNA target sequence is expected to rescue the phenotype caused by siRNA-induced reduction in endogenous gene expression. Alternatively, a phenotype caused by siRNAs that recognize 5′ or 3′ untranslated sequences can be rescued by expressing a cDNA of the target gene that contains only coding sequences [12]. Additionally, bacterial artificial chromosome transgenesis in cultured cells has been used to create RNAi-resistant transgenes [13].

Measure mRNA and protein levels: As mentioned above, confirmation of positive hits involves verifying that the observed phenotype is specifically due to reduced expression of the target gene. Whenever possible, determining siRNA-induced reduction of both mRNA and protein levels is recommended to help interpret your data. In both cases, target mRNA or corresponding protein levels should be assessed relative to those of samples treated with non-targeting negative control siRNAs.

If the siRNA induces a positive result in the assay, but the target mRNA levels are not appreciably reduced, it is possible that the observed phenotype is caused by an off-target effect. In this scenario, the corresponding protein levels may be reduced without observing a reduction in target mRNA levels. There are two possible reasons for this observation: the siRNA could be acting as a microRNA and inhibiting translation rather than targeting its cognate mRNA for destruction, or the RNA assay may not accurately measure target mRNA levels. An RNA assay may fail to accurately reflect mRNA knockdown if the target mRNA cleavage product is relatively stable or if the assay is designed incorrectly (for example, it does not measure one or more targeted splice variants or qRT-PCR normalization was inaccurate). In either case, it is a good idea to measure the reduction in target protein levels after siRNA delivery. If the siRNA induces a distinct phenotype, but knockdown is not observed at either mRNA or protein level, it is more likely that the phenotype is the result of an off-target effect.

One note of caution: we recommend that cell viability (i.e., cell number) be monitored in addition to siRNA-induced reduction of target gene expression levels in case the target gene is essential for cell survival. Low cell viability may complicate mRNA and protein measurements and lead to erroneous results. One way to study essential genes is to use reporter assays as described below. Alternatively, you could monitor the effects of siRNA treatment at shorter time points before significant cell death is observed in your cultures.

Assess mRNA levels: Assessing mRNA levels of the target gene is a direct way to monitor siRNA-induced gene silencing. Quantitative RT-PCR or qRT-PCR, northern blots, branched DNA assays, or microarray analyses can be used to measure mRNA levels. qRT-PCR is the most common method, in part because of its high sensitivity and speed; however, replicates and normalization controls need to be carefully assessed to ensure that slight changes in levels of gene expression are not missed.

Assess protein levels: Measuring protein levels offers another direct way to link reduced gene expression to the loss-of-function phenotype. Remember that the optimal time for measuring protein levels depends on the half-life of the protein. Also, the magnitude of the decrease in protein expression required to detect a loss-of-function phenotype will differ between proteins and may vary with cell cycle. As described in protein-based assays on page 92, there are many assays for quantitating protein levels. The choice of method depends on the specific target and availability of appropriate equipment, assays (e.g., protocols to measure enzyme activity or ion transport), or antibodies.

Reporter gene assays: Whenever possible, detection of endogenous protein levels is preferred because it provides a direct link to changes that occur in your model system. However, reporter assays, which monitor exogenous proteins (expression or enzyme activity), can be a useful alternative for validating candidate hits that have relatively low expression or no available antibodies.

For example, cotransfection of siRNA with plasmids expressing the target transcript may be helpful for studying low-abundance mRNA targets [12]. Because transfection conditions are often not the same for plasmids and siRNA, cell lines stably transfected with the plasmid may be required in some cases to test siRNA effects.

Reporter constructs facilitate the use of fluorescent (e.g., GFP or RFP) [3,13] or enzymatic (e.g., luciferase or β-galactosidase) assays to monitor changes in target gene expression. This approach can involve expression of a fusion protein containing reporter and target sequences [8]. Alternatively, the construct can be designed to generate a chimeric mRNA in which a translation stop codon separates the open reading frame of the reporter gene from the siRNA target sequence so that only the reporter gene is translated.

Instead of using reporter genes, another approach involves fusing epitope tags (e.g., glutathione-S-transferase, Myc, hemagglutinin, 6-histidine, or FLAG™ tags) to the target protein to enable detection with well-characterized commercially available antibodies [8,12,15].

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Section II—Products for RNA interference

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Chapter 7
siRNA technologies

Non-vector methods for transient RNAi

For transient knockdown experiments, synthetic siRNA duplexes offer significant advantages over vector-based methods for RNAi. In particular, synthetic siRNAs are easier to design and perform and typically result in higher levels of transient knockdown. In addition, improvements in RNAi design and application of chemical modification patterns have increased the likelihood of achieving high-level knockdown after testing only a few RNAi molecules. Consequently, using synthetic RNA duplexes is the most popular method for conducting RNAi experiments.

Concerns about nonspecific effects

Although the use of synthetic RNAi molecules is an easy, effective method of inhibiting gene expression, there is some concern about the specificity of these molecules. The introduction of some siRNA duplexes into mammalian cells can result in phenotypic changes unrelated to inhibition of the target gene, thereby generating false-positive results that are nonspecific. Nonspecific effects, which are due to regulation of unintended gene targets, are most commonly referred to as off-target effects, and may be due to sequence-specific motifs that generate aptamer-like effects, such as activation of stress response pathways. siRNA duplexes with partial homology to other targets may also contribute to off-target activity. Gene profiling experiments have shown that duplexes with partial homology to other transcripts can cleave the target or act like microRNA (miRNA) and inhibit translation [1-3]. Specificity studies have revealed that siRNA duplexes can have varying activities depending on the number, position, and base pair composition of mismatches with respect to the target RNA [4].

Unwanted interferon responses

It is generally accepted that siRNA duplexes with fewer than 30 base pairs evade recognition by the proteins mediating the mammalian antiviral response. However, mounting evidence suggests that some siRNAs can activate interferon and stress response pathways. In mammalian cells, double-stranded RNAs (dsRNAs) are recognized by dsRNA-binding proteins and Toll-like receptors, leading to global shutdown of protein synthesis and activation of the interferon response. Some siRNAs containing sequence-specific motifs that induce the interferon response have been identified [5,6]. Eliminating these sequence motifs during the siRNA design process is one strategy to avoid inducing stress response pathways. Nonetheless, other sequence motifs might exist, and methods to screen for inducing these motifs are largely uncharacterized.

Enhancing specificity

Advanced sequence design algorithms can be applied to reduce nonspecific effects due to off-target regulation or sequence-specific activation of stress response pathways. This solution requires a detailed understanding of the rules for designing highly precise duplexes. An alternative approach to enhancing specificity lies in the use of chemical modifications.

Silencer Select siRNAs

RNA interference, the biological mechanism by which double-stranded RNA (dsRNA) induces gene silencing by targeting complementary mRNA for degradation, is revolutionizing the way researchers study gene function. For the first time, scientists can quickly and easily reduce the expression of a particular gene in mammalian cell systems, often by 90% or greater, to analyze the effect of that gene on cellular function.

Proven siRNAs for *in vitro* applications

Invitrogen™ *Silencer*™ Select siRNAs are the best-performing siRNAs for *in vitro* studies and are available in a variety of formats, including preplated collections and custom libraries to simplify screening experiments.

Features of *Silencer* Select siRNAs

- 21-mer siRNAs (including 2 nt 3′ overhang) with enhanced chemical modifications and improved algorithm
- Specific knockdown—novel chemical modifications reduce off-target effects by up to 90%
- Effective knockdown—designed with a next-generation algorithm that typically improves prediction accuracy by more than 25%
- Reliable results—demonstrated improvements in consistency and reliability of phenotypic results
- Unparalleled potency—up to 100-fold more potent than currently available siRNAs
- High confidence—100% guaranteed to silence, the best guarantee in the industry

Cleaner, more consistent data

The *Silencer* Select siRNAs incorporate the latest improvements in siRNA design, off-target effect prediction algorithms, and chemistry. As a result, they allow unrivaled silencing consistency, potency, and specificity, and fewer failed experiments, enabling cleaner, more consistent phenotypic data.

Design algorithm improves siRNA effectiveness

Many siRNA design algorithms predict siRNAs that induce 70% target mRNA knockdown with only ~80% confidence. Many RNAi applications demand better efficiency. The *Silencer* Select siRNA design algorithm was developed using a powerful machine learning method. Performance data from thousands of siRNAs were analyzed to better understand the link between an siRNA's sequence, target location, and thermodynamic properties and its silencing efficiency (Figure 7.1). The result is more effective siRNAs (Figure 7.2).

The *Silencer* Select siRNA design algorithm

The design algorithm incorporates more than 90 different sequence and thermodynamic parameters to increase predictive accuracy by 28% over previous-generation siRNA design algorithms. The result is siRNAs that are up to 100-fold more potent than other siRNAs (modified and unmodified), allowing a higher percentage of "on-target" phenotypes.

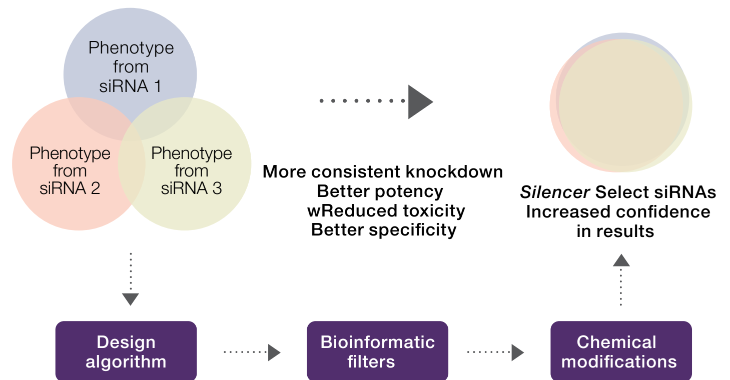


Figure 7.1. The concept behind *Silencer* Select siRNAs.

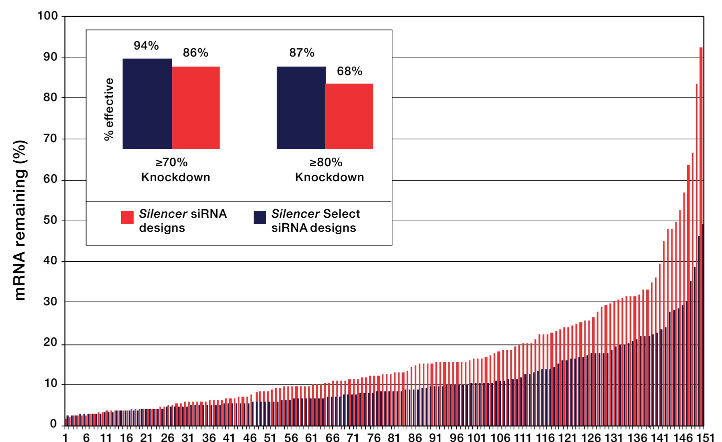


Figure 7.2. *Silencer* Select siRNA design algorithm significantly improves effective siRNA prediction accuracy. The *Silencer* Select siRNA design algorithm was used to design 155 siRNAs for 40 different targets. These siRNAs were tested side by side with siRNAs designed using the previous algorithm at 5 nM in HeLa cells. mRNA knockdown was measured 48 hr post-transfection via qRT-PCR using Applied Biosystems™ TaqMan® Gene Expression Assays. Results are expressed as percent of mRNA remaining compared to Invitrogen™ *Silencer*™ Negative Control No. 1 siRNA–treated cells. The inset shows the percentage of siRNAs that elicited ≥70% and ≥80% mRNA knockdown.

More potent knockdown, fewer off-target effects

Higher siRNA concentrations are known to increase off-target effects [1,7]. The need for more potent siRNAs that can be used at lower concentrations was a key consideration in the siRNA design algorithm improvement process. The *Silencer* Select siRNAs show increased potency and fewer off-target effects compared to first- and second-generation siRNA designs:

- Up to 100x more potent than competitor siRNAs (Figure 7.3)
- Can be routinely transfected at ≤5 nM and retain their silencing power
- Fewer off-target effects when used at these lower concentrations
- Reduced cost per experiment than siRNAs used at higher concentrations

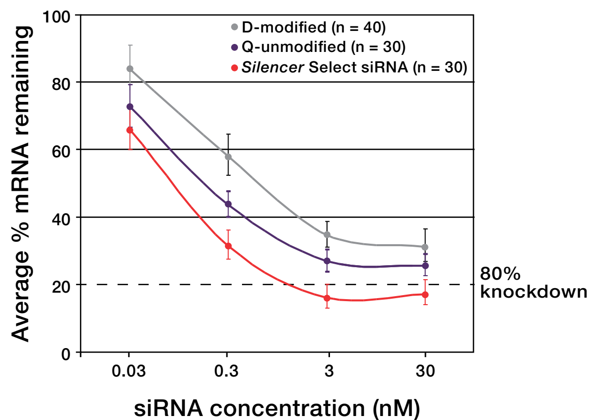


Figure 7.3. *Silencer* Select siRNAs provide up to 100x higher potency compared to other siRNAs. *Silencer* Select siRNAs to 10 different targets and siRNAs from two other suppliers to the same 10 different targets were individually transfected into HeLa cells in triplicate at the indicated siRNA concentration. mRNA knockdown levels were tested 48 hr later as described in Figure 7.4. Average percent mRNA remaining is shown for each set of siRNAs.

More consistent yield of silenced phenotypes

The main goal of an RNAi experiment is to examine the biological effect of knocking down a target of interest, often with a cell-based assay. However, to elicit that phenotype, some minimum threshold level of knockdown is required, and this threshold level will vary depending on the target. *Silencer* Select siRNAs:

- More reliably elicit maximum knockdown levels (Figure 7.4)
- More consistently reach the threshold level of knockdown required to see a loss-of-function phenotype
- In side-by-side tests, result in a higher percentage of expected, silenced phenotypes than siRNAs from other vendors

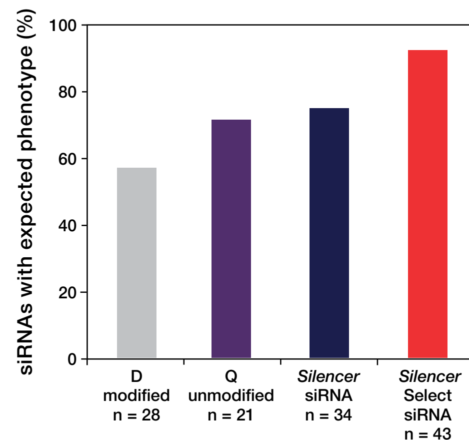


Figure 7.4. *Silencer* Select siRNAs elicit expected phenotype at a higher rate than other siRNAs. siRNAs to 7 gene targets with well-understood RNAi-induced phenotypes were individually transfected at 3 nM and phenotypes measured 48 hr later. Each bar represents the percentage of siRNAs that gave the expected, silenced phenotype. siRNAs to BUB1B, AURKB, WEE 1, and PL K1 were assessed using a multiparametric cell growth/apoptosis assay in U2OS human osteosarcoma cells. siRNAs to HMGCR, LDLR, and FDFT 1 were assessed using an LDL uptake assay in HUH7 human hepatoma cells.

Silencer Select siRNA knockdown guarantee

- Buy 2 *Silencer* Select Predesigned siRNAs to a protein-coding gene target, and both are guaranteed to knock down target gene expression by ≥70%
- *Silencer* Select Validated siRNAs are guaranteed to knock down by ≥80%

For details, go to **thermofisher.com/sirnaguarantee**

Bioinformatic filtering improves siRNA specificity

Although using siRNAs at low concentrations decreases off-target effects, additional specificity can be gained using bioinformatic filtering to predict and eliminate potentially "bad" siRNAs. The *Silencer* Select siRNA design includes a 5-step bioinformatic filtering process (Figure 7.5), which:

- Removes siRNAs with a high propensity for off-target effects
- Uses the Invitrogen™ *Silencer*™ Select siRNA Toxicity Classifier, which eliminates sequences predicted to elicit an off-target apoptotic phenotype
- Minimizes miRNA pathway–related off-target effects by removing siRNAs with seed regions that resemble naturally occurring miRNAs and selecting siRNAs with the fewest seed region matches in the 3′ UTRs of off-target transcripts

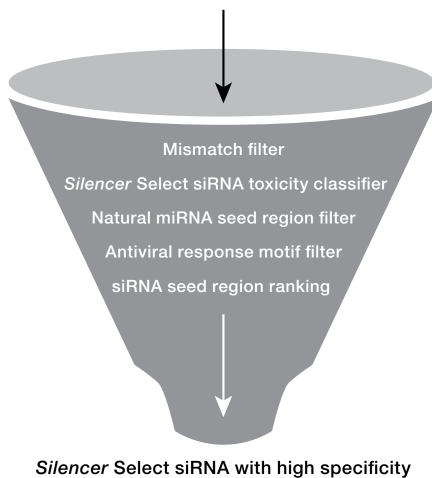


Figure 7.5. Five-step bioinformatic filtering process that eliminates siRNAs predicted to elicit off-target effects.

Chemical modifications enhance antisense strand bias

Strong antisense strand bias, where the antisense (guide) strand of the siRNA is preferentially taken up into the RISC over the sense (passenger) strand, is important both for maximizing siRNA silencing potency and for decreasing passenger strand–related off-target effects (Figure 7.6). Although incorporating the right siRNA design parameters can help, siRNA design alone is not sufficient to ensure strong guide strand bias. The *Silencer* Select siRNA chemical modifications:

- Consistently enhance guide strand bias, which correlates strongly to knockdown efficiency
- Prevent the passenger strand from inducing silencing, which serves to reduce off-target effects
- Result in no loss in siRNA silencing potency; in many cases an improvement is seen

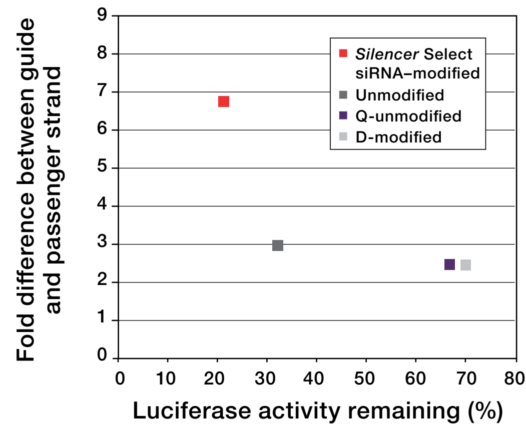


Figure 7.6. *Silencer* Select siRNAs show enhanced guide strand bias. Luciferase reporter gene constructs with siRNA targets cloned in either the sense (guide strand target) or antisense (passenger strand target) orientation were cotransfected with the corresponding siRNA and a β-galactosidase–encoding control vector. Luciferase and β-galactosidase assays were performed 72 hours later, and knockdown for each strand was calculated relative to negative control siRNA–transfected cells. The average ratio of guide strand/passenger strand knockdown is plotted against luciferase knockdown for 6 *Silencer* Select and 36 competitor siRNAs.

More consistent, reliable data

Sequence-specific off-target effects are one of the primary reasons for false-positive results in RNAi experiments. In addition to the potency improvements afforded by the advanced algorithm and state-of-the-art bioinformatic filtering criteria, *Silencer* Select siRNAs incorporate novel modifications that improve siRNA specificity, allowing cleaner, more consistent cell biology data. These modifications:

- Reduce the number of nontargeted, differentially expressed genes detected by gene expression array by up to 90% as compared to unmodified siRNAs (Figure 7.7)
- Result in a dramatic reduction of off-target phenotypes as measured by multiparametric cell-based assays (Figure 7.8)
- Do not negatively impact silencing efficiency and therefore do not compromise the expected on-target phenotypes

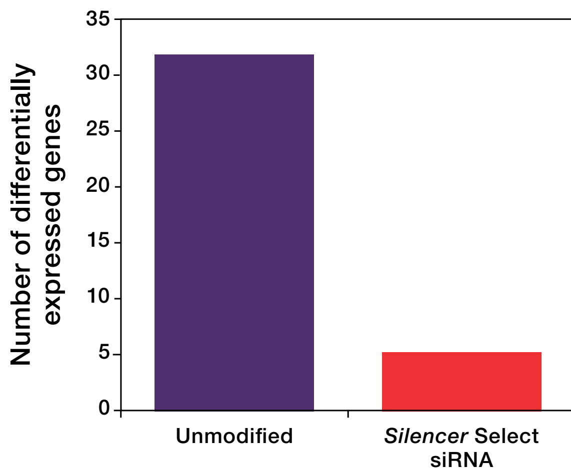


Figure 7.7. *Silencer* Select siRNA modifications reduce the number of differentially expressed genes (off-targets). Three negative control siRNAs without and with the *Silencer* Select siRNA modifications were individually transfected in quadruplicate into HeLa cells at 30 nM. RNA was extracted and analyzed on an Applied Biosystems™ GeneChip™ Human Genome U133 Plus 2.0 Array in triplicate. The y-axis indicates the average number of differentially expressed genes—those showing ≥2-fold change in expression compared to mock-transfected samples.

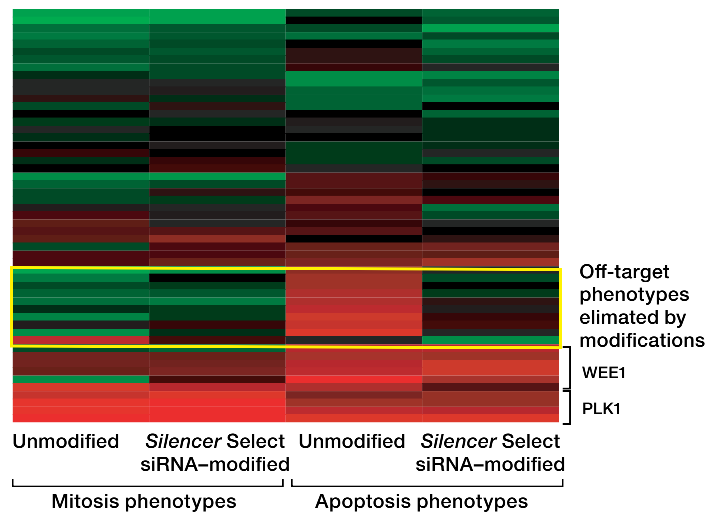


Figure 7.8. *Silencer* Select siRNA modifications reduce off-target effects and yield more reliable phenotypic data. 53 different siRNAs, including older designs previously noted to elicit off-target phenotypes, were transfected into U2OS cells at 30 nM in both unmodified and *Silencer* Select siRNA modified formats. Mitosis and apoptosis were measured 48 hours later. Data are expressed relative to negative control siRNA–transfected cells. Black = mitosis/apoptosis level similar to control. Green = downregulation. Red = upregulation. Note that the expected mitosis and apoptosis phenotypes for PLK and WEE1 siRNAs are preserved with the modifications. In contrast, the off-target apoptotic phenotypes elicited by 10 unmodified siRNAs were completely eliminated with addition of the *Silencer* Select siRNA modifications.

Silencer Select siRNAs for long noncoding RNAs

Long noncoding RNAs (lncRNAs) are a type of noncoding RNAs (ncRNAs), typically over 200 nt, that are abundant in the mammalian transcriptome. lncRNAs have been shown to regulate cellular functions including cell signaling, tumor progression, and metabolic regulation. Knockdown of lncRNAs with siRNA is a useful technique to determine the function and significance of a particular lncRNA. However, this can be challenging due to their cellular location and secondary structure. To help overcome this, Invitrogen™ *Silencer*™ Select lncRNA siRNAs for lncRNA include the following:

- Locked nucleic acid (LNA) technology to help ensure high specificity and reduce off-target effects
- *Silencer* Select technology that designs highly potent siRNA with low cell toxicity for more accurate results

Features of *Silencer* Select siRNAs targeting lncRNAs

- Comprehensive portfolio of *Silencer* Select siRNAs targeting over 5,000 lncRNA targets
- Validated using databases including Gencode v27 for optimal coverage of lncRNA loci, pseudogene loci, and alternatively spliced transcripts
- Predesigned siRNAs closely match existing noncoding TaqMan Assays

Tips for success

lncRNAs typically have lower expression levels compared to coding genes, and expression can vary across cell lines. Improve results for knockdown of lncRNA with these suggestions:

1. Verify expression levels in your cells of interest prior to your experiment.
2. Choose at least three siRNAs for each lncRNA of interest.
3. Use LNA-modified siRNA (e.g., *Silencer* Select siRNA) over unmodified or earlier-generation siRNA for greater potency and lower off-target effects.
4. Use a reliable transfection reagent such as Invitrogen™ Lipofectamine™ RNAiMAX Transfection Reagent for high-transfection efficiency and improved cell viability.

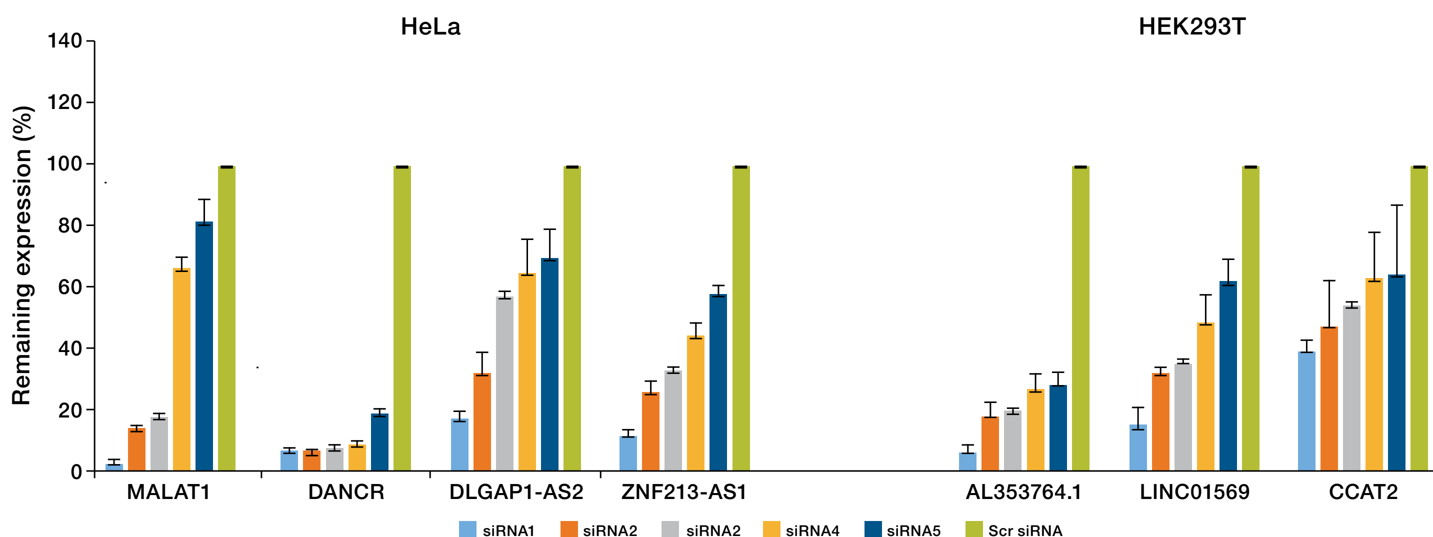


Figure 7.9. Five different siRNAs at a concentration of 25 nM were tested per gene. Transfections were done in duplicate, and each siRNA was delivered into 1 x 10^4 HeLa or HEK293T cells in a 96-well format using Lipofectamine RNAiMAX Transfection Reagent. Cells were harvested 48 hours post-transfection followed by cDNA synthesis and gene expression profiling using Applied Biosystems™ TaqMan® Gene Expression Cells-to-C_T Kit and TaqMan® Real-Time PCR Assays. TaqMan qPCR Assays were performed in triplicate, and remaining expression was normalized to a scrambled (Scr siRNA) negative control (Scr) siRNA transfected sample.

Efficient knockdown of lncRNAs using *Silencer* Select siRNA

Results of targeted knockdown in HeLa and HEK293T cells indicate that two or more siRNAs effected at least 50% knockdown, while at least 80% knockdown was obtained for six out of the seven targets (Figure 7.9). Knockdown was effective for lncRNA with nuclear or cytoplasmic localization. Please note that the *Silencer* Select siRNA performance guarantee is not extended to siRNAs designed to target lncRNA genes.

Cells maintain high cell viability post siRNA transfection

Transfection reagents alone or in combination with siRNAs may lead to a nonspecific decrease in cell viability. This may impact interpretation of knockdown results. Five different siRNAs against seven different lncRNAs were transfected into the indicated cells (Figure 7.10). No significant decrease in viability was detected.

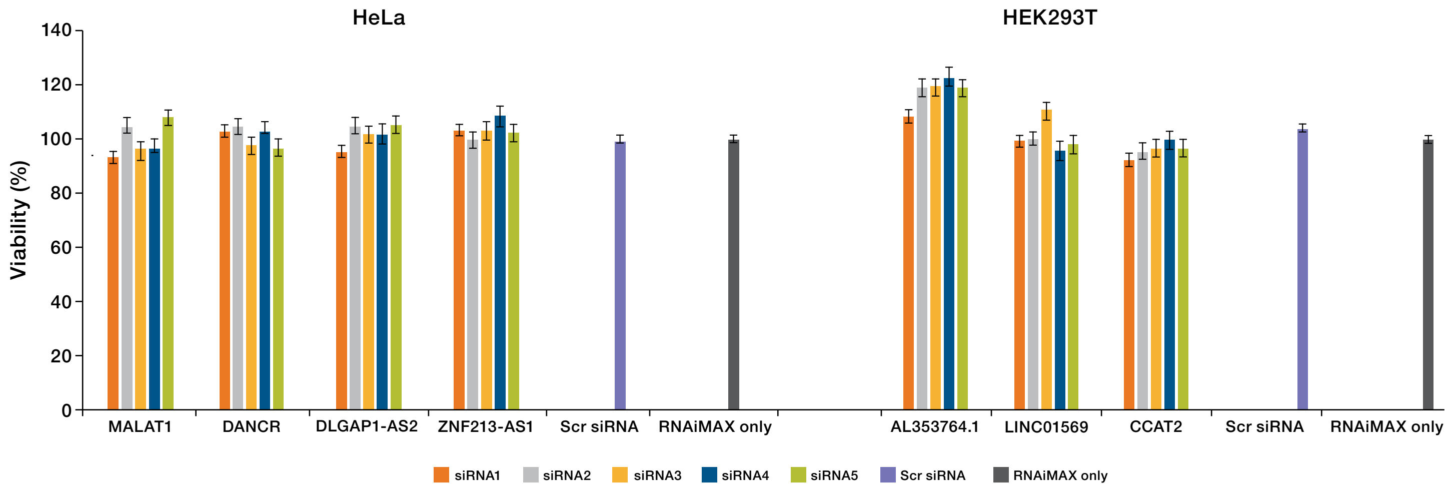


Figure 7.10. siRNAs at a concentration each of 25 nM were used to transfect 1 x 10^4 HeLa or HEK293T cells in a 96-well plate format using Lipofectamine RNAiMAX Transfection Reagent. Forty-eight hours post-transfection and prior to performing TaqMan Real-Time PCR Assay, cell viability was analyzed using the Invitrogen™ PrestoBlue™ Cell Viability assay. Percent cell viability was normalized to samples treated with Lipofectamine RNAiMAX transfection reagent without any siRNA.

