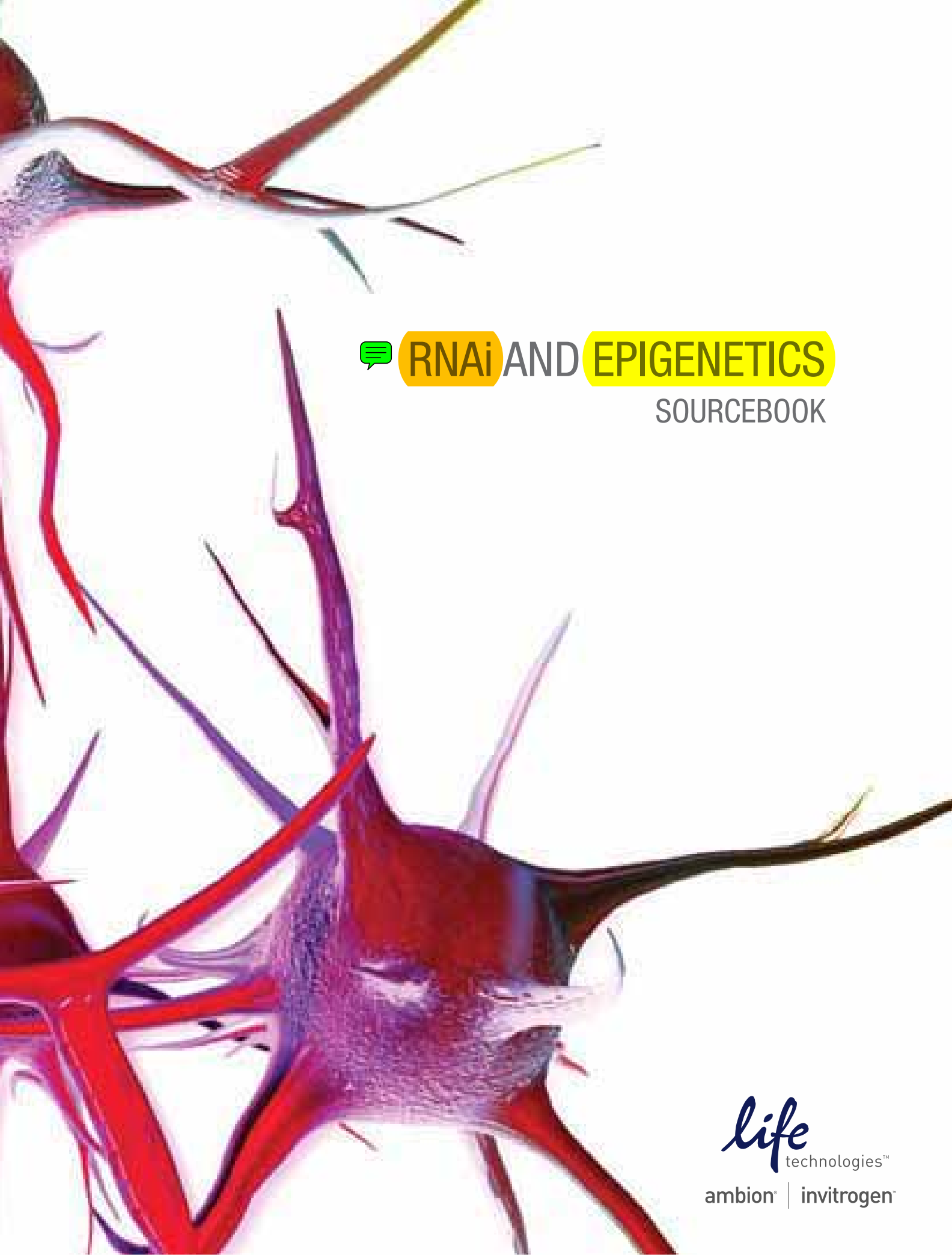

RNAi AND EPIGENETICS

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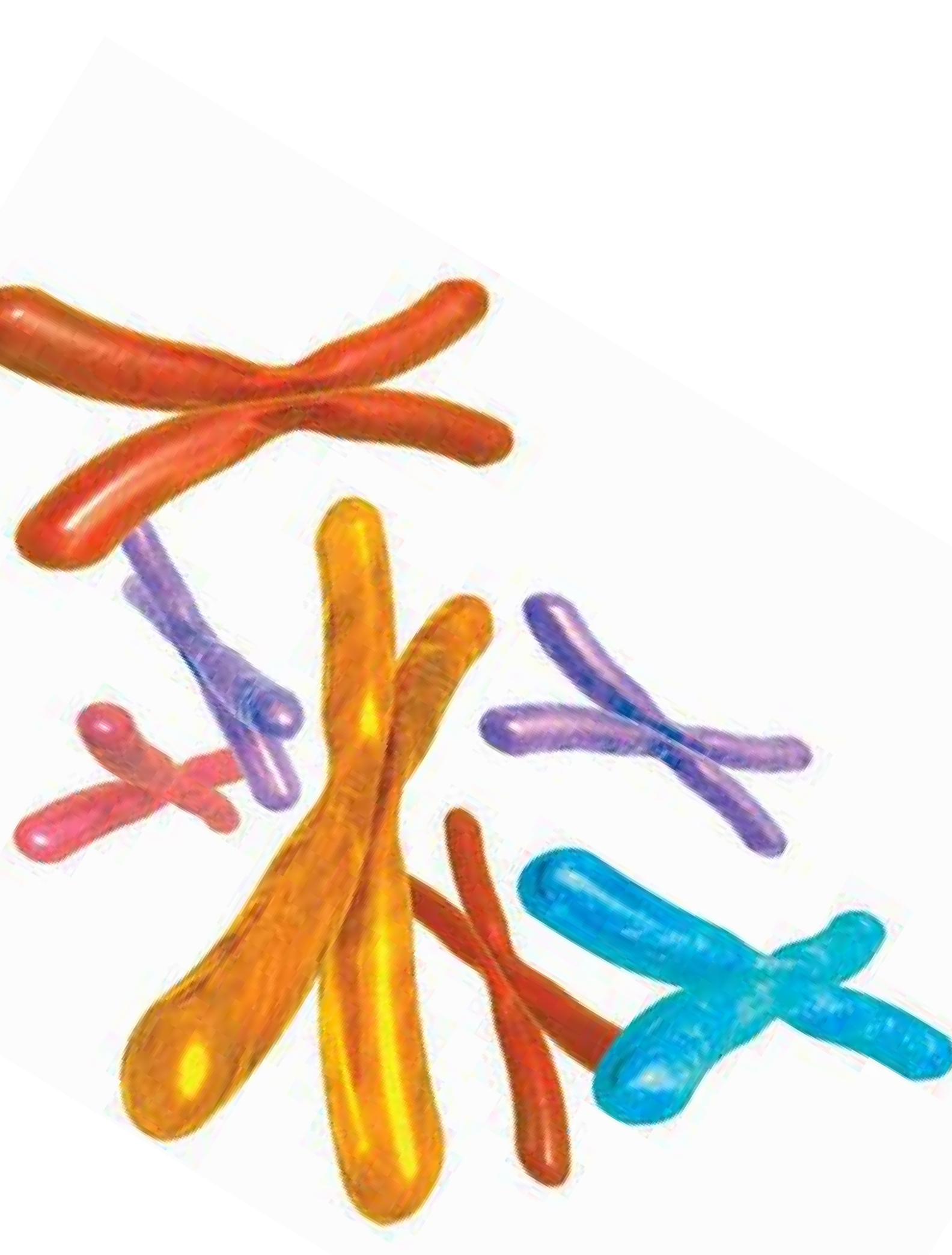

Quick start guide

1. Decide on effector molecule: siRNA or vector—Chapter 1
2. Find your gene online: www.invitrogen.com/findyourgene (shown at right)
3. Choose delivery vehicle: transfection reagent, mechanical method, or viral delivery—Chapter 3
4. Decide on controls—Chapter 2
5. Validate to measure loss of function—Chapter 11