Case study

Robust knockdown of a nuclear localized lncRNA, MALAT-1, with multiple *Silencer* Select siRNAs

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1) is a lncRNA expressed from chromosome 11 and is known to be mis-regulated in several human carcinomas. MALAT-1 lncRNA is expressed in the nucleus, and its overexpression is implicated in the development and progression of numerous malignant cancers.

MALAT-1 expression was confirmed in several common human cell lines (HeLa, A549, HEK293, Jurkat, U2OS, MCF7, and HuH7) at high levels using the MALAT-1 specific noncoding TaqMan Assay (Hs00273907_s1) in quantitative real-time PCR (qRT-PCR).

siRNAs designed against MALAT-1 were transfected into HeLa and A549 cells using Lipofectamine RNAiMAX Transfection Reagent. Knockdown was evaluated by real-time PCR. Our data from HeLa cells showed that the siRNAs are effective at 30 nM to silence MALAT-1 by 80% or greater, and this effect occurred as early as 24 hours post-transfection and persisted up to 5 days. The knockdown was further confirmed by northern blot analysis, which showed a strong reduction in MALAT-1 transcript levels that is consistent with the knockdown determination by qRT-PCR (Figure 7.11).

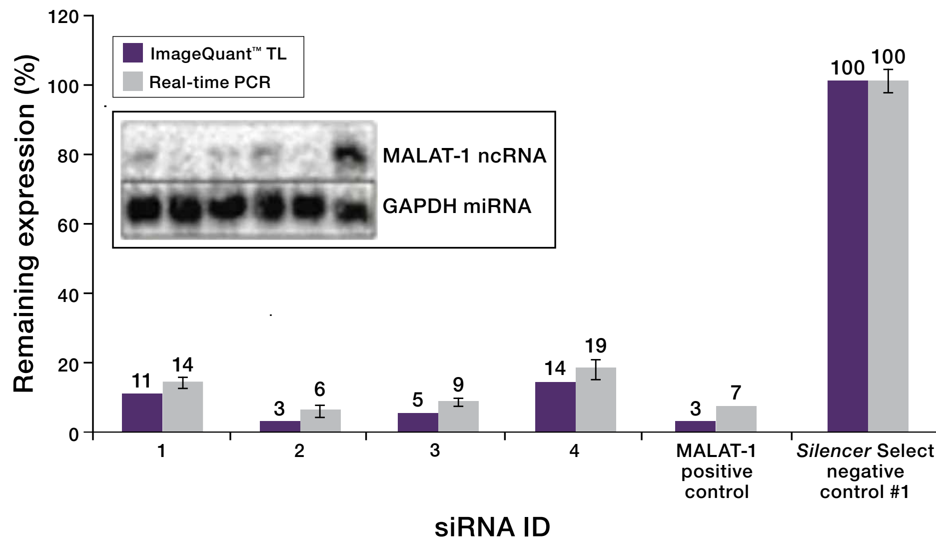


Figure 7.11. Robust knockdown of nuclear localized MALAT-1 ncRNA in HeLa cells by multiple *Silencer* Select siRNAs and confirmation of results by northern analysis.

Silencer Predesigned and Validated siRNAs

Traditional, unmodified siRNAs

For human, mouse, and rat:

- Cost-effective, unmodified siRNAs designed for human, mouse, and rat genes
- Guaranteed silencing
- Provided with free sequence information

Silencer **siRNAs are remarkably effective and guaranteed to silence**

Invitrogen™ *Silencer*™ Predesigned siRNAs—chemically synthesized, unmodified siRNAs available for human, mouse and rat genes—are designed with a rigorously tested siRNA design algorithm (see *Silencer* Select Predesigned siRNAs on page 43 for chemically modified siRNAs designed with an even more effective algorithm). Thermo Fisher guarantees that when three *Silencer* Predesigned siRNAs are obtained to the same target, at least two will reduce target mRNA levels by 70% or more.

Validated *Silencer* siRNAs are individual siRNA duplexes that have already been verified experimentally to reduce the expression of their individual target genes. Each siRNA was designed using the same effective algorithm used to design *Silencer* Predesigned siRNAs. However, each one has also been functionally proven and is guaranteed to reduce target gene expression by at least 70% 48 hours post-transfection.

High-quality synthesis

We synthesize and purify each siRNA in state-of-the-art facilities to meet the highest quality standards. As part of our rigorous quality control procedures, we analyze the mass of each RNA oligonucleotide by MALDI-TOF mass spectrometry and assess the purity of all HPLC-purified oligonucleotides by HPLC. Finally, we analyze each annealed siRNA by gel electrophoresis to confirm that the strands annealed properly. The result is premium quality siRNA that is purified and ready to use. See page 26 for a description of siRNA purity grades.

Silencer **siRNAs are easy to order**

The searchable, online Invitrogen™ siRNA database makes it easy to obtain effective, guaranteed-to-work siRNAs. Simply visit **thermofisher.com/siRNA** to find siRNAs for your human, mouse, or rat gene of interest.

Effective gene silencing for *in vitro* or *in vivo** RNAi applications

- **High stability against nucleases**—duplexes reach their target intact and ready to initiate knockdown
- **No induction of the interferon response**—correlate phenotypes with knockdown activity instead of toxicity
- **Easy tracking of administered RNAi duplexes**—measure biodistribution effectively
- **Knockdown effectiveness and stability**—no need to compromise one for the other

In vivo RNAi experiments are more challenging than their *in vitro* counterparts due to the demands of the cellular environment. These added challenges necessitate the use of the highest-quality materials to obtain meaningful results and a delivery reagent to transfect cells in the targeted organs of the subject animal.

Invitrogen™ Stealth RNAi™ siRNA molecules are chemically modified, blunt-ended, 25-mer double-stranded duplexes that are recognized by the RNA-induced silencing complex (RISC) to mediate inhibition of a target gene. Proprietary chemical modifications allow Stealth RNAi siRNA to overcome many *in vivo*–specific obstacles, allowing effectiveness and stability in *in vivo* applications.

Specific and effective knockdown

In RNAi experiments, it is necessary to minimize off-target effects so that observed phenotypes can be correctly attributed to target knockdown. With standard siRNA duplexes, both strands are able to participate in the knockdown reaction, increasing the chances of off-target effects. With Stealth RNAi siRNA duplexes, the sense strand is chemically modified to prevent it from contributing to RNAi activity. This minimizes the potential for off-target effects and allows a very high percentage of experimentally successful duplexes (Figure 7.12).

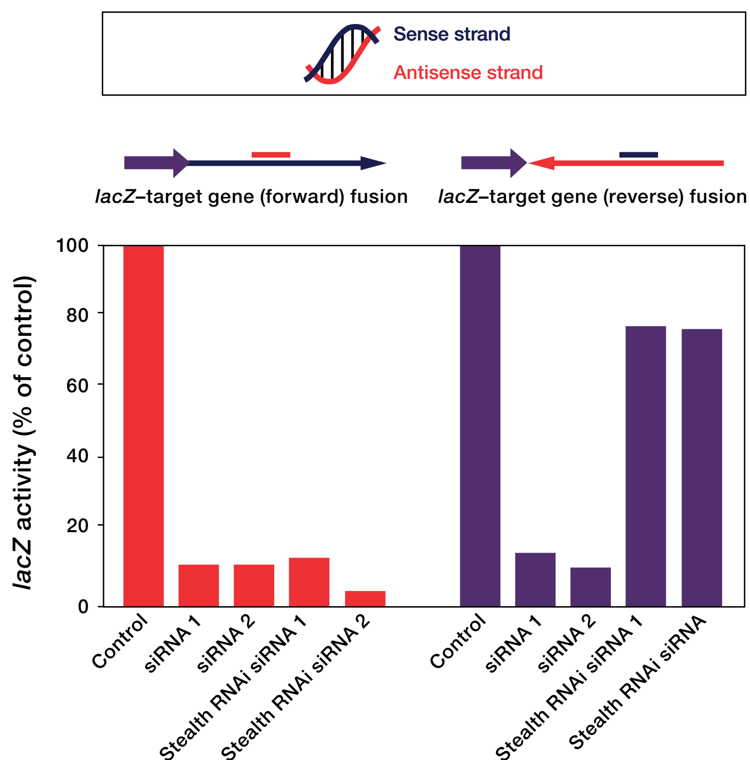


Figure 7.12. Stealth RNAi siRNA exhibits increased target specificity. Sequences 1 and 2 were designed as either siRNA or Stealth RNAi siRNA duplexes against a target gene. Only the antisense strand of a Stealth RNAi siRNA duplex can enter the RISC for specific targeting of mRNA, whereas either strand of siRNA can enter the RISC, increasing the possibility of nonspecific silencing.

* *In vivo* refers to research use in animals. This product is not intended for use in humans.

Stable in the presence of nucleases

Nucleases capable of degrading siRNA duplexes contribute to the challenging *in vivo* experimental environment. Nuclease resistance helps ensure that siRNA duplexes reach their intended location intact and that they are capable of initiating the knockdown reaction. Stealth RNAi duplexes have proprietary chemical modifications that confer remarkable stability for *in vivo* studies (Figure 7.13).

No induction of the interferon response

The interferon response is a powerful immune reaction that can make knockdown analysis nearly impossible due to nonspecific toxic effects. Induction of the interferon response can lead to severely altered global gene activity and make phenotypic assessment difficult. Standard siRNA has the potential to activate this innate response, setting off defense systems usually used to combat viruses. Chemical modifications in Stealth RNAi molecules abolish the immunostimulatory response observed with some sequences. Stealth RNAi complexes do not induce the interferon response (Figure 7.14), providing confidence that observed phenotypes can be attributed to specific target knockdown and not to nonspecific toxicity.

Tracking delivered duplexes

Tracking the biodistribution of administered RNAi duplexes is useful to ensure that the delivered material has targeted the desired tissues. We use the industry-leading Invitrogen™ Alexa Fluor™ fluorescent dyes to label Stealth RNAi duplexes for this purpose. These dyes are ideal for *in vivo* RNAi applications, due to their superior brightness and photostability. Duplexes can be tracked in three different ways: a Stealth RNAi duplex can be directly labeled with an Alexa Fluor dye, it can be biotinylated and detected with labeled streptavidin, or a labeled control duplex can be mixed with a target-specific Stealth RNAi duplex (Figure 7.15). Direct labeling of Stealth RNAi duplexes does not affect their potency.

For more information on these and other products for RNAi studies, go to **thermofisher.com/rnai**

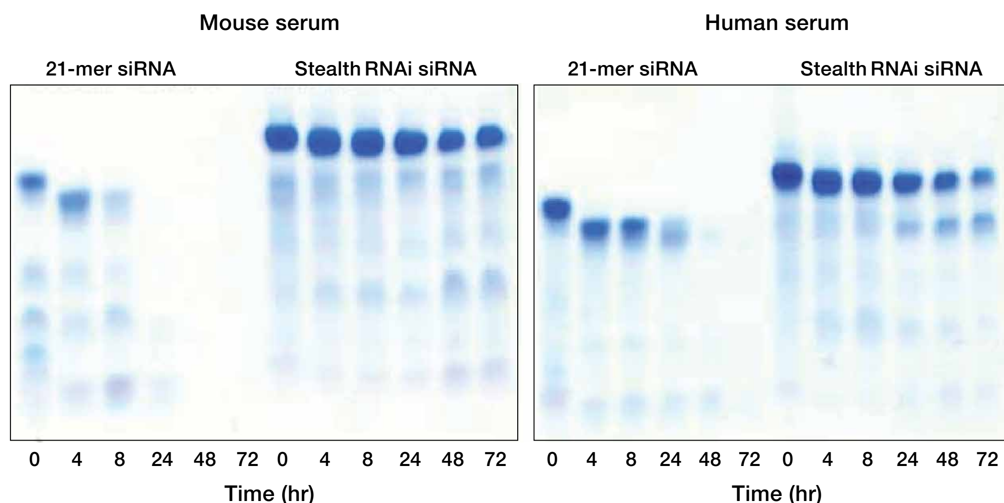
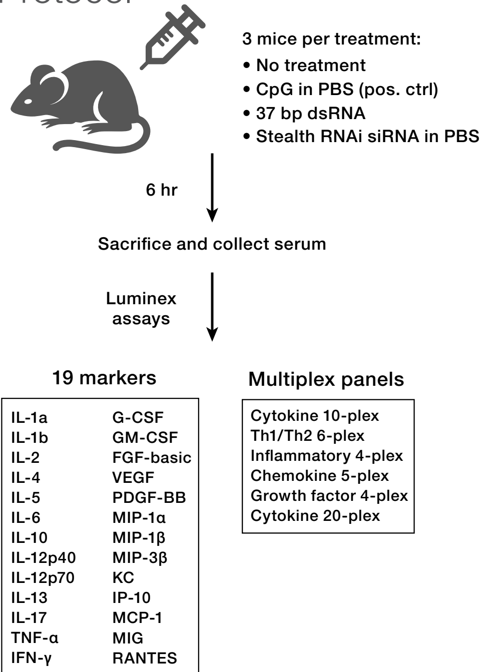


Figure 7.13. Chemical modification makes Stealth RNAi siRNA more stable for *in vivo* applications. Stealth RNAi siRNA duplexes are chemically modified to enhance stability against nucleases in serum. Unmodified 21-mer dsRNA sequences and corresponding Stealth RNAi siRNA sequences were analyzed at 0, 4, 8, 24, 48, and 72 hr following incubation in either 10% mouse or human serum. Samples were separated on an Invitrogen™ Novex™ 15% TBE-urea polyacrylamide precast gel and stained with methylene blue. These data demonstrate that Stealth RNAi duplexes remain intact, whereas unmodified 21-mer dsRNA sequences were susceptible to pronounced degradation.

Protocol



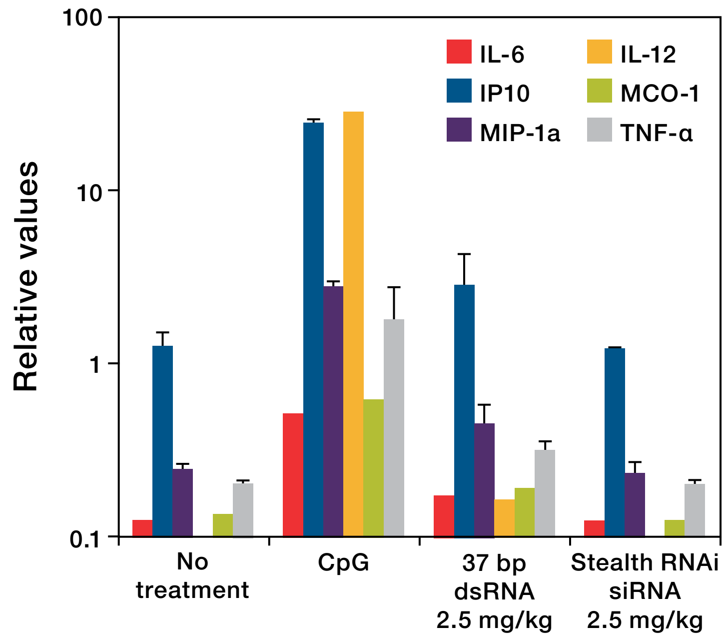


Figure 7.14. Stealth RNAi avoids the interferon stress response so that the data can be confidently correlated with knockdown activity. Tail veins of BALB/c mice (three mice per treatment) were injected at 5 mg/kg and 10 mg/kg of Stealth RNAi duplexes. After 6 hr, serum was collected and processed to measure interferon markers using the Invitrogen™ Cytokine Mouse 20-Plex Panel for the Luminex® instrument.

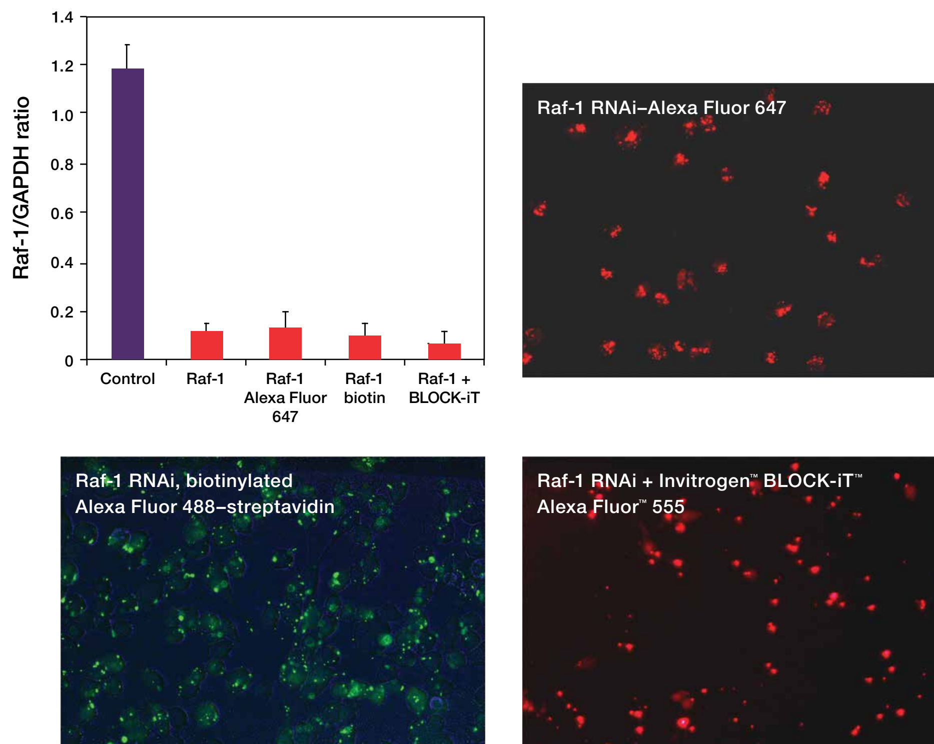


Figure 7.15. Labeled Stealth RNAi exhibits potent knockdown. MDA-MB-435 cells were transfected with 25 nmol unlabeled Stealth RNAi duplexes, or Stealth RNAi duplexes labeled with an Alexa Fluor dye or biotin. In each case the duplexes targeted Raf-1. Knockdown was assessed at >90% by real-time PCR; delivery is demonstrated through fluorescence imaging. Regardless of the labeling strategy, Stealth RNAi knockdown results were consistent.

Ambion *In Vivo* siRNA

When transitioning from cell culture experiments using *Silencer* Select siRNA, the recommendation for an *in vivo* RNAi reagent is Invitrogen™ Ambion™ *In Vivo* siRNA.

Ambion *In Vivo* siRNA molecules were developed as the *in vivo* version of *Silencer* Select siRNAs, with all of the advantages of efficiency and specificity, but suitable for *in vivo* use due to the addition of stabilizing modifications to prevent nuclease-mediated degradation in the presence of serum. They are chemically modified, 21-mer, double-stranded siRNAs with proprietary chemical modifications that allow researchers to overcome many *in vivo*–specific obstacles, ensuring their effectiveness and stability. Ambion *In Vivo* siRNA targeting Factor VII and PPIB have been successfully delivered by mouse tail-vein injection to liver tissue (Figure 7.16), where we demonstrate effective knockdown when measured at the mRNA level.

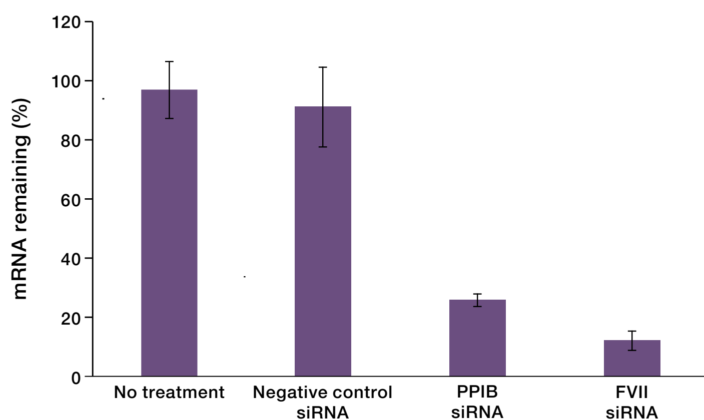


Figure 7.16. Ambion *In Vivo* siRNA complexed with Invivofectamine 3.0 Reagent enables targeted knockdown in the liver after a single intravenous injection. Invivofectamine 3.0 Reagent complexed with Ambion *In Vivo* siRNA targeting mRNA for Factor VII (FVII) or PPIB, injected at doses of 1 mg per kilogram mouse body weight (mg/kg), achieved as much as 85% knockdown of target mRNA levels (knockdown assessed via TaqMan Assay).

Complexes of Invitrogen™ Invivofectamine™ 3.0 Reagent and Ambion *In Vivo* siRNA in a range of amounts were introduced via tail-vein injection. FVII protein levels in the serum were measured using a chromogenic assay 24 hours after injection (Figure 7.17). The amount of knockdown is correlated with the amount of siRNA in the complex. The ED_{50} of Ambion *In Vivo* siRNA with Invivofectamine 3.0 Reagent is 0.1 mg/kg, compared to previous levels of 1.0 mg/kg.

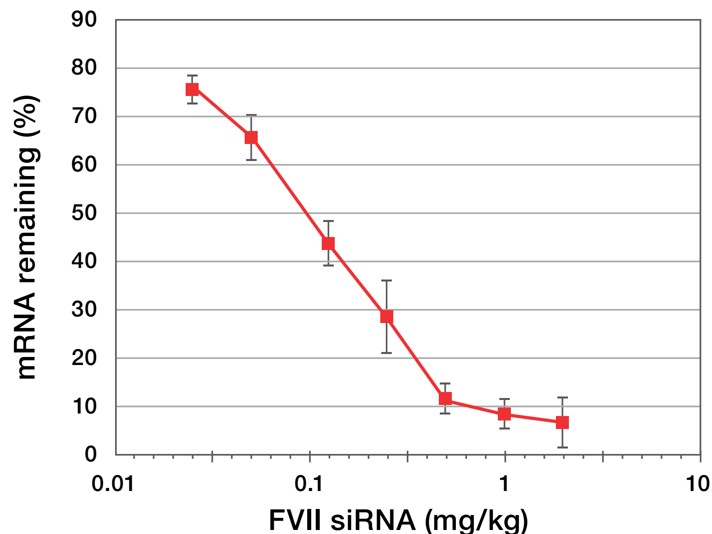


Figure 7.17. Less siRNA required to achieve effective knockdown. Ambion *In Vivo* siRNA targeting FVII delivered with Invivofectamine 3.0 Reagent produce dose-response knockdown in liver after a single intravenous injection. Invivofectamine 3.0 Reagent complexed with Ambion *In Vivo* siRNA targeting FVII was injected at doses ranging from 0.02 to 2 mg/kg. Blood serum was isolated and assayed for FVII protein levels (BIOPHEN™ chromogenic assay).

siRNA selection guide

siRNA type	Description	Technology	Recommended use
Predesigned/validated (tubes)			
***Silencer* Select Predesigned and Validated siRNA**	• Improved potency and guaranteed knockdown • Modified for improved specificity • Predesigned for human, mouse, and rat genes • Validated for some human genes	LNA-modified 21-mer	• Suitable for all applications • Particularly good for cell culture and RNAi screening work
Stealth RNAi and Validated Stealth RNAi	• Modified for serum stability and improved specificity • Predesigned for human, mouse, and rat genes • Validated for some human genes	Modified 25-mer	• Suitable for all applications • Particularly good for *in vivo* applications
***Silencer* Predesigned and Validated siRNA**	• Predesigned for human, mouse, and rat genes • Validated for some human genes	Unmodified 21-mer	• Lower-cost alternative, but without some of the performance benefits of the newer technologies • Suitable for cell culture, not recommended for *in vivo* work
Ambion *In Vivo* siRNA	• Improved potency from advanced algorithm • Modified for improved specificity • Predesigned for human, mouse, and rat genes • Stability-enhanced for *in vivo* applications	LNA-modified 21-mer	• Suitable for *in vivo* applications
Predefined sets/collections			
***Silencer* Select siRNA Libraries**	• Predefined sets of *Silencer* Select siRNAs for the human genome and functional gene classes • 0.25 nmol of each siRNA • Ready for immediate delivery	LNA-modified 21-mer	• For screening of predefined *Silencer* Select siRNA sets such as human kinases, GPCRs, druggable and whole genome
***Silencer* siRNA Libraries**	• Predefined sets of *Silencer* siRNAs for the human and mouse genomes and functional gene classes • 0.25 nmol of each siRNA • Ready for immediate delivery	Unmodified 21-mer	• Lower-cost alternative to *Silencer* Select siRNA Libraries, but without some of the performance enhancements
Customer-defined sets			
Made-to-order *Silencer* Select siRNA Libraries	• Custom sets of Human *Silencer* Select siRNAs at 0.1 to 5 nmol • Priced by quote and ordered offline	LNA-modified 21-mer	• For screening 24 to >10,000 *Silencer* Select siRNAs • Submit a gene list to suit your needs • Multiple sizes and plating options
Made-to-order *Silencer* siRNA Libraries	• Custom sets of *Silencer* siRNAs at 1 to 5 nmol • Priced by quote and ordered offline	Unmodified 21-mer	• Lower-cost alternative to *Silencer* Select siRNA Libraries, but without some of the performance benefits
Custom (you provide the sequence)			
***Silencer* Select siRNA**	• Improved potency from advanced algorithm • Modified for improved specificity	LNA-modified 21-mer	• For when you require a large amount or a custom design; order online **here**
Stealth RNAi siRNA	• Modified for serum stability and improved specificity	Modified 25-mer	• For when you require a large amount, additional chemical modification, *in vivo* processing, or a custom design; order online **here**
Custom siRNA	• Unmodified, but user may request dyes, modified bases, and other modifications	21-mer	• For when you require large amounts, a custom design, or custom modifications; order online **here**
Ambion *In Vivo* siRNA	• Modified for improved specificity • Predesigned for human, mouse, and rat genes • Stability-enhanced for *in vivo* applications	LNA-modified 21-mer	• Suitable for *in vivo* applications • For when you require large amounts, custom design, or *in vivo* processing; order online **here**
Controls			
Stealth RNAi Positive and Negative Controls	• siRNA controls for use with Stealth RNAi • Fluorescent labels available with some	Modified 25-mer	• Any time you are using a Stealth RNAi siRNA
***Silencer* Select Positive and Negative Control siRNAs**	• siRNA controls for use with *Silencer* Select siRNAs	LNA-modified 21-mer	• Any time you are using a *Silencer* Select siRNA
***Silencer* Positive and Negative Control siRNAs**	• siRNA controls for use with *Silencer* siRNAs • Fluorescently labeled controls available	Unmodified 21-mer	• Any time you are using a *Silencer* siRNA

Silencer Select siRNA libraries

Predefined siRNA libraries plated to order

Predefined, made-to-order *Silencer* Select siRNA Libraries are available for the indicated gene families (Table 7.1).

These libraries have been preassembled using the PANTHER Classification System (**pantherdb.org**) and Gene Ontology annotation information to create up-to-date target gene sets.

- Each gene is targeted by three unique siRNAs. Validated siRNAs are included at no extra cost.
- 0.25 nmol of each siRNA is provided dried-down in the wells of barcoded 96- or 384-well plates. This is sufficient for 100–500+ transfections, depending on siRNA working concentration and reaction volume.
- Column 12 of each 96-well plate and columns 23 and 24 in each 384-well plate are left empty to allow for the addition of controls at the time of use.
- Plates are conveniently arranged for pooling of siRNA, if desired.
- The libraries are delivered with plate map files including full siRNA sequence information, as well as each target gene's symbol, full name, aliases, RefSeq accession, and Entrez Gene ID.
- If you would like more information on any of the libraries listed below, email us at **RNAisupport@thermofisher.com**

Table 7.1. List of available predefined *Silencer* Select siRNA libraries.

Cat. No.	Product description	Number of genes*
A30085	*Silencer* Select Human Epigenetics siRNA	521
A30087	*Silencer* Select Human Transcription Factor siRNA	1,819
A30089	*Silencer* Select Human DNA Damage Response siRNA	582
A30091	*Silencer* Select Human Drug Targets siRNA (384-well)	7,926
A30092	*Silencer* Select Human Drug Targets siRNA (96-well)	7,926
A30095	*Silencer* Select Human Apoptosis siRNA	924
A30133	*Silencer* Select Human Drug Transporter siRNA	99
A30136	*Silencer* Select Human Cell Cycle Regulation siRNA	1,471
A30138	*Silencer* Select Human Membrane Trafficking siRNA	142
A30140	*Silencer* Select Human Ubiquitin siRNA	981
A30142	*Silencer* Select Human Cancer Genome siRNA	532
A30144	*Silencer* Select Human Cell Surface siRNA	794
A30146	*Silencer* Select Human Tumor Suppressor siRNA	745
4397918	*Silencer* Select Human Kinase siRNA Library V4	709
4397919	*Silencer* Select Human Phosphatase siRNA Library V4	298
4397916	*Silencer* Select Human GPCR siRNA Library V4	379
4397914	*Silencer* Select Human Nuclear Hormone Receptor siRNA Library V4	47
4397915	*Silencer* Select Human Ion Channel siRNA Library V4	338
4397917	*Silencer* Select Human Protease siRNA Library V4	494
4397920	*Silencer* Select Human Druggable Genome siRNA Library V4; 96-well	9,031
4397927	*Silencer* Select Human Extended Druggable Genome siRNA Library V4; 96-well	10,414
4397922	*Silencer* Select Human Druggable Genome siRNA Library V4; 384-well	9,031
4397923	*Silencer* Select Human Genome siRNA Extension Set V4; 384-well	11,170
4397924	*Silencer* Select Human Druggable Genome siRNA Extension Set V4; 384-well	1,383
4397926	*Silencer* Select Human Genome siRNA Library V4; 384-well	21,584
Human long noncoding RNA (lncRNA)		
A30148	*Silencer* Select Human Long Non-coding RNA (lncRNA) siRNA Library	5,027
A30149	*Silencer* Select Human Long Non-coding RNA (lncRNA) siRNA Library (384-well)	

* Number of genes is subject to change.

Human Genome siRNA Library
(combination of Cat. No. 4397922, 4397923, 4397924)

Increasingly, researchers are performing genome-scale surveys of gene function. The Invitrogen™ *Silencer*™ Select Human Genome siRNA Library V4 is ideal for this purpose. Three unique, nonoverlapping siRNAs are provided for each of 21,584 human targets. This siRNA library is supplied in 384-well plates.

Human Druggable Genome and Human Extended Druggable Genome siRNA Libraries
With siRNAs targeting the most therapeutically relevant human genes and conveniently grouped by target gene class, the Invitrogen™ *Silencer*™ Select Human Druggable Genome siRNA Library V4 and Invitrogen™ *Silencer*™ Select Human Extended Druggable Genome siRNA Library V4 are popular choices for many researchers. The druggable genome set targets 9,031 genes, whereas the extended druggable genome collection targets the same 9,031 genes plus the "Extension Set" for a total of 10,414 genes, including transcription factors. Please contact us for the complete list of targets. These libraries are available in both 96- and 384-well plate formats.

Custom siRNA libraries—the ultimate in flexibility
It is common for a research project to generate a bespoke set of genes for interrogation, so we have the flexibility to generate *Silencer* Select libraries based on your gene list to suit your specific needs. These highly economical plated versions of our guaranteed siRNAs are perfect for pilot studies, primary screen follow-up, or a customized functional genomics screen.

- Minimum order of 20 siRNAs
- Up to 3 siRNAs per gene
- 0.1, 0.25, 0.5, 1, or 2 nmol/well
- Choose from 96-well, 384-well, or 384-well Echo™ Qualified Plates (Labcyte)
- Validated siRNAs are available at no additional cost

To submit a gene list or receive a quote, please contact **RNAiSupport@thermofisher.com**

Information to accelerate discovery
All *Silencer* Select siRNA libraries, whether custom or predefined, are supplied with full siRNA sequence information. Also provided in the data files accompanying the siRNA library are gene annotation information, which includes gene symbol, gene name, aliases, NCBI Entrez Gene ID, and associated RefSeq mRNA accession numbers. Plates are individually barcoded with unique identifiers; this information is also provided with the siRNA and gene information.

Individual siRNAs for enhanced data reliability
All *Silencer* Select siRNA libraries feature three individual siRNAs for each target. Screening with three siRNAs per gene significantly decreases both false-positive and false-negative rates, increasing confidence in RNAi screening data, reducing the risk of missing important genes, and decreasing time spent following up on false-positive hits from the screen.

Chapter references
1. Jackson AL et al. (2003) *Nat Biotechnol* 21:635–637.
2. Saxena S et al. (2003) *J Biol Chem* 278:44312–44319.
3. Scacheri PC et al. (2004) *Proc Natl Acad Sci U S A* 101:1892–1897.
4. Du Q et al. (2005) *Nucleic Acids Res* 33:1671–1677.
5. Hornung V et al. (2005) *Nat Med* 11:263–270.
6. Judge AD et al. (2005) *Nat Biotechnol* 23:457–462.
7. Semizarov D et al. (2003) *Proc Natl Acad Sci U S A* 100:6347–6352.

Chapter 8

Vector-based RNAi technologies

RNAi vectors for more options in your RNAi experiments

Invitrogen™ BLOCK-iT™ RNAi vectors offer a variety of options for long-term or transient RNAi expression. With BLOCK-iT RNAi viral delivery systems, RNAi experiments can be performed in the most biologically relevant cell type rather than in cell lines that are easy to transfect. The BLOCK-iT inducible systems permit RNAi initiation and duration to be controlled, while the BLOCK-iT™ Pol II miR RNAi Expression Vectors limit knockdown to specific tissues or cells. There is a suitable BLOCK-iT RNAi vector for every RNAi application.

We have developed two distinct vector-based RNAi systems for gene knockdown experiments: BLOCK-iT Pol II miR RNAi Expression Vectors and Invitrogen™ BLOCK-iT™ shRNA Vectors. Both Pol II miR RNAi and shRNA vector approaches include the capability for stable and inducible expression and viral delivery.

BLOCK-iT Pol II miR RNAi Expression Vectors have significant advantages over the short-hairpin RNA (shRNA) vector technology currently used for RNAi vector applications (Table 8.1). These vectors include flanking and loop sequences from an endogenous miRNA that directs the excision of the engineered miRNA from a longer Pol II transcript (pri-miRNA). When present in the nucleus, these vectors efficiently use the endogenous cellular machinery to process knockdown sequences that are specifically designed to have 100% homology to the target mRNA and will result in target cleavage. In addition, the loop sequence has a unique restriction site, so that it can be linearized to allow for trouble-free sequencing, as sequencing standard shRNA hairpins is sometimes a challenge.

See Table 8.2 for a comprehensive overview of available vectors for your RNAi experiments.

Table 8.1. Comparison of standard shRNA vectors and BLOCK-iT Pol II miR RNAi Expression Vectors.

BLOCK-iT shRNA vector technologies	shRNA	miR RNAi
Typical knockdown success rate*	~50%	>75%
Expression tracking	No	Yes
Multiple-target knockdown	No	Yes
Tissue-specific expression	No	Yes
Invitrogen™ Gateway™ vector compatibility with most destination (DEST™) vectors	No	Yes

* Rate at which constructs reduce target mRNA expression >70%

Table 8.2. BLOCK-iT vector technologies.

Vector	RNAi vector technology	Transient or stable expression	Selection marker	Delivery method	Constitutive or inducible expression	Reporter	Kits	Page
BLOCK-iT Pol II miR RNAi expression vector kits								
pcDNA6.2-GW/ miR	miR RNAi	Transient and stable	Blasticidin	Transfection	Constitutive Pol II (CMV) Inducible and tissue-specific options	None	BLOCK-iT Pol II miR RNAi Expression Vector Kit (also included in BLOCK-iT Lentiviral Pol II miR RNAi Expression System)	62
pcDNA6.2-GW/ EmGFP-miR	miR RNAi	Transient and stable	Blasticidin	Transfection	Constitutive Pol II (CMV) Inducible and tissue-specific options	Cocistronic Emerald GFP (EmGFP)	BLOCK-iT Pol II miR RNAi Expression Vector Kit with EmGFP (also included in BLOCK-iT Lentiviral Pol II miR RNAi Expression System)	62
BLOCK-iT Pol II miR RNAi lentiviral expression systems								
pLenti6/V5-DEST	miR RNAi	Transient and stable	Blasticidin	Viral transduction (or transfection)	Constitutive Pol II (CMV)	None	BLOCK-iT Lentiviral Pol II miR RNAi Expression System (includes BLOCK-iT Pol II miR RNAi Expression Vector Kit)	63
pLenti6/V5-DEST	miR RNAi	Transient and stable	Blasticidin	Viral transduction (or transfection)	Constitutive Pol II (CMV)	Cocistronic Emerald GFP (EmGFP)	BLOCK-iT Lentiviral Pol II miR RNAi Expression System with EmGFP (includes BLOCK-iT Pol II miR RNAi Expression Vector Kit with EmGFP)	63
pLenti6.4/R4R2/ V5-DEST	miR RNAi	Transient and stable	Blasticidin	Viral transduction (or transfection)	Constitutive (CMV or EF-1α) or other	Cocistronic Emerald GFP (EmGFP)	BLOCK-iT Lentiviral Pol II miR RNAi Expression System with EmGFP (includes BLOCK-iT Pol II miR RNAi Expression Vector Kit with EmGFP)	63
BLOCK-iT shRNA entry vectors								
pENTR/U6	shRNA	Transient	None	Transfection	Constitutive	None	BLOCK-iT U6 RNAi Entry Vector Kit (also included in the BLOCK-iT RNAi Lentiviral Expression System and the BLOCK-iT Adenoviral RNAi Expression System)	69
BLOCK-iT shRNA destination vectors								
pLenti6/BLOCK-iT-DEST	shRNA	Transient and stable	Blasticidin	Viral transduction (or transfection)	Constitutive	None	BLOCK-iT RNAi Lentiviral Expression System BLOCK-iT Lentiviral RNAi Gateway Vector Kit	71–72
pLenti4/BLOCK-iT-DEST	shRNA	Transient and stable	Zeocin	Viral transduction (or transfection)	Inducible or constitutive	None	BLOCK-iT Inducible H1 Lentiviral RNAi System BLOCK-iT Lentiviral RNAi Zeo Gateway Vector Kit	71
pLenti6/TR (for expression of tetracycline repressor only)	shRNA or miR RNAi	Transient and stable	Blasticidin	Viral transduction (or transfection)	Constitutive	None	pLenti6/TR Vector Kit BLOCK-iT Inducible H1 Lentiviral RNAi System	73
pAd/BLOCK-iT-DEST	shRNA	Transient	None	Viral transduction (or transfection)	Inducible or constitutive	None	BLOCK-iT Adenoviral RNAi Expression System pAd/BLOCK-iT-DEST RNAi Gateway Vector	73
pBLOCK-iT 6-DEST	shRNA	Transient and stable	Blasticidin	Transfection	Constitutive	None	pBLOCK-iT 6-DEST	73

Vector	RNAi vector technology	Transient or stable expression	Selection marker	Delivery method	Constitutive or inducible expression	Reporter	Kits	Page
BLOCK-iT shRNA destination vectors (continued)								
pBLOCK-iT 6-DEST	shRNA	Transient and stable	Blasticidin	Transfection	Constitutive	None	pBLOCK-iT 6-DEST	73
pBLOCK-iT 3-DEST	shRNA	Transient and stable	Geneticin	Transfection	Inducible or constitutive	None	pBLOCK-iT 3-DEST	73
Selected Gateway DEST vectors compatible with miR RNAi vector technology*								
pLenti4/TO/ V5-DEST	miR RNAi	Transient and stable	Zeocin	Viral transduction (or transfection)	Inducible (CMV)	Cocistronic EmGFP or none†	ViraPower T-REx Lentiviral Expression System. Needs to be used in a T-REx cell line or in cells that express the Tet repressor protein.	67
pEF-DEST51	miR RNAi	Transient and stable	Blasticidin	Transfection	Constitutive (EF-1α)	Cocistronic EmGFP or none†	No kit available. Vector Cat. No. 12285-011	67
pDEST R4-R3	miR RNAi	Transient	None	Transfection	Your choice	Cocistronic EmGFP or none†	MultiSite Gateway Three-Fragment Vector Construction Kit	67
pLenti6/R4R2/ V5-DEST	miR RNAi	Transient and stable	Blasticidin	Viral transduction (or transfection)	Your choice	Cocistronic EmGFP or none†	ViraPower Promoterless Lentiviral Gateway Expression System with MultiSite Gateway Technology	67
pLenti6/TR (for expression of tetracycline repressor only)	shRNA or miR RNAi	Transient and stable	Blasticidin	Viral transduction (or transfection)	Constitutive	None	pLenti6/TR Vector Kit	73
pcDNA6/TR (for expression of tetracycline repressor only)	shRNA or miR RNAi	Transient and stable	Blasticidin	Transfection	Constitutive (CMV)	None	T-REx Core Kit or T-REx Complete Kit	73
pSCREEN-iT/ *lacZ*-DEST	shRNA or miR RNAi	Transient	None	Transfection	Constitutive	*lacZ*	BLOCK-iT RNAi Target Screening System; BLOCK-iT RNAi Target Screening Kit; pSCREEN-iT/*lacZ*-DEST Gateway Vector Kit	68

* Additional components, such as Invitrogen™ Clonase™ enzyme mixes and Gateway donor vectors, are needed to transfer the miR RNAi cassette into these DEST vectors. The additional components are included in the BLOCK-iT Lentiviral Pol II miR RNAi Expression System but are not included in BLOCK-iT Pol II miR RNAi Expression Vector Kits and must be purchased separately to transfer the miR RNAi expression cassette to alternative DEST vectors.

† Cocistronic EmGFP reporter can be transferred from the original BLOCK-iT Pol II miR RNAi Expression Vector.

BLOCK-iT Pol II miR RNAi Expression Vectors

BLOCK-iT Pol II miR RNAi Expression Vectors combine the advantages of traditional RNAi vectors—stable expression and the ability to use viral delivery—with capabilities for tissue-specific expression and multiple-target knockdown from the same transcript. The pcDNA6.2-GW/miR and pcDNA6.2-GW/EmGFP-miR vectors (Figure 8.1), included in the BLOCK-iT Pol II miR RNAi expression vector kits and the BLOCK-iT Lentiviral Pol II miR RNAi Expression System, are designed for expressing artificial miRNAs that have been engineered to have 100% homology to a target sequence and will result in its cleavage. The BLOCK-iT Pol II miR RNAi Expression Vector technology offers many benefits:

- **Over 75% knockdown success**—screen fewer clones because design predictability is higher than that of other vector-based RNAi methods
- **Easy expression tracking**—assess knockdown of target miRNA simply and reliably with cocistronic expression with EmGFP
- **Multiple-target knockdown**—knock down multiple targets simultaneously or generate synthetic phenotypes
- **Gateway vector compatibility**—use a wide selection of Invitrogen™ destination vectors, including lentiviral vectors, for stable expression in any cell type, such as primary and nondividing cells
- **Tissue-specific experimental options**—select from a variety of destination vectors or Invitrogen™ MultiSite Gateway™ vector applications to add different promoters for cellular applications or *in vivo* knockdown
- **Inducible expression**—regulation of the RNAi response permits the study of changes over time and loss-of-function experiments, and provides an excellent control system to measure phenotypic changes during recovery of gene function

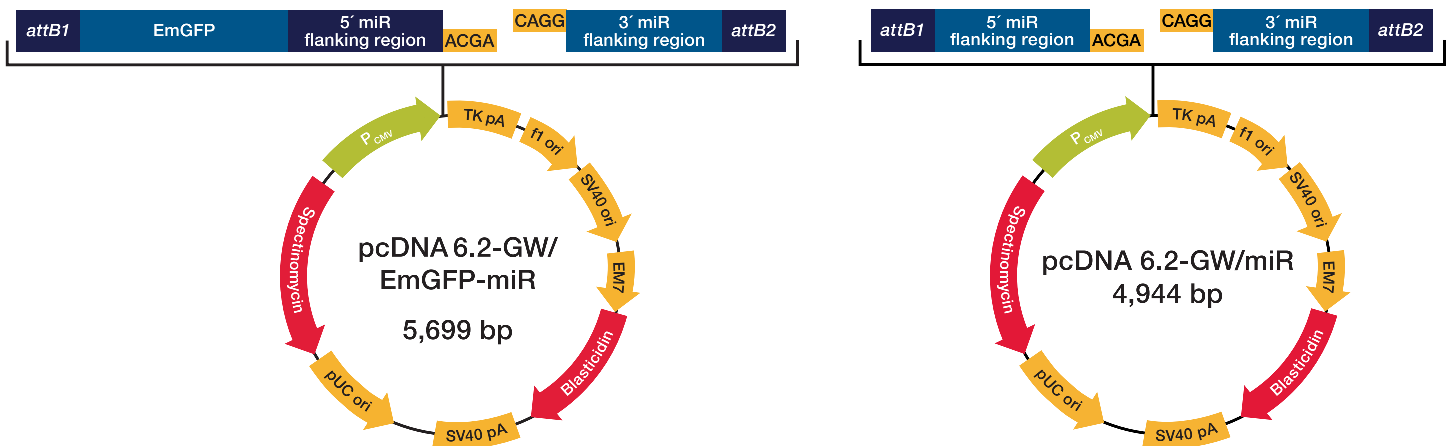


Figure 8.1. The BLOCK-iT Pol II miR RNAi Expression Vectors. The pcDNA6.2-GW/miR vector is driven by the cytomegalovirus (CMV) promoter, has the blasticidin resistance marker, and is available with or without cocistronic EmGFP expression as a reporter.

Combine BLOCK-iT vector kits with lentivirus for stable, long-term expression

BLOCK-iT vectors and Invitrogen™ BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression Systems combine all of the advantages of the BLOCK-iT Pol II miR RNAi Expression Vector Kit with Invitrogen™ ViraPower™ Lentiviral Expression Vectors (Figure 8.2), enabling stable delivery of engineered miRNAs into nondividing, primary, and hard-to-transfect cells. For example, ViraPower Lentiviral Expression Vectors have been used to deliver miR RNAi sequences into rat primary cortical neurons to knock down microtubule-associated protein 2 (Figure 8.3). These vectors deliver miRNA sequences into HT1080 and HeLa cells to knock down *LMNA* (lamin A/C gene) or *lacZ* (Figure 8.4), establishing long-term, stable expression of miRNAs in these cell model systems.

The BLOCK-iT HiPerform Lentiviral Pol II miR RNAi Expression System with EmGFP is the most powerful and flexible RNAi vector offered to date. This technology combines high titers and maximum expression. The ability to incorporate custom promoters makes the system suitable for *in vivo* applications.

The BLOCK-iT HiPerform vector contains an mRNA-stabilizing sequence (WPRE) and a nuclear import sequence (cPPT) that can generate up to 5-fold higher virus titers and EmGFP expression levels in many cell lines. Additionally, MultiSite technology allows you to express the EmGFP/miR RNAi cassette from CMV, EF-1α, your own tissue-specific promoter, or another appropriate *in vivo* promoter:

- Achieve up to 5x higher titers (measured by GFP), allowing more cells to be transduced or higher multiplicities of infection (MOIs) to be employed
- Incorporate your own tissue-specific promoter or another appropriate *in vivo* promoter
- Track miRNA expression through cocistronic expression with EmGFP
- Knock down more than one gene simultaneously through expression of multiple miRNAs from a single transcript

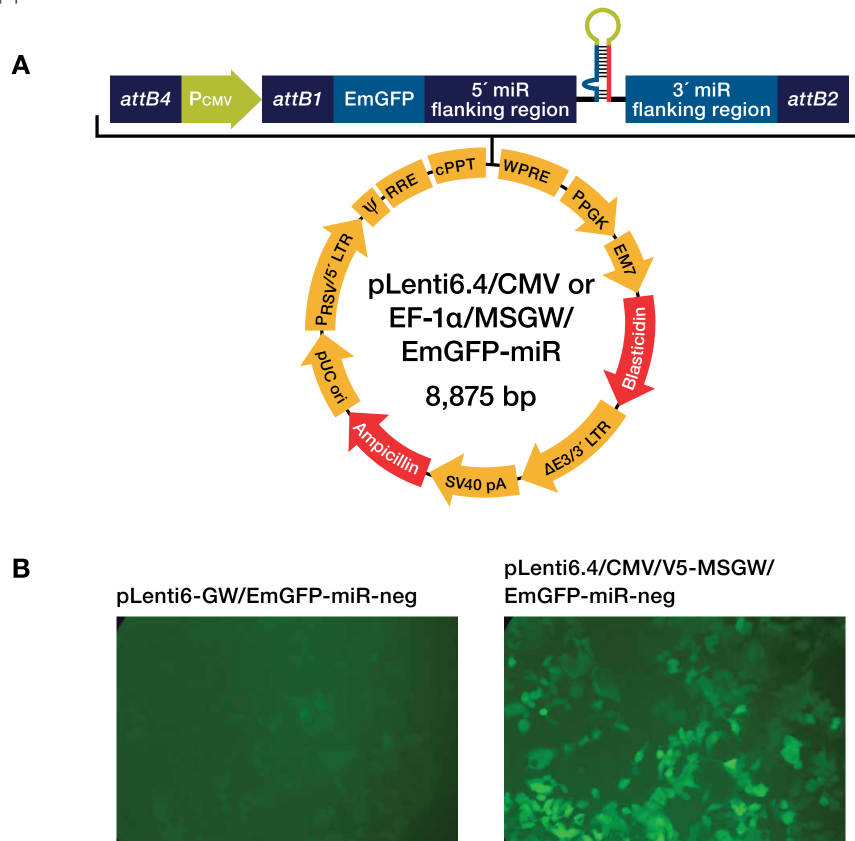


Figure 8.2. pLenti6/V5-DEST vector. (A) The BLOCK-iT Lentiviral Pol II miR RNAi Expression Systems utilize the pLenti6/V5-DEST vector for constitutive expression from the CMV immediate-early promoter, and the blasticidin selection marker for compatibility with a tetracycline-regulated inducible system. **(B)** Images taken 4 days following transduction of Invitrogen™ GripTite™ 293 MSR cells at an MOI of 3 with lentiviral particles generated using the indicated vectors.

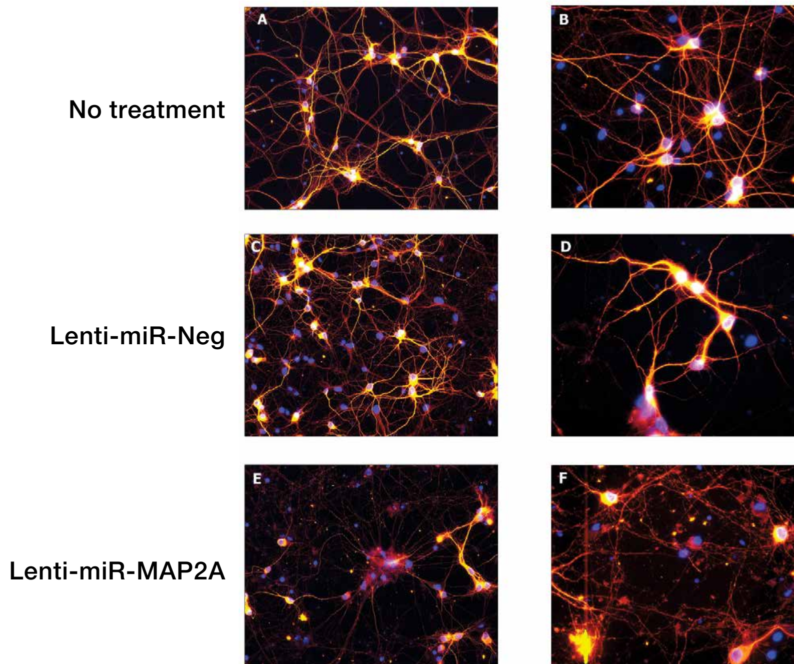


Figure 8.3. Target gene knockdown in primary neurons. Unlike standard retroviruses, lentiviruses can transduce nondividing cells, such as these rat primary cortical neurons. Here, microtubule-associated protein 2 (MAP2A) was targeted, and its expression was dramatically decreased. Untreated cells **(A, B)** and cells treated with Lenti-miR-Neg **(C, D)** or Lenti-miR-MAP2A **(E, F)** were stained for anti-MAP2A and DAPI. Images were acquired with a Nikon™ E800 microscope and the Omega™ XF-32 filter cube for the AF555 channel and the Omega XF136-2 filter cube for DAPI. Panels **A**, **C**, and **E**: 20x magnification. Panels **B**, **D**, and **F**: 40x magnification. There is a visible difference between the transfected control and the lenti control vs. lentiviral delivery of an RNAi knockdown reagent.

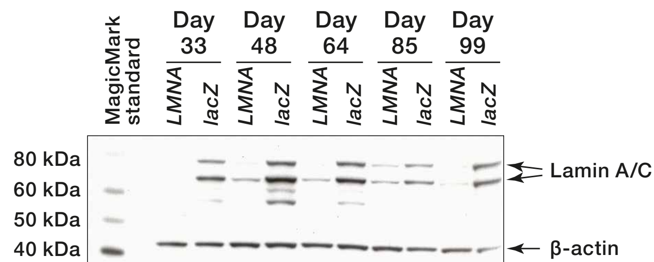


Figure 8.4. Stable lentiviral transduction of miRNA expression cassettes. Western blot analysis of HeLa cell populations stably transduced (MOI = 20) for 33 to 99 days with *LMNA* miRNA– or *lacZ* miRNA–expressing lentiviral particles. The blot was probed with anti–lamin A/C (top half) or anti–β-actin (bottom half) antibodies.

Reliable tracking of the RNAi cassette

Determining which cells are expressing the artificial miRNA of interest is straightforward with cocistronic expression of EmGFP. The pcDNA6.2-GW/EmGFP-miR vector (included in the BLOCK-iT Pol II miR RNAi Expression Vector Kit with EmGFP or the BLOCK-iT HiPerform Lentiviral Pol II miR RNAi Expression System with EmGFP) expresses EmGFP cocistronically with the miRNA of interest, so the expression of EmGFP shows nearly 100% correlation with the expression of the miRNA (Figure 8.5), allowing miRNA expression to be tracked in any cell type.

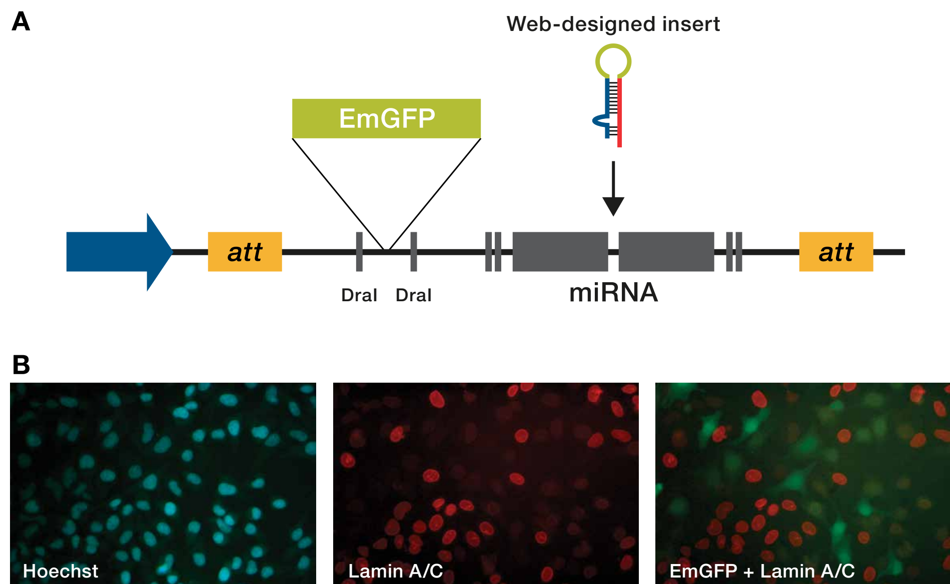


Figure 8.5. 100% correlation of EmGFP and miRNA expression. (A) Map of the BLOCK-iT Pol II miR RNAi expression cassette shows EmGFP between DraI restriction sites for easy removal. **(B)** Cells were transfected with Invitrogen™ Vivid Colors™ pcDNA™ 6.2-GW/EmGFP-miR (directed against lamin) and Invitrogen™ Lipofectamine™ 2000 Transfection Reagent, at an expected 50% efficiency, to demonstrate the 100% tracking of EmGFP and miRNA expression. After 48 hr the cells were stained with Hoechst™ nuclear stain, which stains all cells (left panel), and a red lamin stain (center panel), and monitored for EmGFP expression. Approximately half of the cells highly expressed the lamin protein (compare left and center panels), but red-stained cells were not expressing EmGFP, and EmGFP-expressing cells were not expressing lamin (right panel). This demonstrates the cocistronic expression of EmGFP and the miRNA that greatly reduces lamin expression.

Knockdown of multiple targets for experimental flexibility

The BLOCK-iT Pol II miR RNAi expression vectors are designed for the effective knockdown of more than one target in a single experiment. The Pol II promoter enables cocistronic expression of multiple miRNAs, allowing knockdown of multiple targets from a single construct (Figure 8.7). With a simple restriction enzyme procedure, two or more miRNA sequences can be linked in any order. This process is ideal for knockdown of more than one pathway component or splice variant, or for using knockdown to create synthetic phenotypes.

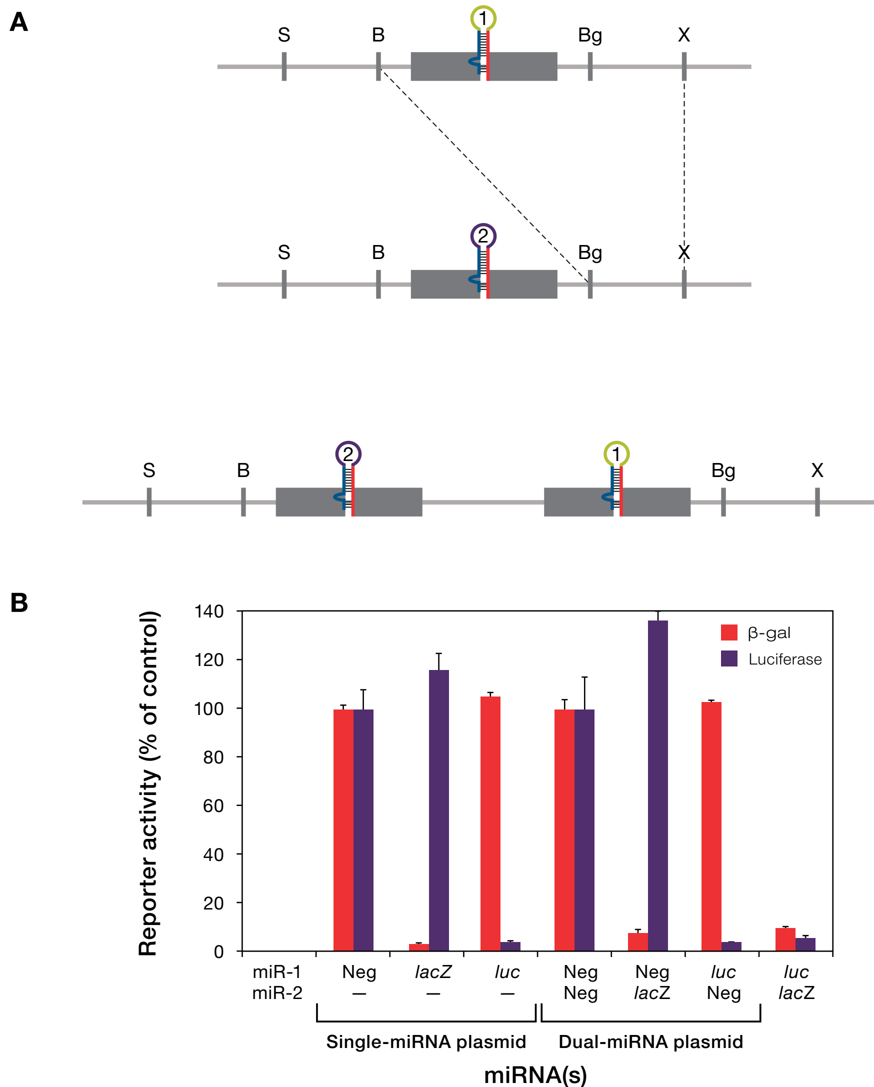


Figure 8.6. Knockdown of multiple targets with BLOCK-iT Pol II miR RNAi expression vectors. (A) Example of a restriction digestion and ligation scheme for combining miRNAs from different vectors. SalI (S), BamHI (B), BglII (Bg), and XhoI sites (X) around the miRNA flanking regions (bars) are indicated. By cloning the BamHI–XhoI fragment containing miRNA 1 into the BglII–XhoI fragment of the vector containing miRNA 2, a dual-miRNA plasmid is created. The original restriction site pattern is recreated (restriction sites between the miRNAs are destroyed) and additional miRNAs can be added in the same manner. Alternatively, miRNAs can be added in front of miRNA 1 by combining SalI–BglII and SalI–BamHI fragments. **(B)** Results following cotransfection of luciferase and *lacZ* reporter plasmids with single- or dual-miRNA vectors with the indicated inserts. Luciferase and β-galactosidase activities are normalized to the single (neg) or dual (neg/neg) miRNA negative control inserts, which form a pre-miRNA but are not predicted to target any known vertebrate genes. Knockdown is slightly attenuated in the dual-miRNA vectors but remains very potent at ≥90%.

Gateway vector compatibility for expanded experimental options

Gateway technology is a fast and efficient way to transfer miR RNAi cassettes into a variety of vectors by homologous recombination. Gateway recombination eliminates tedious and time-consuming subcloning procedures, and the resulting expression cassettes can be easily moved into any of the Gateway DEST vectors. See Table 8.3 for a listing of Gateway DEST vectors that have been functionally tested for compatibility with the BLOCK-iT Pol II miR RNAi Vector Kits.

With the BLOCK-iT Pol II miR RNAi Expression Vector Kits, miRNAs are cloned directly into Gateway expression vectors rather than Gateway entry vectors. As a result, there are two key differences between the pcDNA6.2-GW/miR and pcDNA6.2-GW/EmGFP-miR expression vectors and typical Gateway entry vectors:

1. The miRNA expression vectors include the cytomegalovirus (CMV) promoter. After miRNA sequences are cloned, they are immediately ready for transfection and miRNA expression.

2. *attB* sites flank the miRNA (and EmGFP sequences if using pcDNA6.2-GW/EmGFP-miR). For expression in different DEST vectors, the inserts must first be transferred to a Gateway™ pDONR™ (donor) vector and then to a DEST vector of choice by a dual Invitrogen™ Clonase™ enzyme reaction. The pDONR 221 vector, BP and LR Clonase II enzyme mixes, and pLenti6/V5-DEST vector are included in the BLOCK-iT Lentiviral Pol II miR RNAi Expression System.

Table 8.3. Compatibility of Gateway destination vectors.

Promoter or system	Compatibility	Cat. No.
ViraPower lentiviral vectors, including MultiSite Gateway technology	Bench-tested with pLenti6/V5-DEST and pLenti6/UbC/V5-DEST	V496-10 V499-10
EF-1α promoter	Bench-tested with pEF-DEST51	12285-011
T-REx vector	Bench-tested with pT-REx DEST30	12301-016
Flp-In cell line	Bench-tested with pEF5/FRT/V5-DEST	V6020-20
N-terminal reporter tags	Bench-tested with Vivid Colors pcDNA6.2/N-YFP/DEST	V358-20
MultiSite Gateway technology	Bench-tested with pDEST/R4-R3 using thymidine kinase (TK) poly(A) 3´ element and various 5´ promoter elements	12537-100

Pol II promoter versatility allows tissue-specific expression

Most shRNA vectors are driven by Pol III promoters, significantly limiting the types of promoters that can be used in RNAi experiments and making tissue-specific expression in an *in vivo* system impossible. Other vector approaches use specially modified Pol II promoters, which cannot be easily exchanged for other Pol II promoters. The BLOCK-iT Pol II miR RNAi Expression Vectors include the CMV immediate-early Pol II promoter and are compatible with virtually any other Pol II promoter (Figure 8.7), providing a flexible system to regulate knockdown or to use promoters that are active in specific tissues for *in vivo* studies.

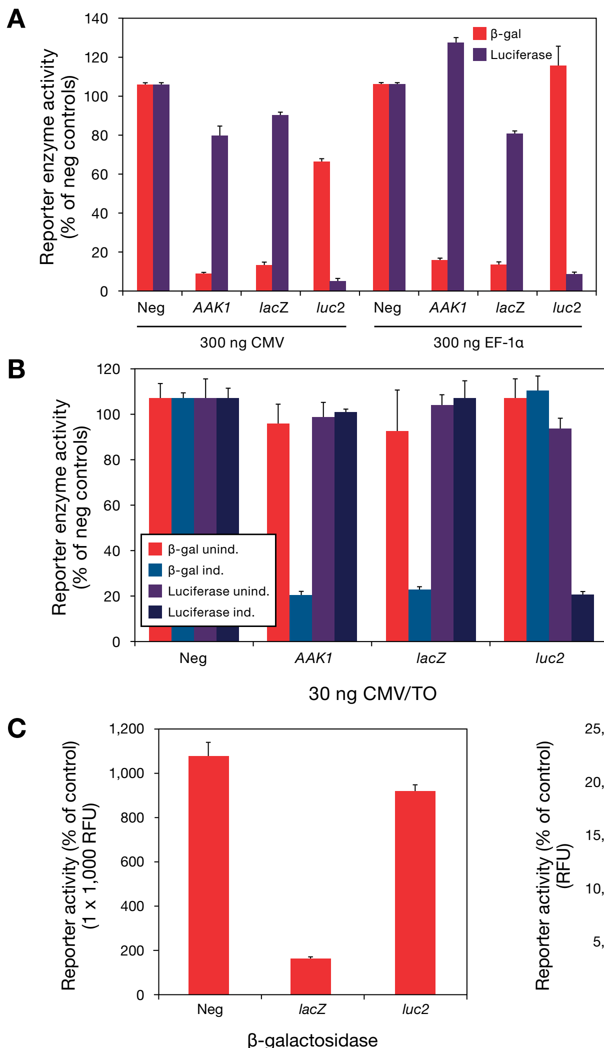


Figure 8.7. BLOCK-iT Pol II miR RNAi Expression Vectors are compatible with multiple promoters. (A) Normalized reporter activities from lysates of GripTite 293 cells cotransfected with 100 ng each of luciferase and *lacZ* reporter plasmids and 300 ng/well of either pcDNA 6.2-GW/EmGFP-miR (CMV) or pEF-GW/ EmGFP-miR (EF-1α) expression vectors. **(B)** Knockdown of pSCREEN-iT/*lacZ-AAK1* vector and luciferase reporters in T-REx 293 cells with and without induction with 1 µg/mL tetracycline at 30 ng of pT-REx-GW/EmGFP-miR (CMV/TO) plasmid per well. **(C)** Knockdown of cotransfected *lacZ* and luciferase reporter genes in HepG2 cells by MultiSite Gateway EmGFP-miR constructs with the anti-trypsin promoter as the 5´ element and the poly(A) signal from the HSV thymidine kinase gene as the 3´ element.

Inducible expression for control over your experiment

The Invitrogen™ BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit with EmGFP enables customers to regulate RNAi experiments (see Figure 8.8B). Now you can observe changes over time by controlling the initiation of the RNAi response with an inducible system. The kit contains the pT-REx-DEST30 Gateway vector which, after simple cloning and shuttling techniques, produces a miR RNAi expression vector suitable for inducible knockdown. The pT-REx-DEST30 Gateway vector contains the CMV promoter with two copies of the tetracycline operator ($TetO_2$) sequence, allowing high-level and regulated expression (Figure 8.8). This permits the study of loss of function in a stably transfected cell line even if the gene of interest is essential. Also, induction of miR RNAi expression can be halted so phenotypic changes can be measured during recovery of gene function.

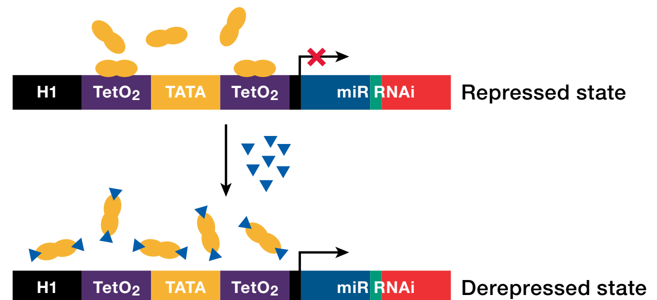


Figure 8.8. Inducible RNAi via tetracycline regulation of the CMV/TO Pol II promoter. When the tetracycline repressor (TR) protein is present in the cell, it tightly binds the two $TetO_2$ sites within the H1 promoter, essentially blocking initiation of shRNA transcription. When tetracycline is added to the culture medium, it binds to, and changes, the conformation of the TR protein, causing the TR protein to release from the two $TetO_2$ sites, allowing transcription to occur.

BLOCK-iT RNAi Entry Vector Kits

pENTR vectors for fast cloning and expression of shRNA cassettes

The Invitrogen™ BLOCK-iT™ Inducible H1 and BLOCK-iT U6 RNAi Entry Vectors (which contain the pENTR™/H1/TO and pENTR™/U6 vectors, respectively) provide a simple and streamlined approach for generating an RNAi cassette that consists of an inducible or constitutive Pol III promoter, the shRNA sequence, and the Pol III termination sequences. The first step for using this technology is to design a double-stranded DNA oligonucleotide with a sense-loop-antisense sequence against the target gene of interest. This sequence encoding the shRNA can be readily designed using the online **BLOCK-iT RNAi Designer**.

Clone the annealed shRNA oligo sequence into the pENTR vector using a convenient 5 min cloning reaction (Figure 8.9). Once the shRNA cassette has been generated in one of the entry vectors, the vector can be delivered directly into mammalian cells for knockdown studies, or the shRNA cassette can be transferred into another BLOCK-iT DEST vector using Gateway technology.

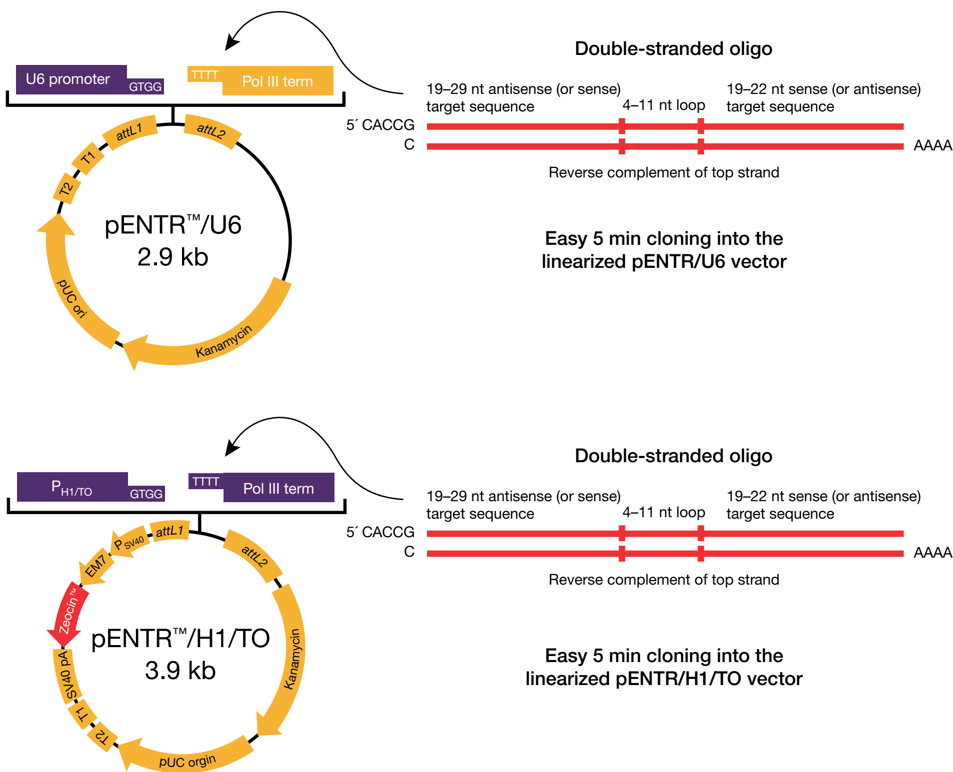


Figure 8.9. Easy shRNA cloning into inducible and constitutive BLOCK-iT entry vectors. A vector expressing the shRNA is generated by designing a short double-stranded DNA oligo containing a sense-loop-antisense sequence against the target. The sequence has 4-nucleotide overhangs at each end that can be ligated into the pENTR/H1/TO or pENTR/U6 entry vector via a brief benchtop reaction. The entry vector contains either the H1 promoter with two tetracycline operator sites flanking the TATA region, or the U6 Pol III type promoter and the Pol III terminator sequence. Cloning the double-stranded oligo sequence into the vector creates an RNAi cassette that expresses the shRNA.

Lentiviral and adenoviral RNAi vectors

Choose any cell type for RNAi

For many disease models, the most appropriate cell types, such as immune system or primary cells, are not amenable to transfection. Viral delivery of RNAi vectors is a powerful alternative to transfection for these cell types, as well as for *in vivo* applications. To accurately determine the efficacy of knockdown from an shRNA/miR RNAi molecule in a population of cells, it is critical to deliver the shRNA/miR RNAi molecule to as many cells as possible. Otherwise, when knockdown is measured by qRT-PCR or western blot analysis, the background of mRNA or protein in untransfected cells will make the knockdown appear less effective than it actually is. Viral delivery can be the best option in virtually any mammalian cell type, including hard-to-transfect, primary, and even nondividing cells. Conveniently, lentiviral delivery systems are available for both shRNA and miR RNAi vectors, and an adenoviral delivery system is available for shRNA vectors (Table 8.4).

The procedure for using both RNAi viral systems is as follows (Figure 8.10):

1. Clone the double-stranded DNA oligo encoding an shRNA or miR RNAi into one of the BLOCK-iT entry (shRNA) or expression (miR RNAi) vectors.
2. Transfer the RNAi cassette into the adenoviral (shRNA only) or lentiviral destination vector by Gateway recombination.
3. Transfect vectors into the appropriate packaging cells provided with the kit (use Invitrogen™ ViraPower™ packaging mix for lentiviral systems only) to produce viral stocks, which can be used immediately or stored at –80°C.
4. Harvest and (for adenovirus only) amplify the viral supernatant and use it for shRNA/miR RNAi delivery to any cell type.

Table 8.4. Choose a lentiviral or adenoviral RNAi system.

Viral system	When to use	Products
Lentiviral RNAi delivery systems	• Stable RNAi in any cell line, even nondividing cells • Inducible or constitutive shRNA or miR RNAi expression • Studies in animal models	• BLOCK-iT Lentiviral Pol II miR RNAi Expression System—a complete lentiviral system with all of the advantages of miR RNAi: multiple-target knockdown and a higher design success rate than conventional shRNA (contains pLenti6/V5-DEST vector) • BLOCK-iT Lentiviral Pol II miR RNAi Expression System with EmGFP—a system with all of the benefits listed above, plus easy expression tracking with cocistronic EmGFP (contains pLenti6/V5-DEST vector) • BLOCK-iT Inducible H1 Lentiviral RNAi System—complete lentiviral system for inducible or constitutive shRNA expression in any cell type (contains pLenti4/BLOCK-iT-DEST vector) • BLOCK-iT Lentiviral RNAi Expression System—complete lentiviral system for constitutive shRNA expression in any cell type (contains pLenti6/BLOCK-iT-DEST vector)
Adenoviral RNAi delivery system	• High-level transient shRNA expression • Effective delivery to a wide range of human cell types • Studies in animal models	• BLOCK-iT Adenoviral RNAi Expression System—complete system for high-level transient expression of shRNA

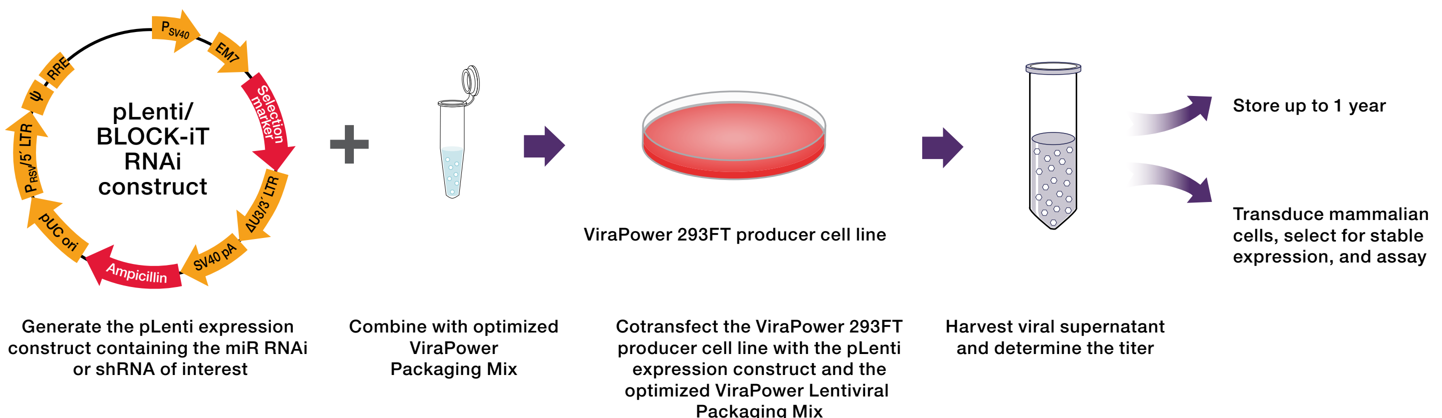


Figure 8.10. How the BLOCK-iT lentiviral system works.

Powerful shRNA delivery with BLOCK-iT viral vectors

The viral delivery systems include these Invitrogen vectors, which are also available separately (Figure 8.11):

- pLenti4/BLOCK-iT-DEST—lentiviral vector with the Invitrogen™ Zeocin™ selection marker for compatibility with a tetracycline-regulated inducible system
- pLenti6/BLOCK-iT-DEST—lentiviral vector with the blasticidin selection marker for fast, efficient selection for constitutive shRNA expression
- pAd/BLOCK-iT-DEST—adenoviral vector expediently produced without the need for a shuttle vector or for performing other labor-intensive methods

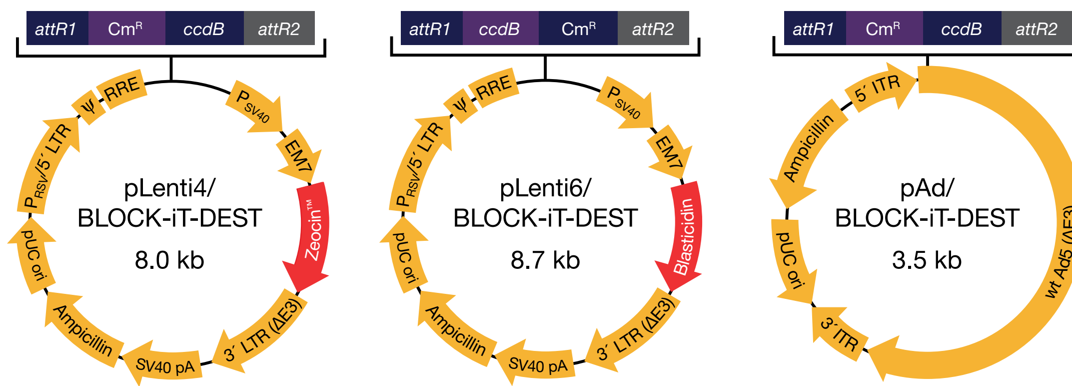


Figure 8.11. BLOCK-iT-DEST viral vectors. The BLOCK-iT Inducible H1 Lentiviral RNAi System can generate long-term inducible knockdown results whether a clonal population is used or individual clones are selected for analysis. The promoter configuration of the H1/TO vector ensures tight regulation; thus, unwanted background from shRNA expression will not interfere with results.

Product	Quantity	Cat. No.
BLOCK-iT Lentiviral Pol II miR RNAi Expression System	20 reactions	K4937-00
BLOCK-iT Lentiviral Pol II miR RNAi Expression System with EmGFP	20 reactions	K4938-00
BLOCK-iT Lentiviral RNAi Expression System	20 reactions	K4944-00
BLOCK-iT Adenoviral RNAi Expression System	20 reactions	K4941-00

BLOCK-iT Lentiviral RNAi System

Stable RNAi expression in nondividing mammalian cells

The RNAi cassette from the Invitrogen™ BLOCK-iT™ U6 entry vector can also be transferred into the pLenti6/BLOCK-iT-DEST or pLenti4/BLOCK-iT-DEST lentiviral RNAi vectors. First, generate lentiviral stocks with the 293FT cell line and associated ViraPower reagents, which are available separately or with the BLOCK-iT Lentiviral RNAi Expression System. Then prepare a lentiviral stock, transduce cells, and perform assay for the RNAi response (Figures 8.12 and 8.13). The BLOCK-iT lentiviral RNAi vectors possess all the required components for efficient packaging of the RNAi shRNA to dividing, hard-to-transfect, and nondividing cells, as well as for *in vivo* delivery to animal models.

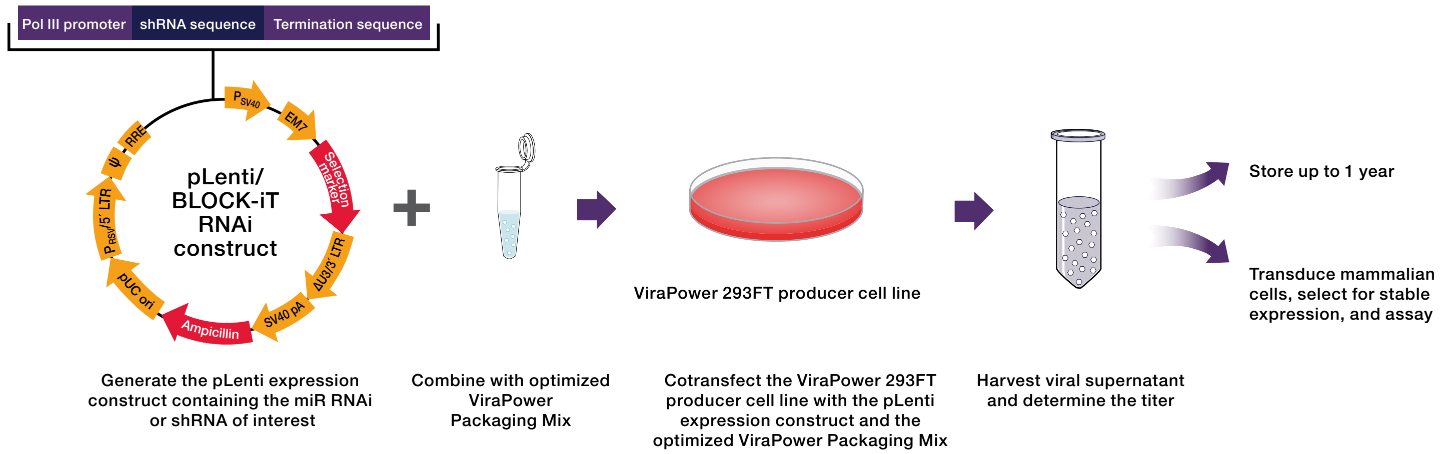


Figure 8.12. Generating BLOCK-iT lentiviral RNAi stock.

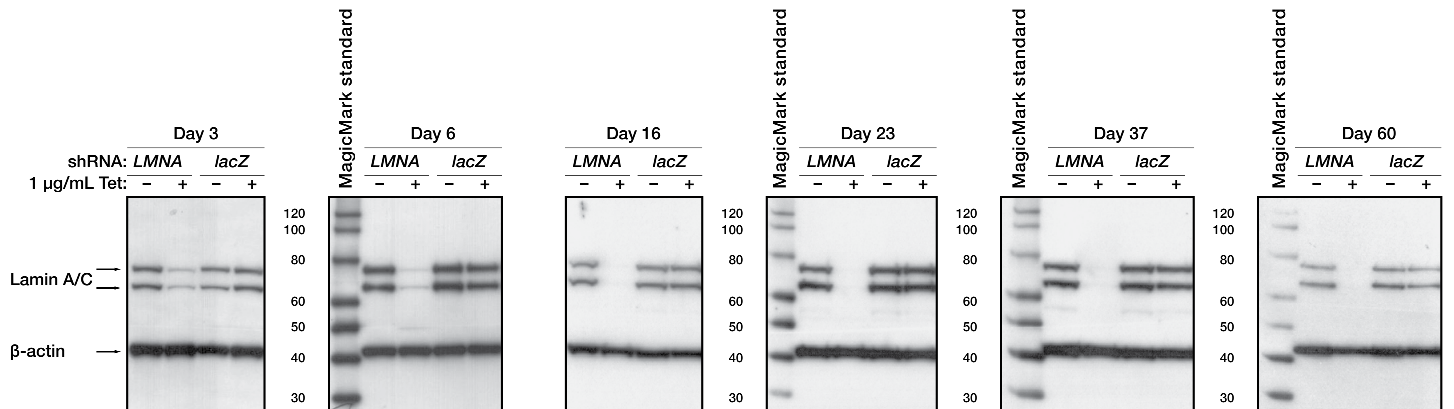


Figure 8.13. Long-term inducible knockdown in stably transduced T-REx HeLa cells. T-REx HeLa cells were transduced with lentiviral particles expressing *LMNA*-directed or control *lacZ*-directed shRNA from the tetracycline (Tet)-inducible H1/TO promoter at an effective MOI of <1. Stable populations of cells were selected with Zeocin reagent for 3 weeks and plated with or without Tet at 1 µg/mL for the number of days indicated. Whole-cell lysates were separated by electrophoresis, followed by western blotting and probing for antibodies against lamin A/C or β-actin (loading control).

BLOCK-iT Adenoviral RNAi Expression System

Efficient transient delivery and shRNA expression in mammalian cells

Adenoviral systems are popular platforms for reliable gene delivery and superior expression of transient shRNA in nearly any mammalian cell type. The key advantage of the Invitrogen™ BLOCK-iT™ Adenoviral RNAi Expression System is Gateway recombination technology, which simplifies the cloning and generation of an adenoviral vector, eliminating the tedious and time-consuming manipulations, screening, and multiple transformations that other adenoviral systems require.

Adenoviruses enter target cells by binding to the coxsackievirus and adenovirus receptor (CAR) [8]. After binding to CAR, the adenovirus is internalized via integrin-mediated endocytosis [9], followed by active transport to the nucleus where the shRNA is expressed. High-level transient shRNA expression is easily achieved with the BLOCK-iT Adenoviral RNAi System (Figure 8.14). Gateway recombination provides a means to quickly generate an adenoviral RNAi construct. For the first time, high-throughput cloning into adenoviral vectors is possible due to the efficacy of this recombination reaction.

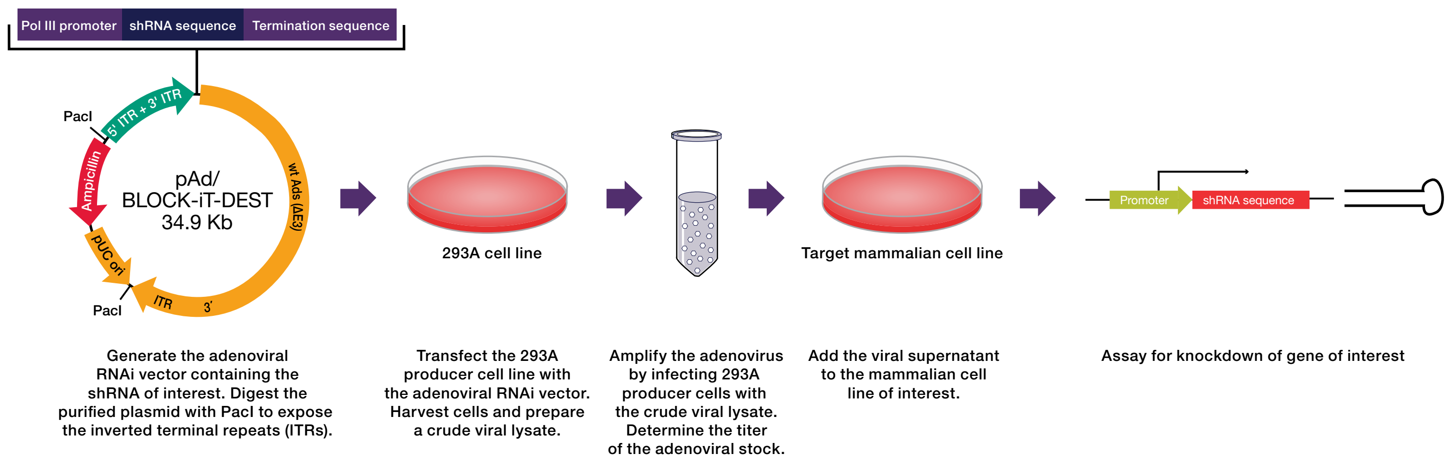


Figure 8.14. How the BLOCK-iT adenoviral RNAi system works.

Product	Quantity	Cat. No.
BLOCK-iT U6 RNAi Entry Vector Kit	20 constructions	K4945-00
T-REx-Jurkat Cell Line	1 x 10^7 cells	R722-07
T-REx-HeLa Cell Line	1 x 10^7 cells	R714-07
T-REx-293 Cell Line	1 x 10^7 cells	R710-07
pLenti6/TR Vector Kit	1 kit	V480-20
pcDNA6/TR	20 µg	V1025-20
BLOCK-iT Adenoviral RNAi Expression System	20 reactions	K4941-00
pAd/BLOCK-iT-DEST RNAi Gateway Vector	6 µg	V492-20
293A Cell Line	1 x 10^7 cells	R705-07

Chapter references

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Chapter 9

RNAi delivery

As introduced in Chapter 3, the delivery of siRNA or an RNAi vector is essential to experimental quality and reproducibility. For the most common RNAi applications, lipid-mediated transfection reagents are the delivery technique of choice.

Since 1993, Invitrogen™ Lipofectamine™ reagents have become the most referenced transfection reagents, with over 50,000 citations to date. They continue to be widely accepted for their overall reliability and ability to consistently transfect a wide variety of cell types with exceptional ease of use. **Cell type–specific protocols** are available to provide guidance for new users or for investigations of new cell types, and our optimization protocols (links in Table 9.1) provide step-by-step guidance to identifying the best conditions for low-toxicity and high-efficiency RNAi-mediated silencing.

Table 9.1. Selecting an Invitrogen™ Lipofectamine™ transfection reagent.

	Lipid-based transfection reagents			
RNAi delivery reagent	Lipofectamine RNAiMAX Transfection Reagent	Lipofectamine 3000 Reagent	Lipofectamine 2000 Transfection Reagent	Lipofectamine Stem Transfection Reagent
Cell types	Workhorse cell lines	Workhorse cell lines	Workhorse cell lines	Workhorse cell lines
	Stem cells	Stem cells		Stem cells
			Neurons	
	Cancer	Cancer	Cancer	
Payload		Plasmid DNA	Plasmid DNA	Plasmid DNA
	siRNA/miRNA			siRNA/miRNA
		Cotransfection	Cotransfection	
	Download protocol	Download protocol	Download protocol	Download protocol

	Viral transduction methods			
Expression system	BLOCK-iT Lentiviral Pol II miR RNAi	BLOCK-iT Lentiviral Pol II miR RNAi, with EmGFP	BLOCK-iT Adenoviral RNAi	BLOCK-iT Lentiviral RNAi
Duration of expression	Transient or stable	Transient or stable	Transient	Transient or stable
Promoter type	Constitutive or inducible	Constitutive or inducible	Constitutive	Constitutive
Expression tracking		EmGFP co-expression		

Lipofectamine RNAiMAX Transfection Reagent

Invitrogen™ Lipofectamine™ RNAiMAX™ Transfection Reagent is a proprietary, siRNA-specific cationic lipid formulation that offers the highest transfection efficiencies with the widest variety of cell types for siRNA gene knockdown experiments:

- Superior transfection efficiency enables use of lower siRNA concentrations and leads to more successful gene knockdown with minimal nonspecific effects (Figure 9.1A)
- Easy optimization due to low cytotoxicity across a 10-fold range of transfection reagent concentrations (Figure 9.1B)
- Versatile approach compatible with a wide range of cell types
- Simple and rapid protocol for consistent results

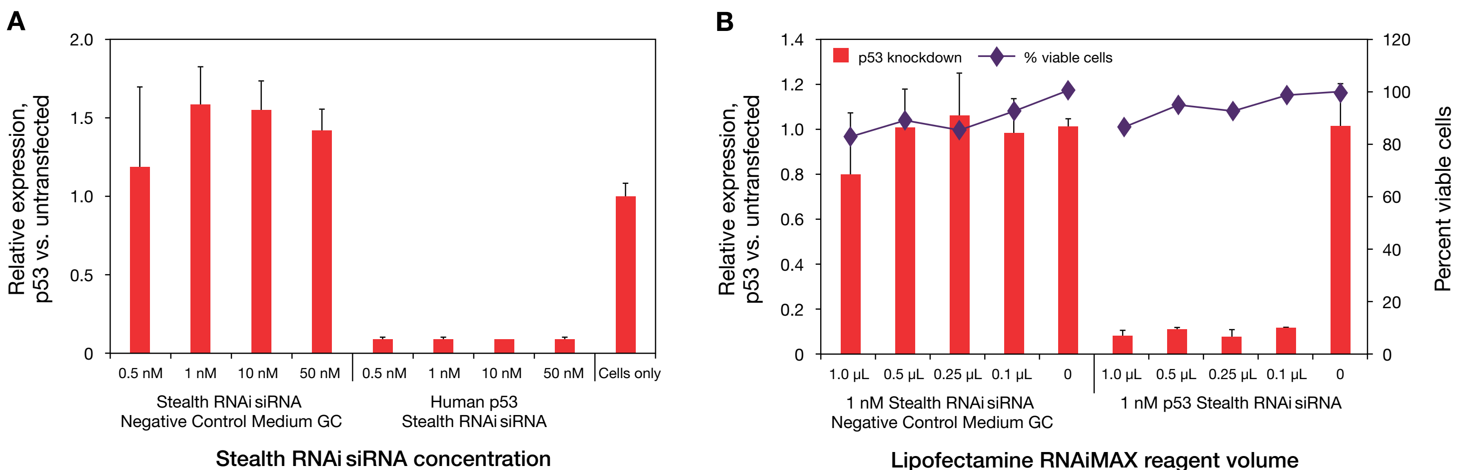


Figure 9.1. Optimal gene silencing and minimal cytotoxicity with Lipofectamine RNAiMAX Transfection Reagent. (A) Superior knockdown levels were observed with as little as 0.5 nM Invitrogen™ Stealth RNAi™ siRNA with Lipofectamine RNAiMAX Transfection Reagent. Transfection complexes containing 0.3 µL of Lipofectamine RNAiMAX Transfection Reagent were prepared in 48-well plates. A549 cells were added to each well to give final Stealth RNAi siRNA concentrations of 0.5 to 50 nM. Twenty-four hours after addition of cells, p53 knockdown was measured by qRT-PCR with Invitrogen™ LUX™ primers and normalized to GAPDH expression. The control duplex was the Stealth RNAi Negative Control Medium GC. **(B)** Minimal cytotoxicity over a 10-fold range of Lipofectamine RNAiMAX Transfection Reagent concentrations. Indicated volumes of transfection reagent were mixed with 10 nM p53 Validated Stealth RNAi siRNA or Stealth RNAi Negative Control Medium GC duplexes in 48-well plates. A549 cells were added to each well for a final siRNA concentration of 1 nM. Knockdown of p53 was measured as described in **A**. Over a 10-fold concentration range of Lipofectamine RNAiMAX Transfection Reagent, high levels of gene silencing were obtained without a dramatic increase in transfection-mediated cytotoxicity.

RNA interference

Simple protocol. The procedure for using the Lipofectamine RNAiMAX Transfection Reagent consists of mixing it with siRNA, adding cells, incubating, and measuring gene knockdown (Figure 9.2). The simplicity and speed, combined with superior transfection efficiency (Figure 9.3), make the Lipofectamine RNAiMAX Transfection Reagent the best choice for high-throughput siRNA transfections. Transfection conditions can be readily established for automated or robotic systems used in such applications. Protocols for using the Lipofectamine RNAiMAX Transfection Reagent can be found at **thermofisher.com/rnaimax**.

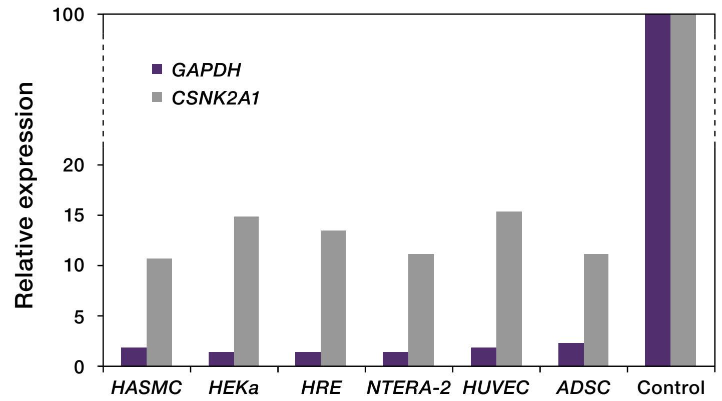


Figure 9.2. Superior knockdown of Invitrogen™ *Silencer*™ Select siRNA with Lipofectamine RNAiMAX Transfection Reagent.

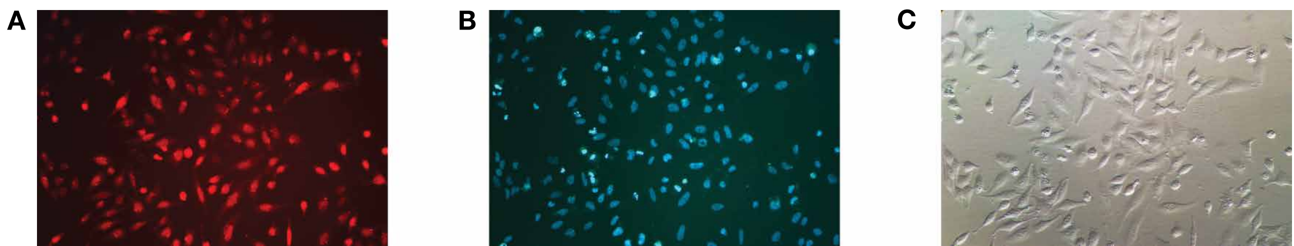


Figure 9.3. Assessing transfection efficiency of the Lipofectamine RNAiMAX Transfection Reagent with the Invitrogen™ BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control. (A) Nuclear localization of the red-fluorescent control oligo is shown after transfection of HeLa cells with the BLOCK-iT Alexa Fluor Red Fluorescent Control (50 nM) using the Lipofectamine RNAiMAX Transfection Reagent. **(B)** Twenty-four hours after transfection, growth medium was removed and replaced with PBS containing 10 mg/mL Hoechst 33342 for visualization of cell nuclei. **(C)** The brightfield image shows that the cells retain a normal morphology after transfection.

Product	Quantity	Cat. No.
Lipofectamine RNAiMAX Transfection Reagent	0.75 mL	13778-075
	1.5 mL	13778-150
BLOCK-iT Alexa Fluor Red Fluorescent Control	2 x 125 µL	14750-100

Note: Lipofectamine RNAiMAX Transfection Reagent is for use with siRNA or miRNA and is not recommended for RNAi vectors.

Lipofectamine 2000 and LTX Transfection Reagents

The Invitrogen™ Lipofectamine™ 2000 Transfection Reagent can be used for delivery of assorted RNAi agents, including shRNA and miR RNAi vectors and synthetic molecules such as siRNA, Stealth RNAi siRNA, and Dicer-generated siRNA pools. Invitrogen™ Lipofectamine™ LTX Reagent, used in combination with Invitrogen™ PLUS™ Reagent, is the choice reagent for delivery of assorted RNAi molecules into hard-to-transfect cell lines, including primary cells, for delivery of assorted RNAi molecules. Lipofectamine 2000 and Lipofectamine LTX reagents for RNAi transfection offer many benefits:

- Effective transfection for shRNA and miR RNAi vectors (Figure 9.4) and synthetics (siRNA and Stealth RNAi siRNA); also works well for cotransfection of synthetics and vectors
- Easy-to-follow protocols; media changes not required
- Convenient optimization of transfection conditions and efficiency with the Invitrogen™ BLOCK-iT™ Fluorescent Oligo
- Excellent performance in a wide variety of cell types

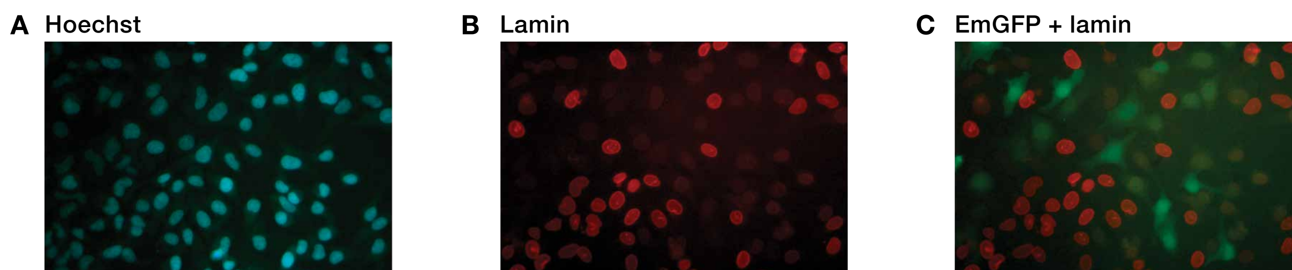


Figure 9.4. Delivery of Invitrogen™ BLOCK-iT™ Pol II miR RNAi expression vectors. Cells were transfected with Invitrogen™ pcDNA™6.2-GW/EmGFP-miR (directed against lamin) and Lipofectamine 2000 Transfection Reagent, at an expected 50% efficiency, to demonstrate the 100% tracking of Emerald Green Fluorescent Protein (EmGFP) and miRNA expression. After 48 hr, cells were stained with **(A)** Hoechst nuclear stain, which stains all cells, and **(B)** a red lamin stain, and monitored for GFP expression. Approximately half of the cells showed high expression of the lamin protein, but red-stained cells did not express EmGFP, and EmGFP-expressing cells did not express lamin **(C)**. This demonstrates the cocistronic expression of EmGFP and the miRNA that greatly reduces lamin expression.

Product	Quantity	Cat. No.
Lipofectamine LTX Reagent	1 mL	15338100
PLUS Reagent	0.85 mL	11514015
Lipofectamine 2000 Transfection Reagent	0.75 mL	11668-027
	1.5 mL	11668-019
BLOCK-iT Fluorescent Oligo for lipid transfection	2 x 125 µL	2013
BLOCK-iT Transfection Kit*	1 kit	13750070

* The BLOCK-iT Transfection Kit contains Lipofectamine 2000 Transfection Reagent and the BLOCK-iT Fluorescent Oligo.

Note: The BLOCK-iT Fluorescent Oligo (Cat. No. 2013) is optimized for use with the Lipofectamine 2000 Transfection Reagent and does not work well with the Lipofectamine RNAiMAX Transfection Reagent.

Neon Transfection System

High transfection efficiency and high cell viability in a broad range of cell lines

The Invitrogen™ Neon™ Transfection System provides:

- **Efficiency**—up to 90% in many cell types, including difficult-to-transfect, primary, and stem cells (Table 9.2)
- **Flexibility**—easily transfect from 2 x 10^4 cells to 6 x 10^6 cells per reaction
- **Simplicity**—single reagent kit for all cell types
- **Versatility**—open system allows electroporation parameters to be optimized freely

The Neon Transfection System is a novel benchtop electroporation device that uses a pipette tip as an electroporation chamber to efficiently transfect mammalian cells, including primary and immortalized hematopoietic cells, stem cells, and primary cells (Figure 9.5). The Neon Transfection System efficiently delivers nucleic acids, proteins, and siRNA into all mammalian cell types with a high cell survival rate. The transfection is performed using between 2 x 10^4 and 6 x 10^6 cells per reaction, in a sample volume of 10 µL or 100 µL, in a variety of cell culture formats (60 mm, 6-well, 48-well, and 24-well).

The Neon Transfection System uses a single transfection kit (Neon™ Kit) that is compatible with various mammalian cell types, including primary and stem cells, thereby avoiding the need to determine an optimal buffer for each cell type.

The Neon Transfection System offers open and transparent protocols that are optimized for ease of use and simplicity. The Neon™ device is preprogrammed with one 24-well optimization protocol to enable you to optimize conditions for your nucleic acid/siRNA and cell type, or you can program and store up to 50 cell-specific protocols in the Neon™ device database. Optimized protocols for many commonly used cell types are also available at **thermofisher.com/neon** for your convenience, to maximize transfection efficiencies for your cell types.

Neon system pipette tip design vs. standard electroporation cuvette

Unlike standard cuvette-based electroporation chambers, the Neon system uses a patented biologically compatible pipette tip chamber that generates a more uniform electric field (Figure 9.6). This design allows better maintenance of physiological conditions, resulting in very high cell survival compared to conventional electroporation [3].

Table 9.2. Examples of cell lines successfully transfected using the Neon Transfection System.

Cell line	Cell type	Transfection efficiency (%)*	Viable cells (%)
MEF primary	Embryonic fibroblast	80	75
293A	Kidney	90	90
3T3-L1	Mouse adipose	85	80
A549	Lung	75	92
Macrophages	Human (peritoneal)	60	60
MCF-7	Breast	70	80
HeLa	Cervical carcinoma	90	87
HL-60	Blood	55	70
PBMC	Blood	23	95
Primary rat cortical cells	Brain, cortical	42	99
Primary rat hippocampal cells	Brain, hippocampal	37	77
Raw 264.7	Blood	74	80

* Transfection efficiency is calculated from total population of live and dead cells. Protocols and reference information for a large number of cell lines are available in the Neon™ cell database at **thermofisher.com/neon**.

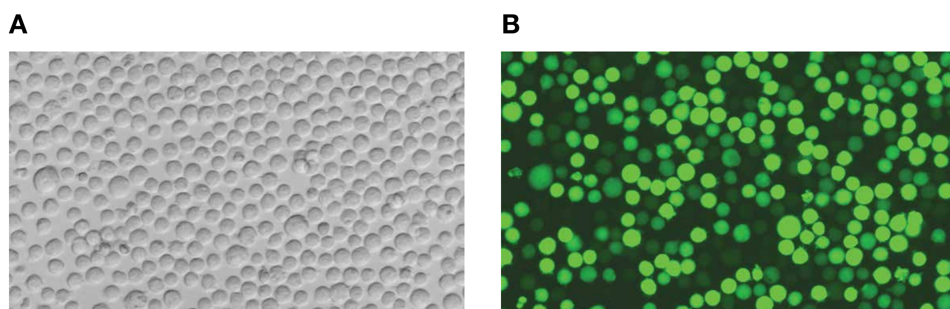


Figure 9.5. High transfection efficiency of Jurkat cells with the Neon Transfection System. **(A)** Intracellular uptake of reporter vector encoded with EGFP at 24 hr following transfection of Jurkat cells using the Neon Transfection System. **(B)** Fluorescence image corresponding to **A**.

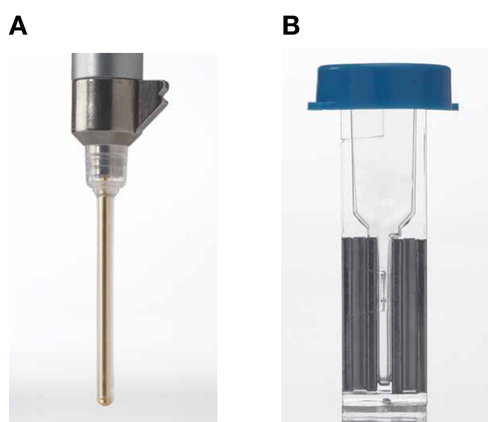


Figure 9.6. (A) Neon system pipette tip compared to (B) a standard electroporation cuvette. The design of the electrode in the pipette has been shown to produce a more uniform electric field. The result is less toxicity to the cells and higher transfection efficiencies.

Plug-and-play out of the box

The transfection processes could not be simpler (Figure 9.7). The Neon system uses a simple 3-step transfection procedure: take up the cells and plasmid mix into the Neon system pipette tip, plug it into the pipette holder, and press Start. The transfection occurs in the Neon system pipette tip. Then just pipette the transfected cells into your culture vessel. No more filling and pulling sample from the cuvette and capping and uncapping.

Simplified transfection kit for all cell types

Avoid the hassle of determining which proprietary buffer kit will work with your favorite cell type. We have simplified your work with just one transfection kit that is compatible with all cell types. Start your transfection with our optimized protocols for popular cell types, or follow our standard simple optimization procedure for all new cell types.

Figure 9.7. Simple 3-step transfection procedure. 1. Load mix of harvested cells and molecules to be delivered (e.g., DNA, RNA, protein) into the Neon system pipette tip. **2.** Plug the pipette into position in the Neon transfection device, select your protocol, and press Start. **3.** Unplug the pipette and transfer your transfected cells into a tissue culture vessel.

Product	Quantity	Cat. No.
Neon Transfection System 100 µL Kit	192 reactions	MPK10096
Neon Transfection System 10 µL Kit	192 reactions	MPK1096
Neon Transfection System	1 each	MPK5000
Neon Transfection System Starter Pack	1 pack	MPK5000S
Neon Transfection System Pipette	1 each	MPP100
Neon Transfection System Pipette Station	1 each	MPS100
Neon Transfection System Extended Warranty	1 each	MPSERV
Neon Transfection Tubes	1 pack	MPT100

Chapter references

1. Elbashir, S.M. et al. 2001. *Nature* 411:494–498.
2. Harborth, J. et al. 2001. *J. Cell. Sci.* 114:4557–4565.
3. Kim, J.A., Cho, K., Shin, M.S. et al. 2008. *Biosens. Bioelectron.* 23(9):1353–1360.

Chapter 10

RNA interference controls

Controls for RNAi experiments

Appropriate controls are essential to success in every RNAi experiment. The number and types of controls chosen depend upon the ultimate research goal [1]. With our RNAi technologies, performing the appropriate control reactions has been simplified (Table 10.1). RNAi control kits are designed to assist researchers in identifying and validating drug targets, generating publishable data, and accomplishing the following:

- Determining which RNAi reagents deliver the best knockdown results
- Achieving greater knockdown by optimizing transfection protocols
- Saving time by confirming cell health early in an experiment
- Proceeding confidently with RNAi experiments by comparing targeted RNAi reagents to a set of reagents optimized for silencing of positive control genes in human cells
- Using nontargeting negative controls that are validated to have no exact matches to human, mouse, or rat genomes

Table 10.1. Control kits ensure successful gene inhibition experiments.

	Components				
Product	**Fluorescent oligo**	**Transfection reagent**	**Dead-cell stain**	**RNAi positive controls**	**RNAi negative controls**
Silencer Select Positive Control siRNA (GAPDH)				•	
Silencer Select Negative Control siRNAs					•
Stealth RNAi siRNA Negative Controls					•
Stealth RNAi siRNA Positive Control (actin, GAPDH, cyclophilin B)				•	
Stealth RNAi siRNA Reporter Control				•	
Silencer Positive Control siRNAs				•	
Silencer Negative Control siRNAs					•
BLOCK-iT Transfection Optimization Kit (Human)	•		•		
BLOCK-iT Alexa Fluor Red Fluorescent Control	•				
BLOCK-iT Fluorescent Oligo	•				
BLOCK-iT Transfection Kit	•	•			
Applications	Track transfection			Monitor experimental variation	Measure the effect of experimental knockdown vs. background
	Optimize transfection conditions				
			Assess cell viability		

Silencer Select positive and negative control siRNAs

- Validated siRNA controls for optimizing siRNA experiments
- GAPDH Positive Control siRNA functionally tested in several common cell lines
- Negative Control siRNA functionally proven to have minimal effects on cell proliferation and viability
- Include *Silencer* Select siRNA modifications for enhanced specificity
- For use in human, mouse, and rat cells

Silencer Select GAPDH Positive Control siRNA

Our extensively validated positive control siRNA to human, mouse, and rat GAPDH serves multiple functions. First, it is an ideal "test" siRNA for those just beginning siRNA experiments, because it is validated to work in multiple cell lines. In addition, because it targets GAPDH mRNA, which is commonly used as an internal control, its effects are easy to assay, and thus it provides an excellent tool to monitor siRNA transfection efficiency by real-time RT-PCR as well as with the Invitrogen™ KDalert™ GAPDH Assay Kit. The Invitrogen™ *Silencer*™ Select GAPDH siRNA includes the same modifications for reducing off-target effects as found in other *Silencer* Select siRNAs.

Silencer Select Negative Control siRNAs

Negative control siRNAs—nontargeting siRNAs with sequences that do not have homology to any gene product—are essential for determining the effects of siRNA delivery on the cell and for providing a baseline to compare siRNA-treated samples. There are two extensively tested Invitrogen™ *Silencer*™ Select Negative Control siRNAs for this purpose. These siRNAs include the same modifications for reducing off-target effects found in other *Silencer* Select siRNAs and have no significant sequence similarity to mouse, rat, or human gene sequences. These negative control siRNAs have been tested by microarray analysis and shown to have minimal effects on gene expression. In addition, multiparametric cell-based assays have confirmed they have no significant effect on cell proliferation, viability, or morphology in the cell lines tested.

Product	Quantity	Cat. No.
Silencer Select GAPDH Positive Control siRNA	5 nmol	4390849
Silencer Select GAPDH Positive Control siRNA	40 nmol	4390850
Silencer Select GAPDH Positive Control siRNA, *In Vivo* Ready	250 nmol	4404024
Silencer Select Negative Control #1 siRNA	40 nmol	4390844
Silencer Select Negative Control #1 siRNA	5 nmol	4390843
Silencer Select Negative Control #1 siRNA, *In Vivo* Ready	250 nmol	4404020
Silencer Select Negative Control #2 siRNA	5 nmol	4390846
Silencer Select Negative Control #2 siRNA	40 nmol	4390847

Stealth RNAi siRNA negative controls

Invitrogen™ Stealth RNAi™ siRNA negative controls provide the means to measure the effect of a Stealth RNAi siRNA duplex targeted to a specific gene, compared to background. These controls have the following features:

- Three levels of GC content (low, medium, high), with three sequences available for matching GC content to that of experimental Stealth RNAi siRNA duplexes. When a single negative control is desired for use with multiple Stealth RNAi siRNAs, the medium GC control is recommended as the most all-purpose.
- No homology to any known vertebrate genes
- Tested sequences do not induce stress response

The Stealth RNAi siRNA Negative Control Kit contains all three controls (low, medium, and high GC content, excluding sequences #2 and #3), and each is also available separately.

Product	GC content	Suitable for use with Stealth RNAi siRNA duplexes with the following GC content	Quantity	Cat. No.
Stealth RNAi siRNA Negative Control Kit			1 kit	12935-100
Stealth RNAi siRNA Negative Control Lo GC	36%	35–45%	250 µL	12935-200
Stealth RNAi siRNA Negative Control Lo GC Duplex #2	36%	35–45%	250 µL	12935-110
Stealth RNAi siRNA Negative Control Lo GC Duplex #3	36%	35–45%	250 µL	12935-111
Stealth RNAi siRNA Negative Control Med GC	48%	45–55%	250 µL	12935-300
Stealth RNAi siRNA Negative Control Med GC Duplex #2	48%	45–55%	250 µL	12935-112
Stealth RNAi siRNA Negative Control Med GC Duplex #3	48%	45–55%	250 µL	12935-113
Stealth RNAi siRNA Negative Control Hi GC	68%	55–70%	250 µL	12935-400
Stealth RNAi siRNA Negative Control Hi GC Duplex #2	68%	55–70%	250 µL	12935-114
Stealth RNAi siRNA Negative Control Hi GC Duplex #3	68%	55–70%	250 µL	12935-115

Stealth RNAi siRNA reporter controls

Invitrogen™ Stealth RNAi™ siRNA reporter controls are ideal for RNAi experiments to optimize your transfection conditions in any vertebrate cell line. These controls are:

- Designed to efficiently knock down their intended targets
- Not homologous to any transcripts in the vertebrate transcriptome

Product	Quantity	Cat. No.
Stealth RNAi siRNA GFP Reporter Control	250 µL	12935-145
Stealth RNAi siRNA Luciferase Reporter Control	250 µL	12935-146
Stealth RNAi siRNA *lacZ* Reporter Control	250 µL	12935-147
Stealth RNAi siRNA β-Lactamase Reporter Control	250 µL	12935-148

Stealth RNAi siRNA positive controls

Invitrogen™ Stealth RNAi™ siRNA positive housekeeping control duplexes (human) are ideal controls for assessing knockdown and optimizing RNAi experiments. These controls are:

- Designed to efficiently knock down their intended targets
- Bench-tested and supplied in a ready-to-use format

Product	Quantity	Cat. No.
Stealth RNAi siRNA GAPDH Positive Control (human)	250 µL	12935-140
Stealth RNAi siRNA Actin Positive Control (human)	250 µL	12935-141
Stealth RNAi siRNA Cyclophilin B Control (human)	250 µL	12935-142

BLOCK-iT Transfection Optimization Kit (Human)

The BLOCK-iT Transfection Optimization Kit is designed to optimize RNAi transfection with Lipofectamine 2000 Transfection Reagent, through the use of controls for transfection and cell viability. Each kit contains a BLOCK-iT Fluorescent Oligo, a dead-cell stain, a positive control Stealth RNAi siRNA duplex directed at human p53, and a scrambled Stealth RNAi siRNA duplex as a negative control.

Product	Quantity	Cat. No.
BLOCK-iT Transfection Optimization Kit (human)	1 kit	13750047

BLOCK-iT Alexa Fluor Red Fluorescent Control and BLOCK-iT Fluorescent Oligo

To achieve significant levels of specific gene inhibition, siRNA must be taken up by target cells. Factors such as poor cell health and high passage number can negatively impact transfection efficiency. The Invitrogen™ BLOCK-iT™ Fluorescent Oligo and the Invitrogen™ BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control allow you to easily visualize transfection results and thus provide key controls for RNAi experiments. These stabilized RNA duplexes help determine transfection efficiency by providing:

- Strong, easily detectable signals that indicate transfection efficiency (Figure 10.1)
- Clear, persistent signals that exceed the intensity of other labeled RNA duplexes
- Predominant nuclear localization to confirm that the control has been internalized
- Unique sequences that have no homology to any known gene, avoiding induction of nonspecific or off-target effects

The BLOCK-iT Fluorescent Oligo and the BLOCK-iT Alexa Fluor Red Fluorescent Control strongly correlate with transfection efficiency of Invitrogen™ Stealth RNAi™, *Silencer*™ Select and *Silencer*™ siRNAs, *mir*Vana™ miRNA mimics and inhibitors, as well as Pre-miR™ miRNA mimics and Anti-miR™ miRNA inhibitors.

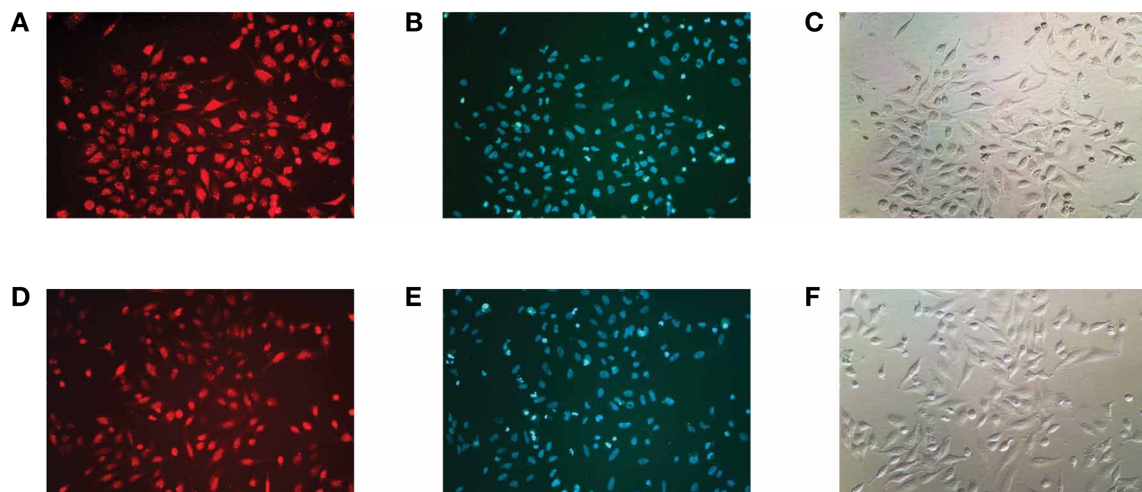


Figure 10.1. BLOCK-iT Alexa Fluor Red Fluorescent Control for clear visualization of transfection results. HeLa cells were transfected with the BLOCK-iT Alexa Fluor Red Fluorescent Control using either **(A–C)** Invitrogen™ Lipofectamine™ Transfection Reagent 2000 or **(D–F)** Invitrogen™ Lipofectamine™ RNAiMAX Transfection Reagent. The recommended HeLa cell transfection protocol was used for each reagent, and the final control concentration was 50 nM. Twenty-four hours after transfection, growth medium was removed and replaced with PBS containing 10 µg/mL Hoechst 33342 for visualization of cell nuclei **(B, E)**. Nuclear localization of the control is seen with both transfection reagents **(A, D)**. Almost 100% of the cells take up the control, and cells retain a normal morphology, as seen in the brightfield images **(C, F)**.

BLOCK-iT Transfection Kit

The BLOCK-iT Transfection Kit enables you to shorten the process of optimizing RNAi transfection conditions and monitoring transfection variation in every experiment. To optimize transfection parameters, the BLOCK-iT Transfection Kit contains Lipofectamine 2000 Transfection Reagent and the BLOCK-iT Fluorescent Oligo (Figure 10.2).

Note: The BLOCK-iT Fluorescent Oligo is optimized for use with Lipofectamine 2000 Transfection Reagent and is not recommended for use with Lipofectamine RNAiMAX Transfection Reagent.

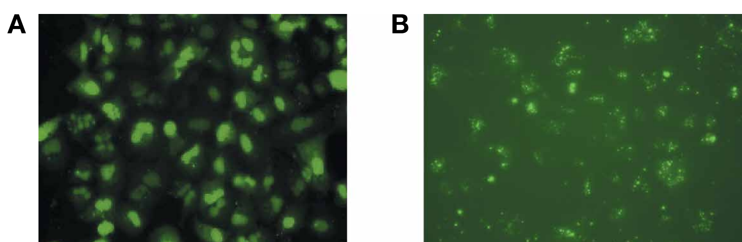


Figure 10.2. Strong fluorescence signal to optimize transfection. A549 cells were transfected with BLOCK-iT Fluorescent Oligo or standard fluorescently labeled RNA, using Lipofectamine 2000 Transfection Reagent. **(A)** Twenty-four hours later, the fluorescent signal had persisted in the cells transfected with the BLOCK-iT Fluorescent Oligo. **(B)** In contrast, the fluorescent signal was largely undetectable in cells transfected with the standard labeled siRNA.

Product	Quantity	Cat. No.
BLOCK-iT Alexa Fluor Red Fluorescent Control (lipid transfection)	2 x 125 µL	14750-100
BLOCK-iT Fluorescent Oligo, for lipid transfection	2 x 125 µL	2013
BLOCK-iT Fluorescent Oligo, for electroporation	75 µL (1 mM)	13750-062
BLOCK-iT Transfection Kit	1 kit	13750-070
Lipofectamine RNAiMAX Transfection Reagent	1.5 mL	13778-150
	0.75 mL	13778-075
Lipofectamine 2000 Transfection Reagent	1.5 mL	11668-019
	0.75 mL	11668-027

Silencer negative control siRNAs

- Nontargeting siRNAs that have limited sequence similarity to known genes
- Validated for use in human, mouse, and rat cells
- Functionally proven to have minimal effects on cell proliferation and viability
- Available individually and as a panel of 7 negative control siRNAs
- HPLC-purified, duplexed, and ready to use

Negative controls

Negative control siRNAs—siRNAs with sequences that do not target any gene product—are essential for determining transfection efficiency and to control for the effects of siRNA delivery. In siRNA screening applications, negative control siRNAs are also critical for setting the "hit" threshold that determines whether an siRNA is considered to have a positive, negative, or neutral effect in a particular assay.

We designed and tested seven negative control siRNAs that have no significant sequence similarity to mouse, rat, or human gene sequences. They have all been tested extensively in cell-based screens to ensure they have no significant effect on cell proliferation, viability, or morphology. Invitrogen™ *Silencer*™ Select Negative Controls #1 and #2, our most popular negative control siRNAs, are available in 40 nmol and 5 x 40 nmol sizes, in addition to the standard 5 nmol size.

***In Vivo* Ready Negative Control**

The "*In Vivo* Ready" Invitrogen™ *Silencer*™ Negative Control #1 siRNA is subjected to an extra level of purification and testing required for the introduction of siRNAs into animals. After HPLC purification and annealing, each siRNA is further purified using a semipermeable membrane to remove excess salt. The result is highly pure siRNA with minimal salt content, suitable for *in vivo* applications. *In Vivo* Ready siRNAs are then filter-sterilized and tested for the presence of endotoxin. At concentrations of 50 µM in the presence of deionized water, *In Vivo* Ready siRNAs contain <0.6 mM Na^+, <2.0 mM K^+, and <0.1 mM Mg^{2+}.

Product	Quantity	Cat. No.
Silencer Negative Control #1 siRNA	5 x 40 nmol	AM4636
Silencer Negative Control #1 siRNA	40 nmol	AM4635
Silencer Negative Control #1 siRNA	5 nmol (50 µM)	AM4611
Silencer Negative Control #1 siRNA, *In Vivo* Ready	250 nmol	4404021
Silencer Negative Control #2 siRNA	5 x 40 nmol	AM4638
Silencer Negative Control #2 siRNA	40 nmol	AM4637
Silencer Negative Control #2 siRNA	5 nmol (50 µM)	AM4613
Silencer Negative Control #3 siRNA	5 nmol (50 µM)	AM4615
Silencer Negative Control #4 siRNA	5 nmol (50 µM)	AM4641
Silencer Negative Control #5 siRNA	5 nmol (50 µM)	AM4642
Silencer Negative Control #7 siRNA	5 nmol (50 µM)	AM4644

Silencer positive control siRNAs

- Validated siRNA controls for optimizing siRNA experiments
- Gene-specific control siRNAs provided with scrambled negative controls
- Functionally tested in several common cell lines
- HPLC-purified, duplexed, and ready to use

Premade, gene-specific siRNA controls

Silencer control siRNAs, which target mRNAs frequently used as internal controls in applications designed to monitor gene expression, such as RT-PCR, northern blot, and RPA, are ideal for developing and optimizing siRNA experiments. Each *Silencer* control siRNA is validated for use in human cell lines, including the GAPDH siRNA, which has also been optimized for mouse and rat. (The cyclophilin siRNA has also been validated in mouse cell lines.) For measuring silencing at the protein level, a selection of antibodies are available, including antibodies against β-actin, cyclophilin, GAPDH, and Ku proteins. Each *Silencer* control siRNA contains 5 nmol of ready-to-use chemically synthesized siRNA. A negative control siRNA (2 nmol) is included with most of these siRNAs. The popular *Silencer* GAPDH siRNAs are also available in 40 nmol and 5 x 40 nmol sizes.

In Vivo Ready Positive Control

Our "*In Vivo* Ready" Invitrogen™ *Silencer*™ GAPDH Positive Control siRNA is subjected to an extra level of purification and testing required for the introduction of siRNAs into animals. After HPLC purification and annealing, each siRNA is further purified using a semipermeable membrane to remove excess salt. The result is highly pure siRNA with minimal salt content, suitable for *in vivo* applications. *In Vivo* Ready siRNAs are then filter-sterilized and tested for the presence of endotoxin. At concentrations of 50 µM in the presence of deionized water, *In Vivo* Ready siRNAs contain <0.6 mM Na^+, <2.0 mM K^+, and <0.1 mM Mg^{2+}.

Product	Quantity	Cat. No.
Silencer c-Myc siRNA	5 nmol (dried)	AM4250
Silencer Firefly Luciferase (GL2 + GL3) siRNA	5 nmol + 2 nmol Neg Control (50 µM)	AM4629
Silencer GAPDH Positive Control siRNA, *In Vivo* Ready	250 nmol	4404025
Silencer GAPDH siRNA (Human)	5 nmol + 2 nmol Neg Control (50 µM)	AM4605
Silencer GAPDH siRNA (Human)	40 nmol	AM4633
Silencer GAPDH siRNA (Human)	5 x 40 nmol	AM4634
Silencer GAPDH siRNA (Human, Mouse, Rat)	5 nmol + 2 nmol Neg Control (50 µM)	AM4624
Silencer GAPDH siRNA (Human, Mouse, Rat)	5 x 40 nmol	AM4632
Silencer GAPDH siRNA (Human, Mouse, Rat)	40 nmol	AM4631
Silencer GFP (eGFP) siRNA	5 nmol + 2 nmol Neg Control (50 µM)	AM4626
Silencer KIF11 (Eg5) siRNA (Human, Mouse, Rat)	5 nmol + 2 nmol Neg Control (50 µM)	AM4639

Chapter reference

1. Editors (2003) Whither RNAi? *Nat. Cell Biol.* 5:489–490.

Chapter 11

Measuring knockdown

Functional validation following RNAi knockdown

For RNAi results to be considered valid, relevant levels of target-specific knockdown must be demonstrated. Ideally, measurement showing a substantial decrease of both the cellular mRNA and the targeted protein would be demonstrated. Knowing the level of knockdown is critical for interpretation of functional and phenotypic RNAi effects. Typically, 70% knockdown, as measured by quantitative reverse transcription PCR (qRT-PCR), is considered the minimum knockdown required for a successful RNAi experiment.

A knockdown level equal to or exceeding 70% might give a negative phenotypic result (i.e., no change in phenotype relative to control), but this outcome does not necessarily mean that the gene of interest is not functionally relevant. Some genes may not show a phenotypic response even at greatly reduced levels of mRNA. This lack of an observable response can be due to the protein having a long half-life (thus, measuring knockdown by western blotting might be desirable) or due to a very small amount of protein being sufficient to achieve normal cellular function. If the latter is the case, it is imperative to generate the highest possible level of knockdown to see an effect.

TaqMan Gene Expression Assays

- Gene-specific Applied Biosystems™ TaqMan® probe and primer sets for quantitative gene expression studies in human, mouse, rat, and 30 other species
- Convenient single-tube format and 20X or 60X formulation
- Universal thermal cycling conditions

Applied Biosystems™ TaqMan® Gene Expression Assays (Figure 11.1) are a comprehensive collection of more than 1.2 million predesigned primer and TaqMan probe sets designed to quickly and easily perform quantitative gene expression studies on human, mouse, rat, and 30 other species. Each gene expression assay consists of an Applied Biosystems™ FAM™ dye–labeled TaqMan® MGB probe and a pair of PCR primers designed to amplify the target of interest from cDNA. Assays are supplied as preformulated mixes (20X or 60X) in ready-to-use single tubes. Every assay has been optimized to run under universal thermal cycling conditions at a final reaction concentration of 250 nM for the probe and 900 nM for each primer. We have combined a comprehensive selection of assays with a streamlined approach to provide you with a convenient, standardized process for quantitative gene expression.

For straightforward access to TaqMan Gene Expression Assays to detect siRNA-mediated silencing, simply access the Related Assays section (Figure 11.1) from any siRNA search result on **thermofisher.com/siRNA**. You can also search for your assay using public ID numbers (including RefSeq ID, Entrez Gene ID, or Unigene ID), common gene names, symbols, or aliases, and functional categories and groups (such as kinases, cytokines, transcription factors, etc.). Visit **thermofisher.com/taqman**.

Figure 11.1. Screenshot of how to access TaqMan qPCR Assays specific to each predesigned siRNA.

Custom TaqMan Gene Expression Assays

- Available for any species or organism
- Use the target sequence of your choice
- Provided in a convenient single-tube format

Custom TaqMan Gene Expression Assays are available for any species, any splice variant, or any novel gene. Simply use the Custom TaqMan Assay Design Tool (**thermofisher.com/cadt**) to format and submit your target sequence, or to search for specific sequences or predesigned assays for your gene of interest. The software easily guides you through the ordering process, from selecting the assay size, formatting your target sequence to identifying the ideal location of the probe and submitting your order via email. All file submissions are done in a secure format to ensure that your target sequences and the associated assays that are designed remain confidential. With Custom TaqMan Gene Expression Assays, you benefit from our proprietary software algorithms for primer and probe design, which enable you to obtain optimal assays for each target sequence. Assays are delivered in a single-tube, ready-to-use format, along with the primer and probe sequences designed from your submitted sequence.

Description	Concentration	# of 20 µL reactions	Applied Biosystems™ dye labels	Universal formulation	Delivery time	Cat. No.
TaqMan Gene Expression Assays						
Inventoried	20X	250	FAM dye	Yes	3–5 days	4331182
Made-to-Order	20X	360	FAM dye	Yes	5–10 days	4351372
Custom TaqMan Gene Expression Assays	20X	360	FAM dye	Yes	10–14 days	4331348
	20X	750				4332078
	60X	2,900				4332079
TaqMan Endogenous Controls						
Not primer-limited			FAM dye	Yes		Various
Primer-limited			VIC dye	Yes		
Custom TaqMan Probes			FAM dye VIC dye TET dye NED dye	No	4–7 days	Various: see page 91

Custom TaqMan probes and primers

- Choice of dye labels, quenchers, and synthesis scale
- Available for any species or organism
- For use in quantitative gene expression, SNP genotyping, other allelic discrimination applications, and pathogen detection

When you know the exact sequences you need for your TaqMan probes and primers, we can synthesize them for you (Table 11.1). As the industry leader in real-time PCR, we provide high-quality custom products that can be used in all your real-time and endpoint applications. These products offer you the ideal in flexibility if you prefer to optimize your own reaction formulation or you simply prefer to buy in bulk.

Table 11.1. TaqMan probe usage chart for gene expression. For gene expression, the minimum numbers of reactions obtained from our TaqMan probe products were calculated based on universal assay conditions, primer concentrations of 900 nM, and probe concentrations of 250 nM. The numbers are shown for 50 µL and 20 µL reactions.

TaqMan probe quantity	Number of 50 µL reactions (96-well plates)	Number of 20 µL reactions (384-well plates)
6,000 pmol	480	1,200
20,000 pmol	1,600	4,000
50,000 pmol	4,000	10,000

Product	Delivery time	Quantity	Cat. No.
Applied Biosystems™ TaqMan® TAMRA™ Probes	4–5 days	6,000 pmol*	450025
	4–5 days	20,000 pmol*	450024
	4–5 days	50,000 pmol*	450003
TaqMan Probes are available with a choice of 5′ fluorescent label—6-FAM, VIC, or TET dye*—and 3′ Quencher TAMRA probe. All probes are HPLC-purified and sequence-verified by mass spectrometry.			
Applied Biosystems™ TaqMan® MGB Probes	6–7 days	6,000 pmol*	4316034
	6–7 days	20,000 pmol*	4316033
	6–7 days	50,000 pmol*	4316032
TaqMan MGB probes are available with a choice of 5′ fluorescent label—6-FAM, VIC, TET,* or NED dye**—and a 3′ minor groove binder (MGB)/nonfluorescent quencher (NFQ). All probes are HPLC-purified and sequence-verified by mass spectrometry. For Research Use Only. Not for use in diagnostic procedures.			

* Please note that filter-based instruments such as the Applied Biosystems™ ABI PRISM™ 7000 Sequence Detection System and Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR Systems are not supplied with calibration plates for TET dye. These instruments may be custom calibrated to use TET dye in a singleplex reaction, but TET dye should not be used in a multiplex reaction with either FAM or VIC dyes, as the TET dye will not be distinguished by these instruments.

** Please note that the Applied Biosystems 7500 Real-Time PCR System is optimized for use with NED dye–labeled probes. Probes labeled with NED dye will give lower signal intensities on other real-time instruments than probes labeled with 6-FAM, VIC, or TET dye. 3′ label: MGBNFQ (minor groove binder/nonfluorescent quencher).

qRT-PCR directly from cells

Cells-to-C_T family of kits

- Complete, preoptimized expression workflows in a single box produce robust and reliable results
- Prepare samples and perform DNase treatment at room temperature in 10 minutes or less
- Results equivalent to purified RNA with validated accuracy, reproducibility, and sensitivity
- Validated with hundreds of primer sets and TaqMan Gene Expression Assays

Invitrogen™ Cells-to-C_T™ kits enable you to quickly and easily transform cultured cells into real-time RT-PCR results. A breakthrough cell lysis and RNA stabilization technology eliminates the need for RNA purification. Because samples can be processed directly in culture plates (96- or 384-well), sample handling and the potential for sample loss or transfer error are minimized, resulting in higher reproducibility. However, Cells-to-C_T kits do not stop at sample preparation. The lysis technology is integrated into a complete workflow that includes reverse transcription reagents and high-performance Applied Biosystems™ TaqMan® Assay– or SYBR™ Green I–based PCR master mixes (Figure 11.2). For more information, visit **thermofisher.com/cellstoct**.

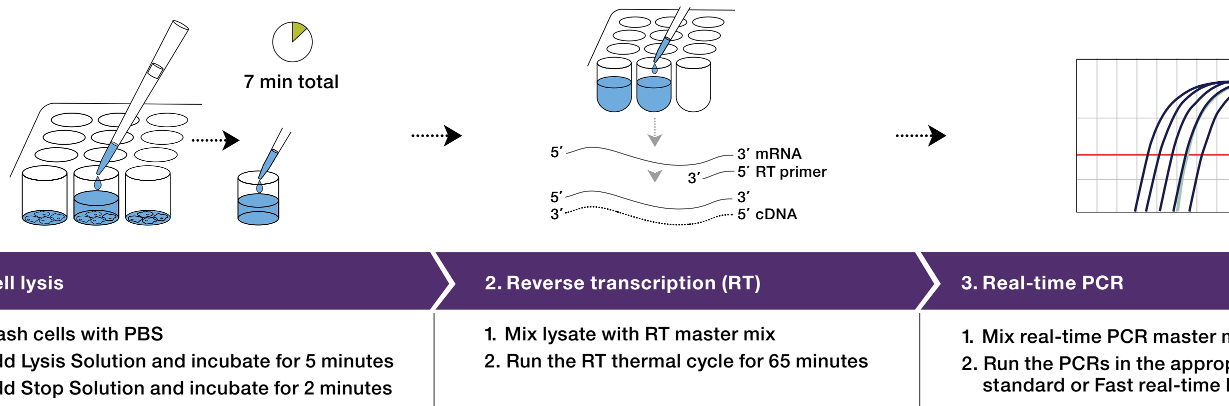


Figure 11.2. Cells-to-C_T kits provide a complete workflow from cells to qRT-PCR. Cells-to-C_T kits require 10 min or less to release nucleic acids into a cell lysate at room temperature. This cell lysate is compatible with the included reverse transcriptase and real-time PCR reagents.

Protein separation and western blotting

To verify that RNAi-mediated knockdown of a gene results in a decrease in the target protein, we recommend analysis of protein from RNAi-treated cells by protein separation and western blotting techniques. We supply kits and reagents as well as services for protein separation, staining, blotting, and detection. Popular Invitrogen™ products include the NuPAGE™ gel system, SimplyBlue™ SafeStain, MagicMark™ XP Western Protein Standard, and WesternBreeze™ Immunodetection Kits.

The NuPAGE™ Novex™ precast gel system is a revolutionary high-performance polyacrylamide gel system. The NuPAGE™ system consists of NuPAGE™ Novex™ Bis-Tris gels and buffers for small to mid-sized proteins, and NuPAGE™ Novex™ Tris-acetate gels for larger proteins. The unique formulation and neutral operating pH of NuPAGE™ gels during electrophoresis offer significant advantages over other gel systems:

- Longest shelf life—up to 1 year
- Best resolution, sharpest bands
- Fastest run times and most efficient transfers
- Highest protein capacity and greatest protein stability

For a complete list of products or custom protein services, visit **thermofisher.com/proteomics**.

Antibodies and immunodetection

Invitrogen™ immunodetection offerings include research and pathology antibodies, flow cytometry instruments and reagents, cytokine and signaling products, and labeling and detection products. Find high-quality antibodies, assays, and kits for immunodetection at **thermofisher.com/antibodies**.

Nucleic acid purification and quantification

Invitrogen™ nucleic acid purification technologies, formats, and kits help meet the challenges posed by particular nucleic acid types, sample sources and volumes, throughput levels, and downstream applications. Featured products include the Invitrogen™ TRIzol™ line of products and Invitrogen™ PureLink™ DNA and RNA purification systems. For quantifying DNA, RNA, and proteins, Invitrogen™ Qubit™ technology provides the most accurate method. Qubit technology uses sensitive dyes that become highly fluorescent upon binding to their targets. Unlike other assays that rely solely on absorbance, assays using these dyes are extremely selective for the molecule being quantified. Our products for nucleic acid purification and quantification comprise one of the most comprehensive collections available:

- **Technologies**—reagents, filter columns, plates, and beads (magnetic and nonmagnetic)
- **Formats**—manual, high-throughput, and automatable
- **Sample types**—cells, blood, and tissue, including formalin-fixed, paraffin-embedded (FFPE) forensic samples and more
- **Macromolecules**—purification and quantification of DNA, RNA, and protein

Visit **thermofisher.com/nap** for a complete description of nucleic acid purification and quantification technologies.

Chapter 12
RNAi services

RNAi services

Access years of scientific experience

Strategic outsourcing can dramatically increase the pace of discovery and decrease the cost of product development. An outsourcing partner contributes specialized scientific expertise and state-of-the-art technology—whether in molecular biology, RNAi delivery, or development of cell-based or biochemical assays. Allowing researchers to focus their efforts on determining the best targets and compounds to pursue, our scientists provide the expertise required to take a project from beginning to end in a timely, professional manner.

Our custom RNAi Services utilizes our strengths in synthetic siRNA chemistry, screening, and downstream assays to help customers achieve robust knockdown and to further the understanding of complex biological systems. RNAi Services offers many advantages:

- State-of-the-art technologies and equipment, and high-throughput capabilities
- Quality RNAi reagents with proven performance—Invitrogen™ *Silencer*™ Select siRNA, Ambion™ *In Vivo* siRNA, and *mir*Vana™ miRNA reagents
- A specialized scientific team with years of experience in gene knockdown studies
- Fast, reliable results that are competitively priced
- Professional customer support and consulting

Each RNAi Services project can be customized to fit specific scientific goals. Whether you need assistance in designing RNAi reagents or you desire a more expansive service such as high-throughput screening, we have the resources to accelerate your research.

RNAi design services

Successful RNAi experiments start with well-designed molecules. Our RNAi Design Services uses a proprietary algorithm to generate highly effective siRNA designs. This service can accommodate a variety of sequence requests:

- Design of large numbers of RNAi sequences for one or numerous genes
- Design of sequences to target different splice variants or multiple species
- Design of siRNAs targeting virtually any species

siRNA and miRNA custom synthesis

A wide variety of chemical modifications on siRNA or microRNA reagents are available to suit your experimental needs.

- Invitrogen™ Alexa Fluor™ dyes or other fluorophores
- Biotin conjugation
- 2´-O-methyl or 2´-fluoro bases
- Phosphorothioate linkages

We will assess feasibility on any modified siRNA or microRNA reagent, simply send your request to **RNAiSupport@thermofisher.com**.

Delivery optimization services

Achieving robust gene inhibition begins with efficient delivery of RNAi reagents with minimal toxicity. Optimizing the RNAi transfection conditions for a particular cell line is the first step in any RNAi experiment, and we have been optimizing transfection in a broad range of cell lines for nearly two decades. For easy-to-transfect cell lines, we typically use cationic lipid transfection reagents. For difficult-to-transfect cell lines, we recommend viral delivery. The Delivery Optimization Services uses our extensive knowledge and expertise with viral vectors and nonviral reagents to test a matrix of delivery parameters to find the optimal delivery conditions for the cell line of interest.

RNAi functional validation services

Invitrogen can rapidly identify effective RNAi reagents that can be used in further functional studies. Understanding that the goal of each RNAi experiment is unique, Invitrogen offers a wide array of approaches to screen for the most effective RNAi reagents.

Quantitative RT-PCR (qRT-PCR)—measure mRNA knockdown

This method sensitively measures endogenous mRNA levels. If the target gene is expressed at a reasonable level in the specified cell line, qRT-PCR is fast and reliable.

RNAi target screening system—use a reporter-based system for preliminary tests

This method is preferred for high-throughput applications or to screen for effective RNAi reagents before moving experiments into a difficult cell line. The Stealth RNAi™ siRNA or siRNA duplex is cotransfected with the pSCREEN-iT™/lacZ reporter vector, and β-galactosidase levels are used to quantitatively determine the effectiveness of the RNAi duplex.

Western blot analysis—measure protein knockdown

This method is best for clearly showing that loss of function is due to specific endogenous protein knockdown, and removes concerns that protein half-life is affecting phenotype.

Dose response/IC_{50} assays—determine optimal conditions for a chosen system

Dose response data show the dynamic range of RNAi suppression and enable the most appropriate siRNA concentration to be selected for a chosen cell type. IC_{50} is the concentration of the siRNA duplex that is required for 50% inhibition of the target and is commonly used as a measure of drug effectiveness. The dose response is also a good way to determine conditions that show optimal knockdown and minimal toxicity associated with the delivery method. Invitrogen can evaluate siRNA duplexes at various time points in a particular cell line.

Phenotypic assay development and high-throughput screening services

The ultimate goal of gene knockdown is to observe a change in phenotype. Having many years of experience with RNAi experiments and phenotypic assays, we will use this experience and knowledge to help develop and conduct assays tailored to specific research projects. The RNAi services focus on delivering the most reliable data utilizing the most appropriate RNAi technology for phenotypic assays. A wide range of phenotypic assays can be performed for a variety of target classes—kinases, phosphatases, proteases, nuclear receptors, G-protein–coupled receptors (GPCRs), ion channels, and cytochrome P450s—including assays of the following types:

- Morphological
- Enzymatic
- Biochemical
- Cell-based
- Customized

RNAi custom collaborative research

Using a fully collaborative approach, we can work with you to custom-design and execute an RNAi approach to fit your needs. From simple custom assays to long-term arrangements for target discovery and validation, we have the history, experience, and technology to help you succeed. Contact us to discuss your vision.

Note: Find information on RNAi services online at **thermofisher.com/customservices**.

Chapter 13
MicroRNA modulation and analysis

As the complexity of a genome increases, so does the ratio of noncoding to coding RNAs [1]. Noncoding RNAs appear to be involved in a variety of cellular roles, ranging from simple housekeeping functions to complex regulatory functions. Of the various subclasses of noncoding regulatory RNAs, microRNAs (miRNAs) are the most thoroughly characterized. These RNAs are typically 19 to 22 nucleotides long and are thought to regulate gene expression posttranscriptionally by binding to the 3´ untranslated regions (UTRs) of target mRNAs and inhibiting their translation [2]. Version 22 of the MirBase database suggests that the number of unique miRNAs in humans exceeds 2,500, though several groups have hypothesized that there may be up to 20,000 noncoding RNAs that contribute to eukaryotic complexity [3,4].

MicroRNA genesis and function

Both RNA polymerase II and III transcribe miRNA-containing genes, generating long primary transcripts (pri-miRNAs) that are processed by the RNase III–type enzyme Drosha, yielding hairpin structures 70 to 90 bp in length (pre-miRNAs). Pre-miRNA hairpins are exported to the cytoplasm, where they are further processed by the RNase III protein Dicer into short double-stranded miRNA duplexes 19 to 22 nucleotides long. The miRNA duplex is recognized by the RNA-induced silencing complex (RISC), a multiple-protein nuclease complex. One of the two strands, the guide strand, assists this protein complex in recognizing its cognate mRNA transcript. The RISC:miRNA complex often interacts with the 3´ UTR of target mRNAs at regions exhibiting imperfect sequence homology, inhibiting protein synthesis by a mechanism that has yet to be fully elucidated (Figure 13.1).

Plant miRNAs can bind to sequences on target mRNAs by exact or nearly exact complementary base pairing and thereby direct cleavage and destruction of the mRNA [5,6]. Similar to the mechanism employed in RNA interference (RNAi), the cleavage of a single phosphodiester bond on the target mRNA occurs between bases 10 and 11 [7]. In contrast, nearly all animal miRNAs studied so far do not exhibit perfect complementarity to their mRNA targets, and seem to inhibit protein synthesis while retaining the stability of the mRNA target [2]. It has been suggested that transcripts may be regulated by multiple miRNAs, and an individual miRNA may target numerous transcripts. Research suggests that as many as one-third of human genes may be regulated by miRNAs [8]. Several unique physical attributes of miRNAs, including their small size, lack of polyadenylated tails, and tendency to bind their mRNA targets with imperfect sequence homology, have made them elusive and challenging to study.

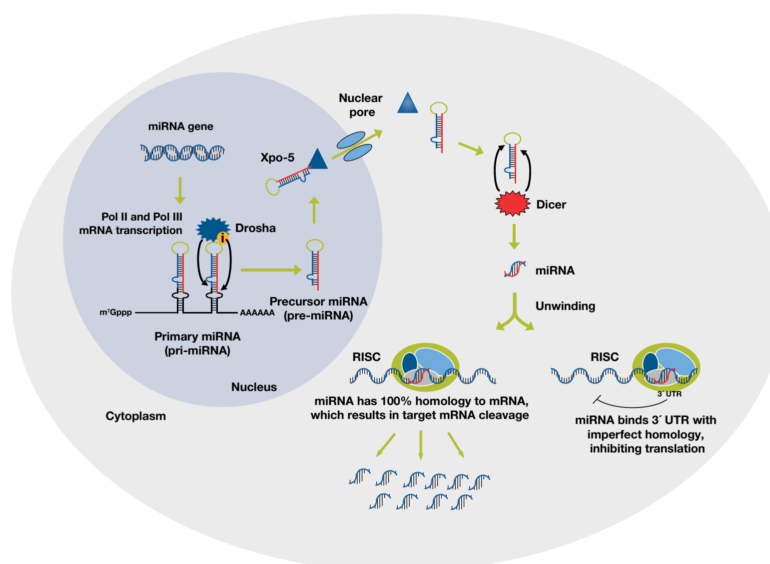


Figure 13.1. Biogenesis and function of miRNA. MicroRNA transcripts, generated by RNA polymerases II and III, are processed by the RNase III enzymes Drosha (nuclear) and Dicer (cytoplasmic), yielding 19–22 nucleotide miRNA duplexes. One of the two strands of the duplex is incorporated into the RISC complex, which regulates protein expression.

MicroRNA expression profiling

Profiling the differences in global miRNA expression between samples is a useful first step in identifying specific miRNAs that influence a biological process. For example, a researcher might compare the miRNA profiles in diseased vs. healthy tissue, compound-treated vs. untreated samples, or different organs from a single subject with the intention of identifying those miRNAs that are expressed at different levels between the sample types.

Since miRNAs can influence cellular function, even at very low concentrations, and can be expressed over an extremely wide range, miRNA quantitation requires tools with high sensitivity and a broad dynamic range. Applied Biosystems™ Megaplex™ Primer Pools, in conjunction with Applied Biosystems™ TaqMan® Array MicroRNA Cards, are ideal for such experiments. Using these tools, researchers can generate an expression profile for 754, 518, or 303 miRNAs from human, mouse, or rat, respectively, in a single working day from as little as 1 ng of input total RNA. Taking full advantage of the gold-standard sensitivity, specificity, and dynamic range afforded by TaqMan® Assay chemistry, and incorporating our innovative stem-loop RT primer design for PCR of tiny targets, Megaplex Primer Pools provide significant benefits over microarrays, which require several days and hundreds of nanograms of input RNA to generate data.

MicroRNA profiling using TaqMan Assay technology

Features of the workflow:

- Results the same day—complete an experiment profiling hundreds of miRNAs in as little as 5 hours (Figure 13.2)
- Ideal for human, mouse, and rat profiling
- Requires only minute sample amounts—as little as 1 ng of total RNA input—making it suitable for FFPE, FACS, biopsy, and other very small samples
- Comprehensive coverage of known miRNAs consistent with Sanger miRBase v14 and v10, for human and mouse, respectively

How it works

Up to 381 miRNAs are reverse transcribed in a single reaction using Megaplex™ RT Primers, a mixture of miRNA-specific stem-loop primers. Next, an optional amplification step can be performed using Megaplex™ PreAmp Primers. This unbiased amplification step significantly increases the concentration of miRNAs in the sample enabling maximum sensitivity and detection by real-time PCR. For the final quantitation step, the TaqMan® Universal PCR Master Mix is added to each sample and the mixtures are pipetted into the sample loading ports of a TaqMan Array MicroRNA Card—a preconfigured microfluidic card containing 384 TaqMan MicroRNA Assays. The real-time PCR is run on Applied Biosystems™ Real-Time PCR Systems.

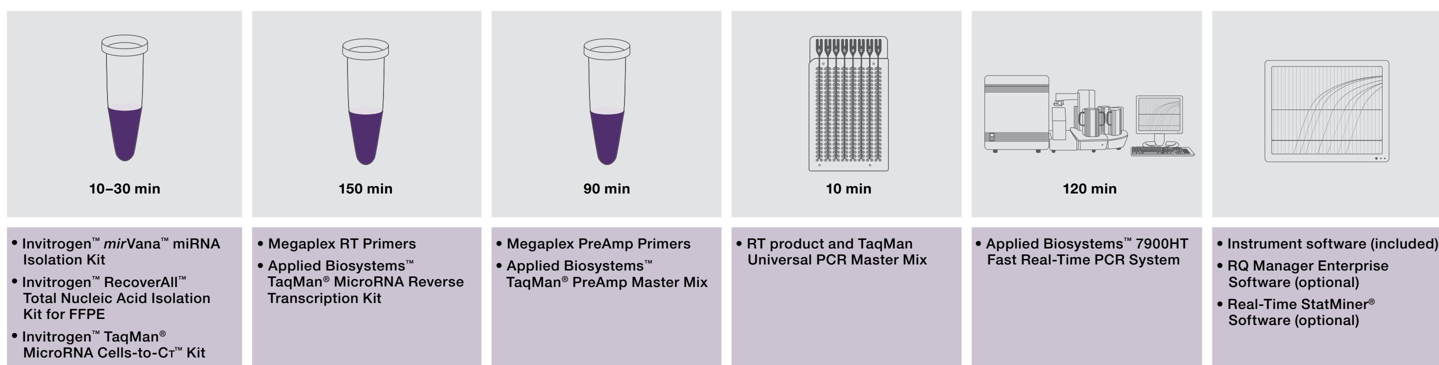


Figure 13.2. MicroRNA profiling workflow using TaqMan Assay technology.

Megaplex Primer Pools

Whether your profiling experiment requires ultimate sensitivity, broad coverage, or both, Megaplex Primer Pools offer the flexibility to enable you to accomplish your research goals (Figure 13.3). Providing comprehensive coverage of Sanger miRBase v14 and v10, for human and mouse, respectively, when used with TaqMan MicroRNA Arrays, Megaplex Primer Pools deliver the ideal miRNA profiling solution for both human and rodent species.

- Megaplex RT Primers—Designed to streamline miRNA profiling, Megaplex RT Primers are pools of RT primers identical to those found in individual TaqMan MicroRNA Assays. Megaplex RT Primers are ideal for global miRNA expression profiling; just two pools provide comprehensive coverage of the Sanger miRBase v10 database content, and their content matches the corresponding TaqMan MicroRNA Array Human or Rodent MicroRNA Card. However, they can also be used to prepare cDNA for individual TaqMan MicroRNA Assays.
- Megaplex PreAmp Primers—When samples are limiting or assay sensitivity is of utmost importance, Megaplex PreAmp Primers significantly help enhance the ability to detect human and rodent miRNAs with low expression levels or from limited amounts of sample. Megaplex PreAmp Primers enable the generation of a comprehensive miRNA expression profile, using as little as 1 ng of input total RNA (Figure 13.4). With content matched to the Megaplex RT Primers and the TaqMan Array Human or Rodent MicroRNA Cards, cDNA prepared by reverse transcription with Megaplex RT Primers can be preamplified using Megaplex PreAmp Primers and TaqMan PreAmp Master Mix to uniformly amplify all miRNAs that were present in the original sample.

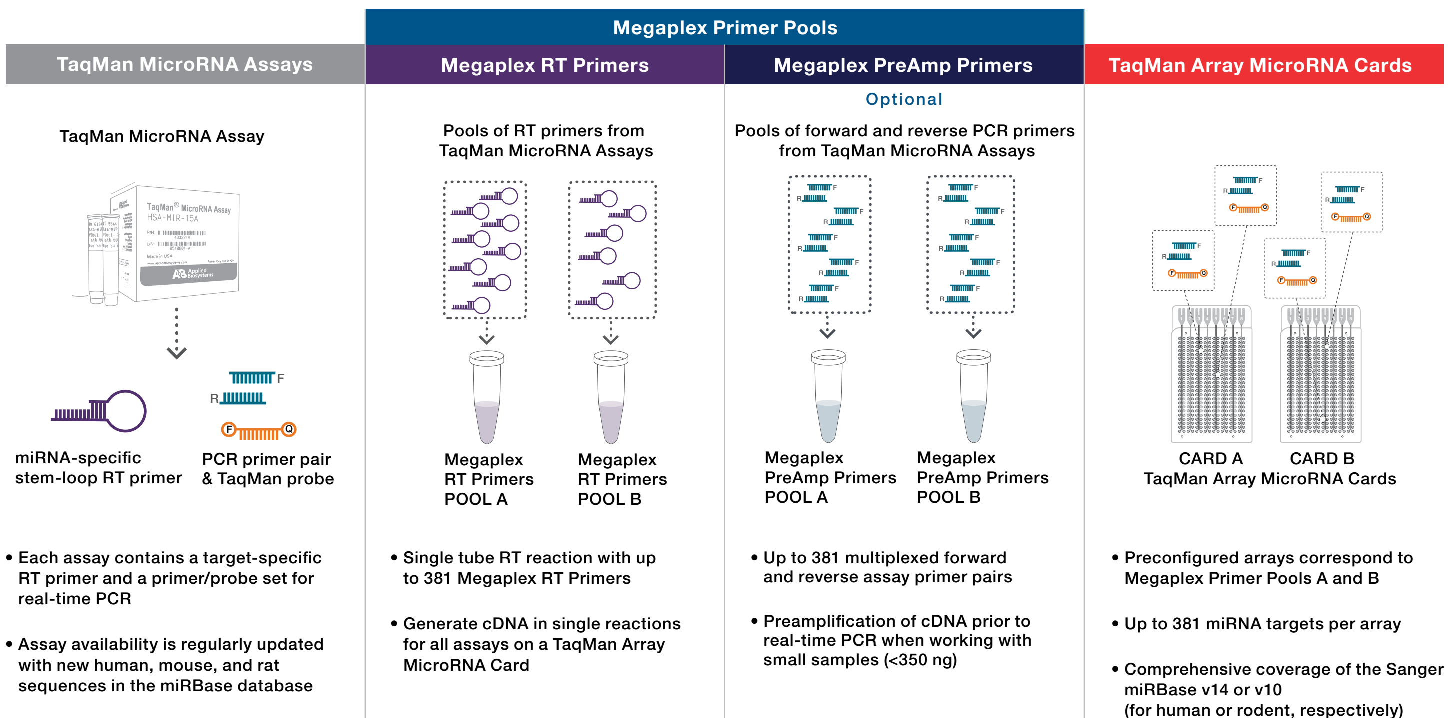


Figure 13.3. Megaplex Primer Pools bring the power of real-time PCR to microRNA profiling experiments.

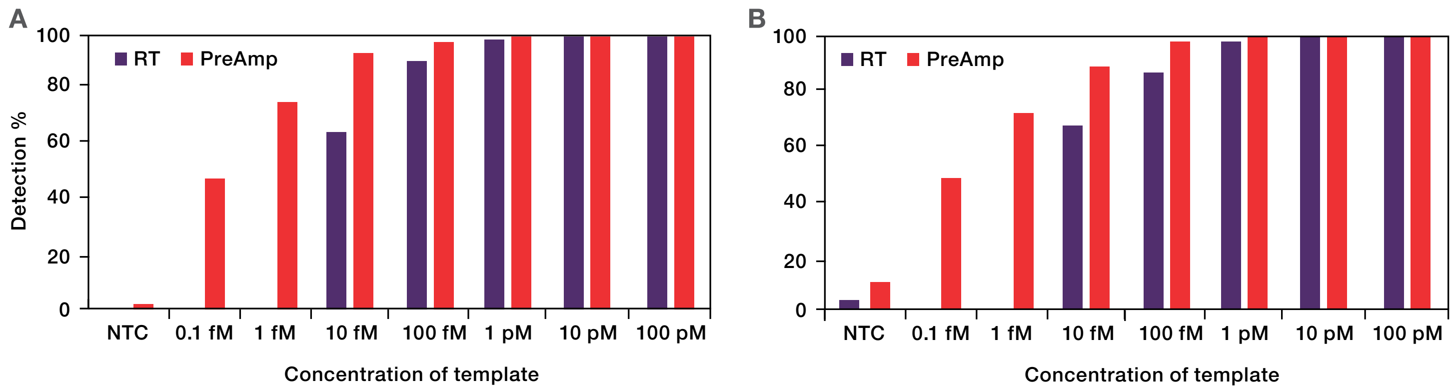


Figure 13.4. MicroRNA detection using Megaplex Primer Pools and TaqMan Array MicroRNA Cards. Synthetic artificial targets (10 pM) for each assay represented on the A and B TaqMan Array MicroRNA Cards were spiked into a complex total RNA background (10 ng/µL). The mixture was serially diluted across a range of 6 or 4 orders of magnitude, and the artificial targets were detected using the Megaplex primer pools workflow with and without the preamplification step. In addition, no-template control (NTC) reactions were performed to confirm assay specificity. Real-time PCR quantitation was performed using corresponding TaqMan Array Human or Rodent MicroRNA Cards, on a 7900HT Fast Real-Time PCR System. **(A)** Detection rate of Megaplex Human Pool A content. **(B)** Detection rate of Megaplex Human Pool B content.

TaqMan Array MicroRNA Cards

Ideal for human or rodent profiling, TaqMan Array MicroRNA Cards provide all the advantages of TaqMan MicroRNA Assays in a convenient, preconfigured microfluidic card. The content of each card is matched to the respective Megaplex Primer Pools and contains up to 381 unique TaqMan MicroRNA Assays, reducing setup time and experimental variability. Following reverse transcription of miRNA targets using Megaplex RT Primers, and optional preamplification with Megaplex PreAmp Primers, TaqMan Universal Master Mix II is simply combined with each reaction and pipetted into each of the eight sample loading ports of the TaqMan Array cards. This simplifies sample handling and helps increase sample throughput. A set of two TaqMan MicroRNA Arrays for each species provides assays to cover Sanger miRBase v14 or v10 (for human or rodent, respectively) database content. When used in conjunction with Megaplex RT Primers and optional Megaplex PreAmp Primers, a comprehensive dataset can be generated in as little as five hours.

TaqMan MicroRNA Reverse Transcription (RT) Kit

The Applied Biosystems™ TaqMan® MicroRNA RT Kit provides all the necessary components for optimal TaqMan MicroRNA Assay performance. Components of this kit are used with the single RT primer provided with each individual TaqMan MicroRNA Assay, or with Megaplex RT Primers, to convert miRNA to cDNA prior to real-time PCR quantitation.

Detect and quantify specific microRNAs

Detailed studies on specific miRNAs are often conducted to validate previous results or to learn more about these miRNAs. Through the use of novel adaptations in assay design, we bring the benefits of TaqMan Assays and quantitative real-time PCR—unparalleled sensitivity, specificity, and dynamic range—to miRNA detection and quantitation. Applied Biosystems™ TaqMan® MicroRNA Assays incorporate a target-specific stem-loop reverse transcription primer. This innovative design addresses a fundamental problem in miRNA quantitation: the short length of mature miRNAs (~22 nt). The stem-loop structure provides specificity for only the mature miRNA target and forms an RT primer/mature miRNA chimera that extends the 3′ end of the miRNA. The resulting, longer RT product presents a template amenable to standard TaqMan® real-time PCR assay. To facilitate accurate results, every TaqMan MicroRNA Assay is functionally validated under laboratory conditions. We offer a comprehensive collection of TaqMan MicroRNA Assays covering a broad collection of species, including but not limited to human, mouse, rat, *Arabidopsis*, *C. elegans*, and *Drosophila*. In addition, new assays are added on a regular basis in accordance with updates to the Sanger miRBase Registry.

Targeted microRNA quantitation workflow

Features of the workflow (Figure 13.5):

- Ideal for quantitating individual miRNAs from a broad assortment of species, including human, mouse, rat, *Drosophila*, *C. elegans*, and *Arabidopsis*
- Each kit includes the TaqMan Assay and reverse transcription primer specific for the mature miRNA target of interest
- Assays are continually added for human, mouse, and rat species to stay aligned with the Sanger miRBase Registry

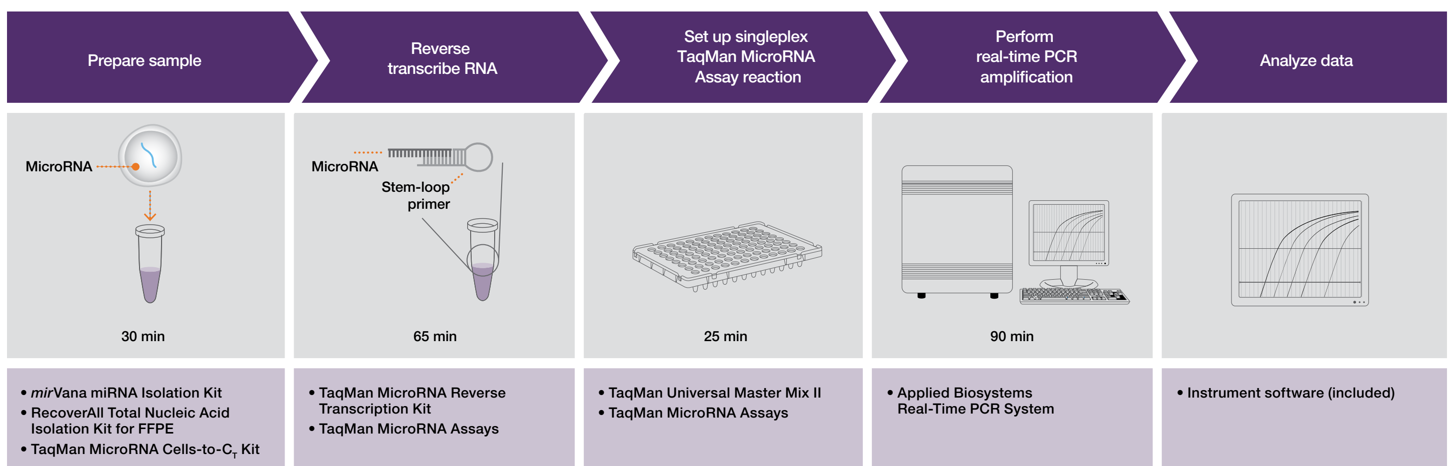


Figure 13.5. Targeted microRNA quantitation workflow.

How it works

TaqMan MicroRNA Assays employ an innovative target-specific stem-loop reverse transcription primer to address the challenge of the short length of mature miRNA. The primer extends the 3′ end of the target to produce a template that can be used in standard TaqMan Assay–based real-time PCR (Figure 13.6). Also, the stem-loop structure in the tail of the primer confers a key advantage to these assays: specific detection of the mature, biologically active miRNA.

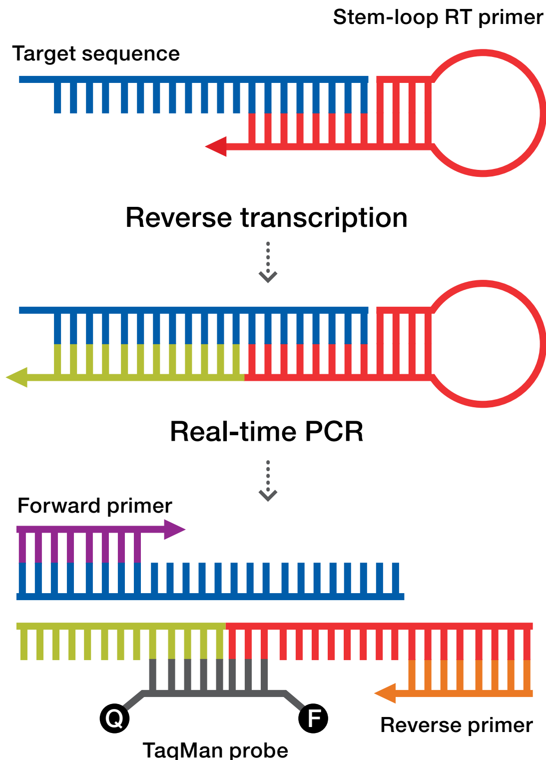


Figure 13.6. TaqMan MicroRNA Assay approach. A simple two-step process brings the advantages of real-time PCR to miRNA research.

Individual TaqMan MicroRNA Assays

Two-step TaqMan MicroRNA Assays, containing the TaqMan Assay and reverse transcription primer specific for the target of interest, are designed for individual miRNA targets found in human, mouse, rat, *Drosophila*, *C. elegans*, and *Arabidopsis* species. We are continually increasing the number of assays for these species to remain aligned with the Sanger miRBase Registry. Due to the high degree of miRNA conservation between species, coverage is observed to extend well beyond these core species.

TaqMan MicroRNA Assay Endogenous Controls

This selection of endogenous control assays for human, mouse, rat, *Arabidopsis*, *C. elegans*, and *Drosophila* simplifies data normalization. Designed against carefully selected small noncoding RNAs that are unrelated to miRNAs, these controls are expressed at consistent levels across a wide variety of cell types, tissues, and experimental conditions.

TaqMan MicroRNA Reverse Transcription (RT) Kit

The TaqMan MicroRNA RT Kit provides all the necessary components for optimal TaqMan MicroRNA Assay performance. Components of this kit are used with the single RT primer provided with each individual TaqMan MicroRNA Assay, or with Megaplex RT Primers, to convert miRNA to cDNA prior to real-time PCR quantitation.

TaqMan technology goes small with big benefits for miRNA research

- **Highly specific**—quantitate only the biologically active mature miRNAs, not precursors—with single-base discrimination of homologous family members (Figure 13.7)
- **Sensitive**—requires only 1–10 ng of total RNA or equivalent, conserving limited samples
- **Wide dynamic range**—up to 9 orders of magnitude—detect high and low expressors in a single experiment (Figure 13.8)
- **Fast, simple, and scalable**—two-step real-time RT-PCR assay quickly helps provide high-quality results
- **Custom assays available**—you specify the sequence, and we will design an assay

miRNA assays		Mature miRNAs			Precursors	
		Perfectly matched C_t	Mismatched C_t	ΔC_t (mismatch vs. match)	C_t	ΔC_t (precursor vs. mature)
let-7a	Looped	16.5	33.1	16.6	29.5	13.0
	Linear	23.6	38.3	14.7	30.4	6.8

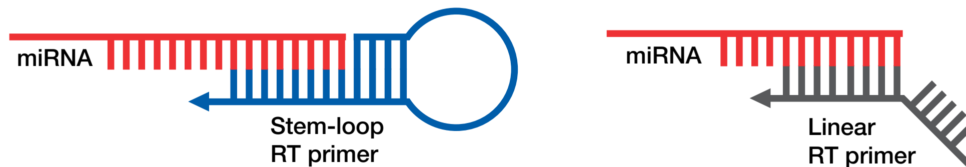


Figure 13.7. The stem-loop primer strategy for reverse transcription in TaqMan MicroRNA Assays confers specificity for biologically active mature microRNA. An off-the-shelf TaqMan MicroRNA Assay for let-7a, containing a stem-loop RT primer, was compared with a comparable-sequence linear RT primer/PCR primer/TaqMan probe set. Next, 1.5×10^8 copies of synthetic miRNA mimicking mature let-7a, mature let-7e (a closely related miRNA differing at only two base positions), and the stem-loop let-7a precursor were added to RT reactions primed with either the stem-loop TaqMan MicroRNA Assay RT primer or linear RT primer of comparable sequence. The cDNA was then amplified using real-time PCR. The data indicate that the stem-loop RT primer confers better discrimination between mature and precursor miRNAs and closely related targets.

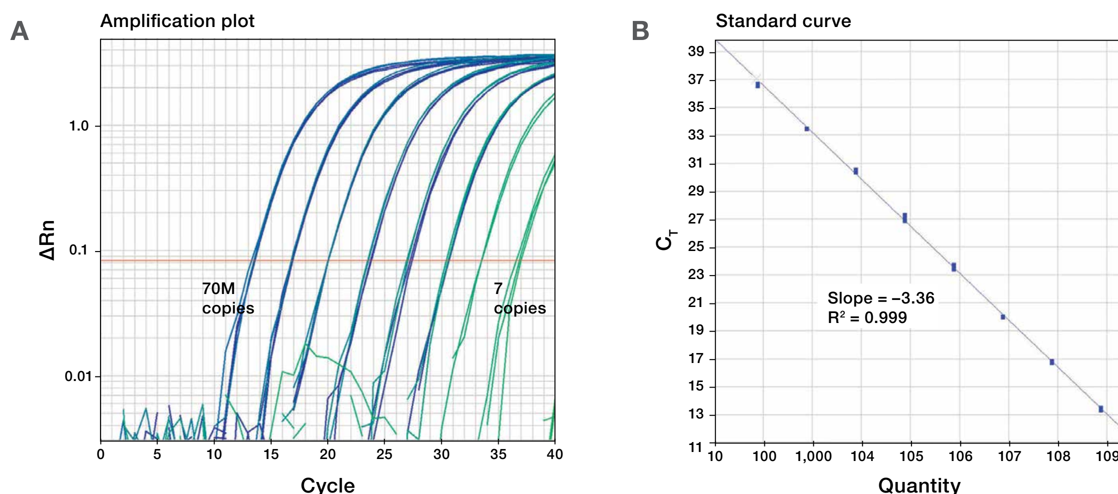


Figure 13.8. TaqMan MicroRNA Assays provide wide dynamic range. This wide dynamic range enables miRNA targets that vary in abundance from a few copies to millions of copies to be accurately quantitated in the same experiment—an important factor given the wide range of miRNA concentrations within and across different cells, tissue types, and disease states. To illustrate the dynamic range and sensitivity of TaqMan MicroRNA Assays, a synthetic lin-4 miRNA was serially diluted and amplified using the lin-4 TaqMan MicroRNA Assay. **(A)** Amplification plot of synthetic lin-4 miRNA over seven orders of magnitude. Synthetic RNA input ranged from 1.3×10^{-3} fM (equivalent to 7 copies per reaction) to 1.3×10^{4} fM (equivalent to 7×10^{7} copies per reaction) in PCR. **(B)** Standard curve of synthetic lin-4 miRNA amplification.

Analyze microRNA function

Analyses of miRNA function are performed using strategies that are similar to those used for protein-coding genes. Transfecting cultured cells with miRNA mimics can help identify gain-of-function phenotypes; downregulation or inhibition experiments using miRNA inhibitors can be conducted to identify loss-of-function phenotypes. The combination of upregulation and downregulation can be used to identify genes and cellular processes that are regulated by specific miRNAs. Further investigation of miRNA function includes studies of miRNA-mRNA target interaction and the impact on target mRNA levels and concomitant protein expression.

Selecting a miRNA mimic or inhibitor

We recommend Invitrogen™ *mir*Vana™ Mimics and Inhibitors for anyone getting started with functional analysis of endogenous microRNA and looking for the latest advances in specificity and potency. Invitrogen™ Pre-miR™ and Anti-miR™ reagents remain relevant for ongoing studies that have benefited from these original product formats.

MicroRNA functional analysis workflow

Features of the workflow (Figure 13.9):

- Gain- and loss-of-function phenotypes identify genes and processes regulated by miRNAs
- Western blotting and/or real-time RT-PCR are used to validate miRNA targets
- Target site interaction and impact on protein expression can be evaluated using reporter gene and immunodetection systems

How it works

*mir*Vana miRNA Mimics and Inhibitors are designed to mimic or inhibit specific miRNAs for artificial upregulation and downregulation of target mRNA translation, respectively. miRNA targets are validated by quantitating target protein and/or messenger RNA levels in response to miRNA upregulation or downregulation. Western blot analysis is used to investigate the impact on protein expression.

	miRNA mimics for gain-of-function studies		miRNA inhibitors for loss-of-function studies	
	Recommended *mir*Vana Mimics	Pre-miR Mimics	Recommended *mir*Vana Inhibitors	Anti-miR Inhibitors
Content database coverage	miRBase v.22	miRBase v.15	miRBase v.22	miRBase v.15
Available formats	Individual tubes, custom plated collections, or whole-genome library	Individual tubes	Individual tubes, custom plated collections, or whole -genome library	Individual tubes
Product summary	• Chemical modifications prevent passenger strand entry into RISC • Highly specific microRNA mimics	• RNA duplexes mimic native microRNA	• Patented design and chemical modifications • Potent, long-lasting microRNA inhibition	• Single-stranded inhibitors of endogenous microRNAs

Search for your miRNA mimic or inhibitor of interest at **thermofisher.com/mirna**

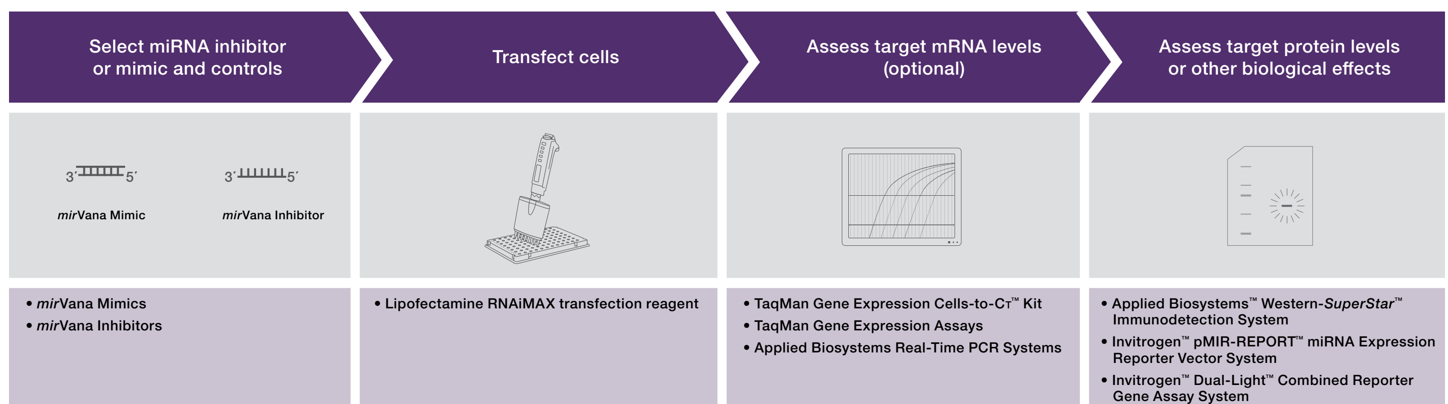


Figure 13.9. MicroRNA functional analysis workflow.

*mir*Vana miRNA Mimics and Inhibitors

*mir*Vana miRNA Mimics and Inhibitors provide a means to study the function of specific miRNAs in a range of organisms, and to validate their role in regulating target genes. We released the second generation of these products, which are more specific (mimics) and potent (inhibitors) than their predecessors. *mir*Vana oligonucleotides, available in both *in vitro* and *in vivo* formulations, reflect the most up-to-date Sanger miRBase content.

*mir*Vana miRNA mimics are small, chemically modified double-stranded RNA molecules that are designed to mimic endogenous mature miRNAs, resulting in artificial downregulation of target mRNA translation and accompanied, in some cases, by reduction in transcript levels. Like natural miRNAs, these mimics have two strands—the mature strand, which is functional and used by the Argonaute (Ago) protein to target mRNAs; and the star strand, which is nonfunctional and is cleaved and expelled from the complex. The chemical modifications in *mir*Vana miRNA mimics inactivate the star strand to help ensure that the guide strand (representing the desired mature miRNA) is taken up by Ago to produce the miRNA effect (Figure 13.10). *mir*Vana miRNA mimics are designed to produce maximum and consistent effects at concentrations as low as 0.3 nM and are available individually or as libraries.

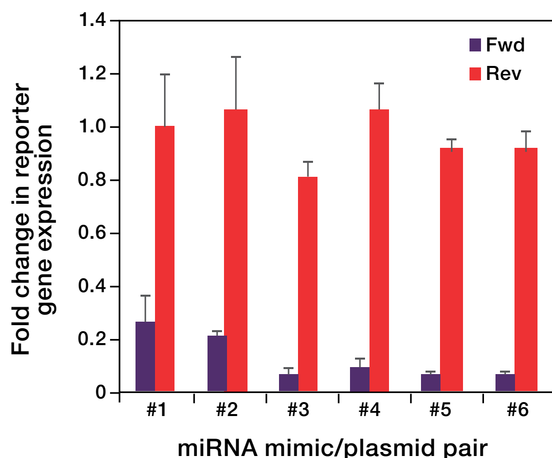


Figure 13.10. High specificity of *mir*Vana miRNA mimics. HeLa cells were cotransfected with one of six different miRNA mimics at 3 nM concentration and a corresponding reporter plasmid. The fold change in reporter gene expression for each miRNA mimic strand was determined by measuring expression in the presence of mimic relative to that of a negative control (set at 1.0). Each plasmid has the reporter gene cloned both in forward orientation (Fwd) to measure activity of the miRNA mimic mature strand, and in reverse/complement orientation (Rev) to test activity of the nonfunctional star strand. For all six mimics, mature strand activity is high (reporter gene expression reduced 5- to 10-fold compared with negative control), and star strand activity is low or absent (similar to negative control).

Inhibitors with excellent potency

mirVana miRNA inhibitors are chemically modified, single-stranded oligonucleotides designed to specifically bind to and inhibit endogenous miRNAs, resulting in artificial upregulation of target mRNA translation. Compared with competitors, these inhibitors have the highest-potency *in vitro* inhibition at the lowest miRNA inhibitor concentration (Figure 13.11). Like the mimics, the *mir*Vana miRNA inhibitors are available individually or as libraries.

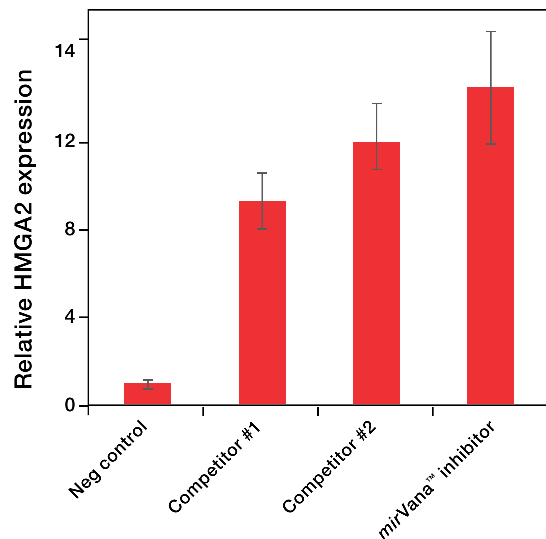


Figure 13.11. *mir*Vana miRNA inhibitors are more potent than products from leading competitors. *mir*Vana miRNA inhibitors and two other commercially available let-7 inhibitors were each transfected into HeLa cells at 10 nM concentration using Invitrogen™ Lipofectamine™ RNAiMAX™ reagent. Twenty-four hours later, HMGA2 mRNA levels were measured by qRT-PCR using TaqMan Assays.

Take advantage of *in vivo*–ready tools

*mir*Vana miRNA mimics and inhibitors are compatible with both *in vitro* and *in vivo* applications; they have been validated with Lipofectamine RNAiMAX Transfection Reagent for use in cell-based systems (Figure 13.12), and with Invitrogen™ Invivofectamine™ 2.0 Reagent for *in vivo* delivery (Figure 13.13). These oligonucleotides are nontoxic and do not induce an immune response in the animal models tested. *In vivo*–ready *mir*Vana miRNA mimics and inhibitors have been purified by HPLC and dialysis and are ready for immediate *in vivo* use.

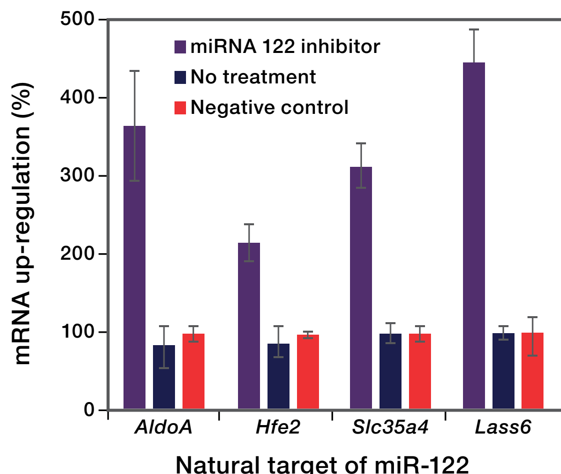


Figure 13.12. *mir*Vana miRNA inhibitors effectively suppress miRNA *in vivo*. miR-122 or Negative Control #1 *mir*Vana miRNA inhibitors were complexed with Invivofectamine 2.0 Reagent and injected into the tail veins of BALB/c mice on 3 consecutive days at 7 mg per kg body weight. Twenty-four hours after the last injection, expression levels of four natural targets of miR-122 were measured in the liver using TaqMan Assays. Significant upregulation of all four mRNAs was detected in mice treated with miR-122 inhibitor, as compared with mice that received no treatment or the negative control. Results indicate that *mir*Vana miRNA inhibitors were efficiently delivered to the liver with Invivofectamine 2.0 Reagent, where they inactivated miR-122, leading to upregulation of genes naturally suppressed by miR-122.

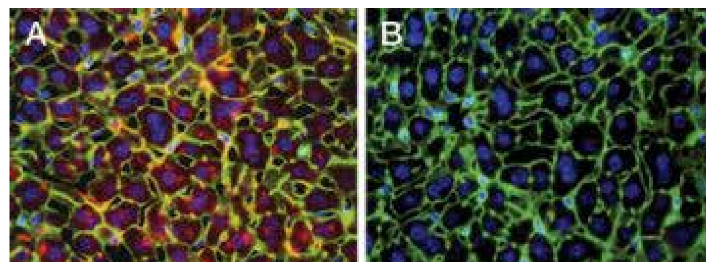


Figure 13.13. Fluorescently labeled *mir*Vana miRNA mimics and inhibitors enable tracking *in vitro* and *in vivo*. (A) Invitrogen™ Alexa Fluor™ 647 dye–labeled inhibitor was complexed with Invivofectamine 2.0 Reagent and injected into the tail veins of BALB/c mice. Twenty-four hours later, animals were sacrificed, and their livers sectioned and prepared for analysis. *mir*Vana miRNA inhibitors are readily visualized in the liver section (red, Alexa Fluor™ 647 dye–labeled miRNA; green, Alexa Fluor™ 488 Phalloidin; blue, DAPI), indicating efficient uptake into hepatocytes. **(B)** Liver section from a mouse treated with unlabeled *mir*Vana miRNA inhibitor. Fluorescently labeled mimic and inhibitors can be custom-ordered upon request; note that fluorescent labels may affect the biological activity of mimics or inhibitors.

Validated positive and negative controls

The Invitrogen™ *mir*Vana™ miRNA mimic miR-1 Positive Control is routinely used in experiments utilizing mirVana miRNA mimics. This mimic effectively downregulates the expression of twinfilin-1, also known as PTK9, at the mRNA level (60–95% reduction). The Invitrogen™ *mir*Vana™ miRNA mimic Negative Control #1 has a unique sequence designed such that it does not target any human, mouse, or rat genes; it has been tested in human and mouse cell lines and tissues and validated to not produce identifiable effects on known miRNA functions.

The *mir*Vana miRNA inhibitor let-7 Positive Control is validated for experiments using *mir*Vana miRNA inhibitors. Endogenous let-7 miRNA negatively regulates *HMGA2*, which encodes a ubiquitously expressed nonhistone chromatin protein that modulates gene expression through changes in chromatin architecture. Upon introduction of the let-7 inhibitor, significant elevation in *HMGA2* mRNA can be measured by qRT-PCR (Figure 13.11). The *mir*Vana miRNA inhibitor Negative Control #1 has a unique sequence designed such that it does not target any human, mouse, or rat genes; it has been tested in human and mouse cell lines and tissues and validated to not produce any measurable effects on known miRNA functions.

Product	Quantity	Cat. No.
*mir*Vana miRNA Mimic, Negative Control #1	5 nmol	4464058
*mir*Vana miRNA Mimic, Negative Control #1	20 nmol	4464059
*mir*Vana miRNA Mimic, Negative Control #1	50 nmol	4464060
*mir*Vana miRNA Mimic, Negative Control #1	250 nmol	4464061
*mir*Vana miRNA Mimic, miR-1 Positive Control	5 nmol	4464062
*mir*Vana miRNA Mimic, miR-1 Positive Control	20 nmol	4464063
*mir*Vana miRNA Mimic, miR-1 Positive Control	50 nmol	4464064
*mir*Vana miRNA Mimic, miR-1 Positive Control	250 nmol	4464065
*mir*Vana miRNA Inhibitor, Negative Control #1	5 nmol	4464076
*mir*Vana miRNA Inhibitor, Negative Control #1	20 nmol	4464077
*mir*Vana miRNA Inhibitor, Negative Control #1	50 nmol	4464078
*mir*Vana miRNA Inhibitor, Negative Control #1	250 nmol	4464079
*mir*Vana miRNA Inhibitor, let-7c Positive Control	5 nmol	4464080
*mir*Vana miRNA Inhibitor, let-7c Positive Control	20 nmol	4464081

Predefined *mir*Vana miRNA mimic and inhibitor libraries

Thermo Fisher Scientific offers the most comprehensive miRNA collections available.

- 100% coverage of miRBase Sequence Database version 21
- Coverage to miRBase version 22 available as custom fulfillment
- 96-well or 384-well plate format for high-throughput screening
- Microplates qualified for use with Labcyte Echo™ acoustic liquid handlers available upon request
- Complete plate layout information and miRNA annotation provided

Predefined collections for human and mouse miRNA	Amount/well	# of miRNAs	Cat. No.
*mir*Vana miRNA Mimics, Human v 21 – 384- or 96-Well Plates	0.25 nmol	2,565	4464074
*mir*Vana miRNA Inhibitors, Human v 21 – 384- or 96-Well Plates	0.25 nmol	2,565	4464092
*mir*Vana miRNA Mimics, Human v 21 – 384- or 96-Well Plates	1 nmol	2,565	4464075
*mir*Vana miRNA Inhibitors, Human v 21 – 384- or 96-Well Plates	1 nmol	2,565	4464093
*mir*Vana miRNA Mimics, Mouse v 21 – 384- or 96-Well Plates	0.25 nmol	1,900	4464074
*mir*Vana miRNA Inhibitors, Mouse v 21 – 384- or 96-Well Plates	0.25 nmol	1,900	4464092
*mir*Vana miRNA Mimics, Mouse v 21 – 384- or 96-Well Plates	1 nmol	1,900	4464075
*mir*Vana miRNA Inhibitors, Mouse v 21 – 384- or 96-Well Plates	1 nmol	1,900	4464093

Custom miRNA mimic and inhibitor libraries

In addition to our predefined genome-wide libraries, *mir*Vana mimics and inhibitors are available as custom collections.

- 96-well, 384-well, or Echo-qualified 384-well plates compatible with acoustic liquid handlers
- Perfect for pilot studies, primary screen follow-up, or a customized functional genomics screen
- Minimum order of 20 wells

Request a quote for a custom miRNA library

Product lines for custom library fulfillment	Quantity per well
***mir*Vana miRNA mimics–human, mouse, or rat, miRBase v22**	0.25 or 1.0 nmol/well
***mir*Vana miRNA inhibitors–human, mouse, or rat, miRBase v22**	0.25 or 1.0 nmol/well

Publications featuring *mir*Vana miRNA libraries

The following selected peer-reviewed citations demonstrate the utility of *mir*Vana mimic and inhibitor libraries in high-throughput screening applications to assess microRNA involvement in biological pathways such as those of development and growth, metabolic pathways and diseases, and cancer progression.

Lopes IR et al. (2019) Shedding light on microRNA function via microscopy-based screening. *Methods* 152:55-64.

This comprehensive overview of miRNA mimic and inhibitor screening provides a detailed case study of the identification of miRNAs that control infection by the bacterial pathogen *Salmonella*. It includes methodologies that are readily adaptable for screens to address a wide variety of biological questions.

Takagawa Y et al. (2020) miR-1293, a candidate for miRNA-based cancer therapeutics, simultaneously targets BRD4 and the DNA repair pathway. *Mol Ther* 28(6):1494-1505.

A genome-wide human *mir*Vana miRNA mimic screen carried out in 10 cancer cell lines uncovered three tumor-suppressive microRNAs which target the gene *BRD4*, suggesting potential candidate for miRNA-based cancer therapeutics.

Meyer H-J et al. (2017) Identification of a novel miRNA that increases transient protein expression in combination with valproic acid. *Biotechnol Prog* 33(4):1139-1145.

A custom collection of 875 *mir*Vana mimics was screened to successfully identify two human miRNAs that increase recombinant protein titers for the purpose of large molecule therapeutic development.

Pal AS et al. (2021) Identification of microRNAs that promote erlotinib resistance in non-small cell lung cancer. *Biochem Pharmaco*l 189:114154.

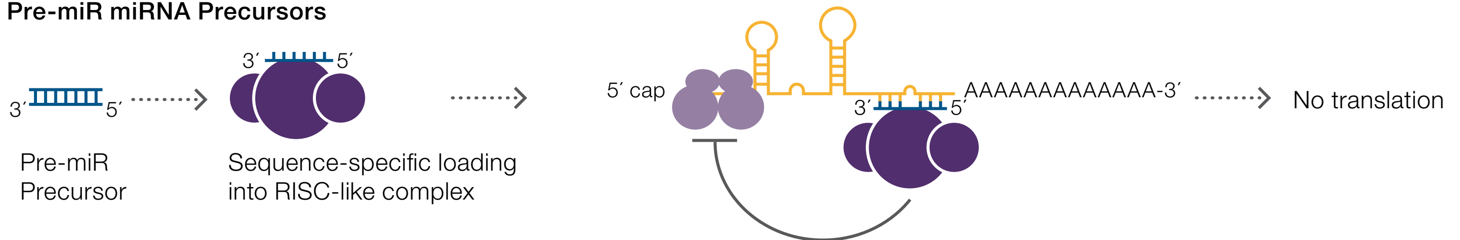
Multiple microRNAs that drive EGFR-inhibitor resistance were identified in this genome-wide screen using *mir*Vana miRNAs, uncovering a model for how miRNA dysregulation may contribute to drug resistance.

Pre-miR miRNA Precursors and Anti-miR Inhibitors

Invitrogen™ Pre-miR™ miRNA Precursors and Anti-miR™ miRNA Inhibitors are designed to mimic or inhibit specific miRNAs for gain-of-function or loss-of-function studies, respectively (Figure 13.14). Both can be introduced into cells using transfection or electroporation methods similar to those used for siRNAs.

Pre-miR miRNA Precursors are small, chemically modified, double-stranded RNA molecules that are similar to, but not identical to, siRNAs, and are designed to mimic endogenous miRNAs. Thanks to their small size, they are easier to transfect than vectors, and can be delivered using conditions similar to those used for siRNAs by transfection or electroporation. In contrast to miRNA expression vectors, these synthetic molecules can be used in dose response studies.

Anti-miR miRNA Inhibitors are chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNAs (Figure 13.15). When tested using individual reporter constructs containing the appropriate miRNA binding site, these inhibitors induced, on average, an approximately 4-fold increase in the expression of the reporter relative to cells cotransfected with a negative control Anti-miR miRNA inhibitor, indicating their strong inhibitory properties.



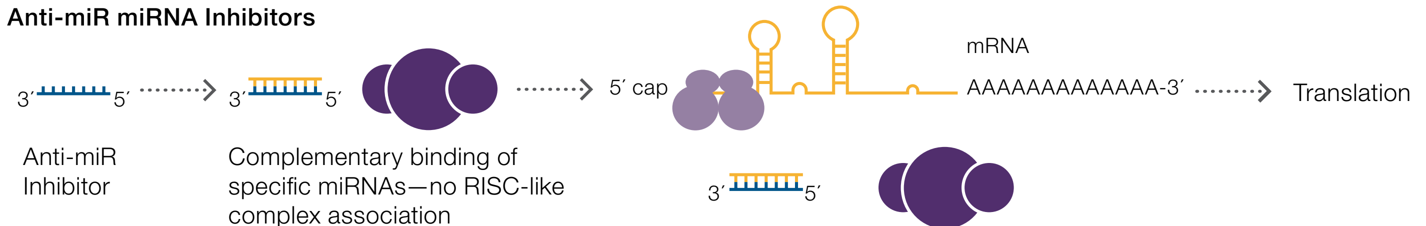


Figure 13.14. Activity of Pre-miR miRNA Precursors and Anti-miR miRNA Inhibitors.

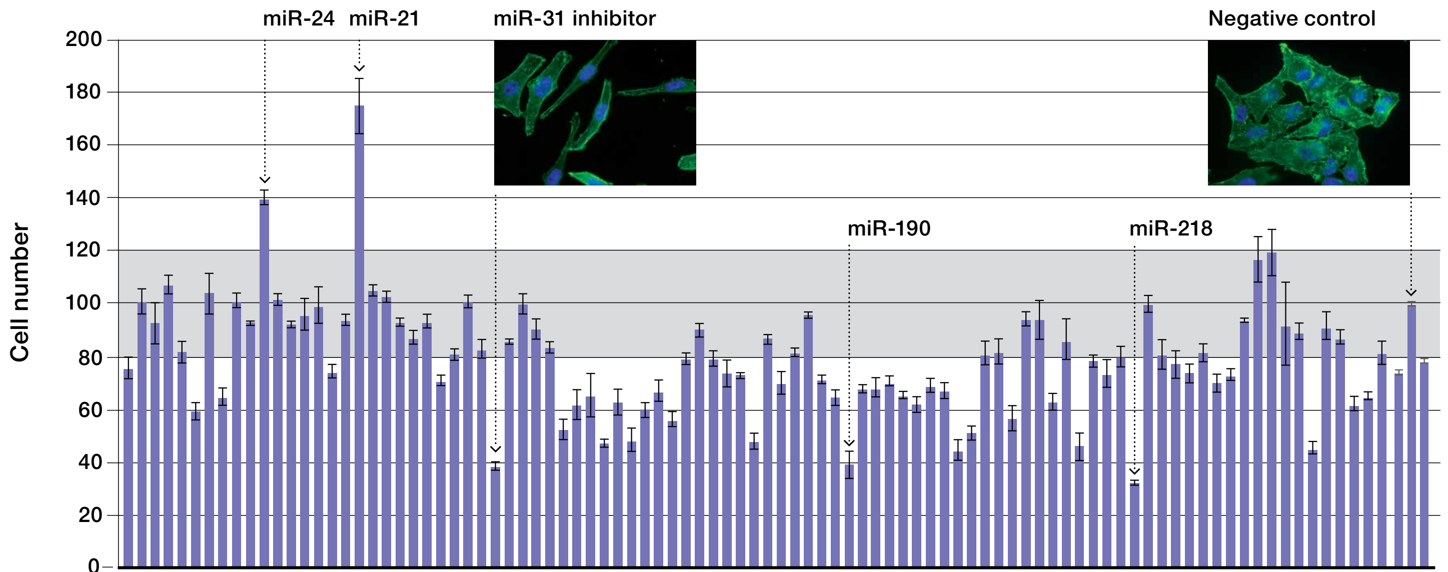


Figure 13.15. Identification of miRNAs involved in cell proliferation by screening with Anti-miR miRNA Inhibitors. HeLa cells were transfected with individual Anti-miR miRNA Inhibitors. After 72 hours, cells were fixed, stained with propidium iodide, and treated with an antibody to detect β-actin. Cell number was analyzed using an Acumen Explorer™ (TTP LabTech) plate reader. The horizontal shaded area represents the normal range of cell number for this type after treatment with Anti-miR Negative Control #1. The right inset shows the morphology of these control cells. The left inset shows the morphology of cells transfected with an Anti-miR Inhibitor to miR-31. Artificial upregulation of miR-31 resulted in elongated cells and reduced cell numbers.

Product	Quantity	Cat. No.
Pre-miR miRNA Precursor Negative Control #1	5 nmol	AM17110
Pre-miR miRNA Precursor Molecules—Negative Control #2	5 nmol	AM17111
Pre-miR hsa-miR-1 miRNA Precursor Positive Control	5 nmol	AM17150
Cy®3 Dye-Labeled Pre-miR Negative Control #1	5 nmol	AM17120
FAM™ Dye-Labeled Pre-miR Negative Control #1	5 nmol	AM17121
Anti-miR miRNA Inhibitor Negative Control #1	5 nmol	AM17010
Anti-miR hsa-let-7c miRNA Inhibitor Positive Control	5 nmol	4392431
Cy3 Dye-Labeled Anti-miR Negative Control #1	5 nmol	AM17011
FAM Dye-Labeled Anti-miR Negative Control #1	5 nmol	AM17012

Reporter vector and combined chemiluminescent assay systems

To evaluate the interaction between miRNAs and their target sites, the Invitrogen™ pMIR-REPORT™ miRNA Expression Reporter Vector System provides a simple solution. This validated reporter gene system contains two mammalian expression vectors, pMIR-REPORT™ Luciferase and pMIR-REPORT™ β-Galactosidase Control Vector. pMIR-REPORT Luciferase features the firefly luciferase reporter gene under the control of a CMV promoter and a cloning region for miRNA target sequences or a 3′ UTR with one or more putative miRNA binding sites downstream of the luciferase-coding sequence. After cloning, pMIR-REPORT Luciferase can be cotransfected into mammalian cells with the pMIR-REPORT β-Galactosidase Control Vector to evaluate the effects of endogenous miRNA expression on the target.

In addition, the pMIR-REPORT System can be used to monitor downregulation or upregulation of reporter gene expression after transfection with *mir*Vana miRNA Mimics or Inhibitors, respectively. With a *mir*Vana miRNA Mimic Library, pMIR-REPORT Luciferase is an ideal screening tool to study miRNA-mediated regulation of a target gene.

TaqMan Gene Expression Assays

A number of miRNAs have been reported to directly affect target mRNA levels. Others are thought to target the expression of transcription factors, indirectly affecting the expression levels of many genes. Either way, TaqMan Gene Expression Assays can be used with miRNA gain-of-function and loss-of-function experiments to quantitate effects on target mRNA expression.

We offer more than 1 million TaqMan Gene Expression Assays for 30 species, the most comprehensive set of predesigned real-time PCR assays available. All TaqMan Gene Expression Assays are designed using our validated bioinformatics pipeline, and run with the same PCR protocol, eliminating the need for primer design or PCR optimization.

MicroRNA sample preparation kits

Most RNA isolation kits were developed to recover messenger RNA, and eliminate smaller molecules such as miRNAs. We offer a range of products that were designed for optimal recovery of miRNA and other small RNAs from a wide variety of sample types (Figure 13.16). The selection guide (Table 13.1) is designed to help researchers decide which product best fits their needs. The following pages provide more detailed information on the sample preparation products mentioned in the workflows for miRNA discovery, profiling, targeted analysis, and functional analysis.

We offer a variety of Invitrogen™ kits for the isolation and analysis of miRNA and other small RNAs. Each kit is ideal for use in miRNA analysis because it is optimized to enable:

- Quantitative recovery of small RNA (<200 nt)
- Maintenance of representative amounts of small RNA (eliminating experimental bias)

*mir*Vana miRNA Isolation Kit

Samples are lysed in a denaturing lysis solution that both stabilizes RNA and inactivates RNases. The lysate is then extracted with acid-phenol/chloroform, yielding a semipure RNA sample. The RNA is further purified over a glass-fiber filter to yield either total RNA or a size fraction enriched in miRNAs. The kit reagents are specifically formulated for miRNA retention to avoid the loss of small RNAs that is typically seen with standard glass-fiber filter methods. This quick and easy procedure is compatible with virtually all cell and tissue types, and can be used for efficient isolation of total RNA containing small RNA, or for enrichment of the small RNA fraction (<200 nt), to increase sensitivity in downstream analyses.

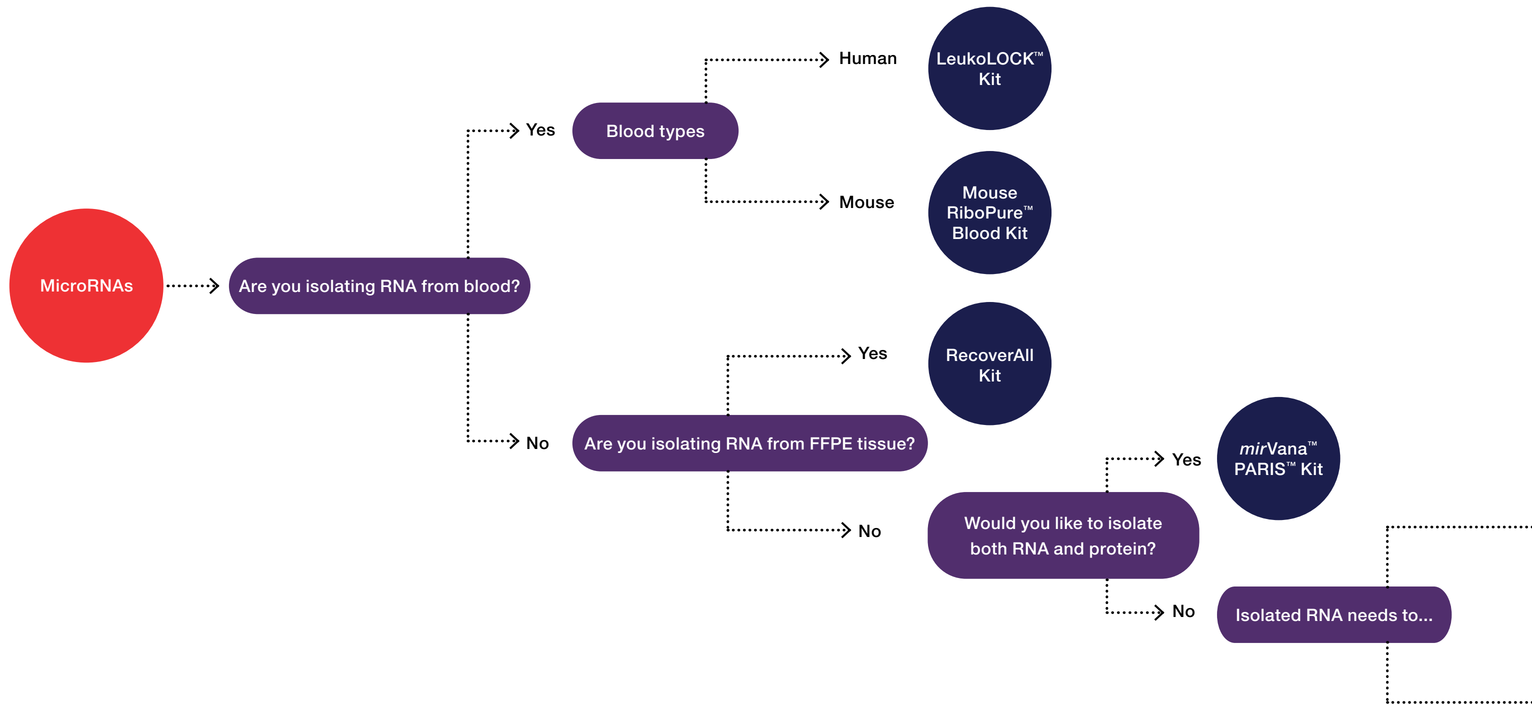


Figure 13.16. MicroRNA sample preparation selection guide featuring Invitrogen™ products.

RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues

The Invitrogen™ RecoverAll™ Kit procedure requires about 45 minutes of hands-on time and can easily be completed in less than 1.5 hours when isolating RNA. FFPE samples are deparaffinized using a series of xylene and ethanol washes and are then subjected to a rigorous protease digestion with an incubation time tailored for recovery of either RNA or DNA. Nucleic acids are purified using a rapid glass-filter method that includes an on-filter nuclease treatment, and are finally eluted into either water or the low-salt buffer provided. Any RNA fragmentation that has already occurred in FFPE tissues cannot be reversed. However, the protease digestion conditions of the RecoverAll kit are designed to release a maximal amount of RNA fragments of all sizes, including miRNA, in a relatively short amount of time. This RNA can be readily analyzed by real-time RT-PCR and generates profiles equivalent to those seen in RNA isolated from fresh or flash-frozen samples.

TaqMan MicroRNA Cells-to-C_T Kit

Start with 10–100,000 cultured cells per sample, either in multiwell plates or individual tubes, and be ready for RT-PCR in 10 minutes. Cells are washed in PBS and lysed for 8 minutes at room temperature; DNase treatment can be performed concurrently. Lysis is terminated by adding Stop Solution and incubating for two additional minutes at room temperature. Because samples can be processed directly in culture plates (96- or 384-well), sample handling is reduced, and the risk of sample loss or transfer error is minimized. No heating, washing, or centrifugation is required; the Invitrogen™ TaqMan® MicroRNA Cells-to-C_T™ Kit greatly reduces a traditionally time-consuming, labor-intensive process to just 10 minutes. Also included in the kit are TaqMan MicroRNA Reverse Transcription Reagents and TaqMan® Universal PCR Master Mix to complete the gold-standard TaqMan® miRNA profiling workflow.

TaqMan Gene Expression Cells-to-C_T Kit

Similar to the Cells-to-C_T Kit for microRNA, the Invitrogen™ TaqMan® Gene Expression Cells-to-C_T™ Kit enables real-time RT-PCR directly in cultured cell lysates without isolating RNA. This kit, however, includes reverse transcription and real-time PCR reagents that are optimized for quantitation of mRNA expression. It is ideal for validation studies that analyze the effects of miRNA on their mRNA targets.

invitrogen

Table 13.1. Invitrogen™ microRNA sample preparation kits: quantitative recovery of small RNAs from a variety of sample types.

	TaqMan MicroRNA Cells-to-C_T Kit	*mir*Vana miRNA Isolation Kit	*mir*Vana PARIS Kit	RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues	MagMAX™-96 for Microarrays Total RNA Isolation Kit
Technology	Cells-to-C_T technology, with optional DNase treatment for preparation of cultured cell lysates that can be used in real-time RT-PCR without RNA isolation	Acid-phenol and rapid, enhanced glass-fiber filter purification	Cell Disruption Buffer combined with acid-phenol/chloroform extraction and glass-fiber filter purification	Deparaffinization, protease digestion, and glass-fiber filter purification	Cell lysis using TRI Reagent™ and magnetic bead purification
Sample input amounts	10 to 10^5 cultured cells	10^2–10^7 cultured cells or 0.5–250 mg tissue	10^2–10^7 cultured cells or up to 100 mg tissue	Up to four 20 µm FFPE sections	Up to 5 x 10^6 cultured cells or up to 100 mg tissue
Features	• Go from cells in culture to RT-PCR, typically in 10 min at room temperature • Simple procedure with no sample transfers, no centrifugation, and no vacuum manifold needed • For real-time RT-PCR • Superior results when used with TaqMan® MicroRNA Assays and Arrays	• Fast, easy isolation of small RNA from cultured cells and most tissues (including tissues with high levels of ribonucleases) • Ideal for miRNA profiling experiments and other gene expression applications	• Simple, 30 min procedure • Protein can also be recovered • Optional small RNA enrichment procedure • Ideal for correlating mRNA, miRNA, and/or siRNA with protein levels	• Isolate total nucleic acids, including DNA and microRNAs, from FFPE samples • No overnight proteinase K digestion required—deparaffinize in the morning and perform qRT-PCR in the afternoon • Routinely obtain yields >50% that of unfixed tissue from the same sample source • For real-time RT-PCR and PCR, mutation screening, and microarray analyses	• Highly consistent results from experiment to experiment • Streamlined RNA purification • Requires less hands-on time than competitor kits • Walk away—integrate with established robotic platforms • Modified protocol to recover small RNAs
Kit sizes	100 lysis reactions/500 PCRs 400 lysis reactions/2,000 PCRs	Up to 40 purifications	Up to 40 purifications	40 purifications	96 purifications
Cat. No.	4391848 4391996	AM1560	AM1556	AM1975	AM1839

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