Steps of an siRNA experiment

Have on hand:

→ Transfection/electroporation agent and protocol
→ Assays to assess knockdown and other RNAi effect(s)
→ Positive and negative control siRNAs
→ Two or more siRNAs to gene of interest

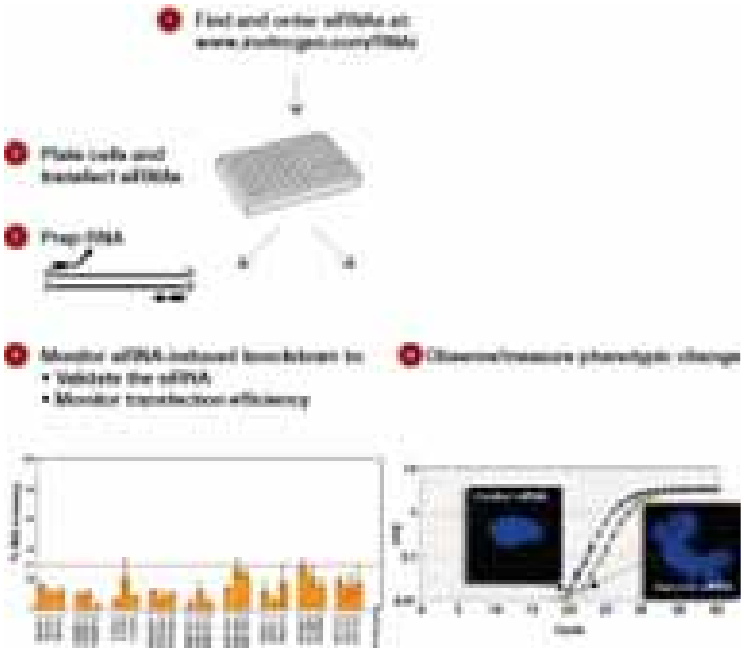

Figure 1. RNAi workflow diagram. RNAi experiment workflow following siRNA design and synthesis.

Glossary of common RNAi terms

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

siRNA
Short interfering RNA. siRNAs are 21–25 bp dsRNA with dinucleotide 3' overhangs that are processed from longer dsRNA by Dicer in the RNA interference pathway. Introduction of synthetic siRNAs can induce RNA interference in mammalian cells. siRNAs can also originate from endogenous precursors.

shRNA
Short hairpin RNA; also short interfering hairpin. 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 target a sequence designed to form hairpins and loops of variable length, which are processed by cellular siRNA machinery. Once in the cell the shRNA can decrease the expression of a gene with complementary sequences by RNAi.

miR RNAi
Vectors that express microRNAs for RNAi. miRNAs are 19–23 nt single-stranded RNAs, originating from single-stranded precursor transcripts that are characterized by imperfectly base-paired hairpins. miRNAs function in a silencing complex that is similar, if not identical, to RISC.

Chemically modified siRNA
siRNA molecules which have chemical modifications.

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

Off-target effects
Effects that occur when one or a few nontarget genes not specifically targeted show loss of gene function following the introduction of an siRNA or d-siRNA pool. 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.

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CHAPTER 1

Introduction to RNAi

Make your RNA interference experiments simple, stress-free, and successful

RNA interference (RNAi) is one of the most important technological breakthroughs in modern biology, allowing us to directly observe the effects of the 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 (RNAi). While studies in *C. elegans* were encouraging, the use of RNAi was limited to lower organisms because delivering long dsRNA for RNAi was nonspecifically inhibitory in mammalian cells. In 2001, shorter RNAs (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 siRNA, 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, 21 nucleotides in length, which are processed internally by an enzyme called Dicer. The Dicer cleavage products were first referred to as short interfering RNA, now known as siRNA. 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 (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 message is targeted for degradation, which ultimately results in the loss of protein expression.

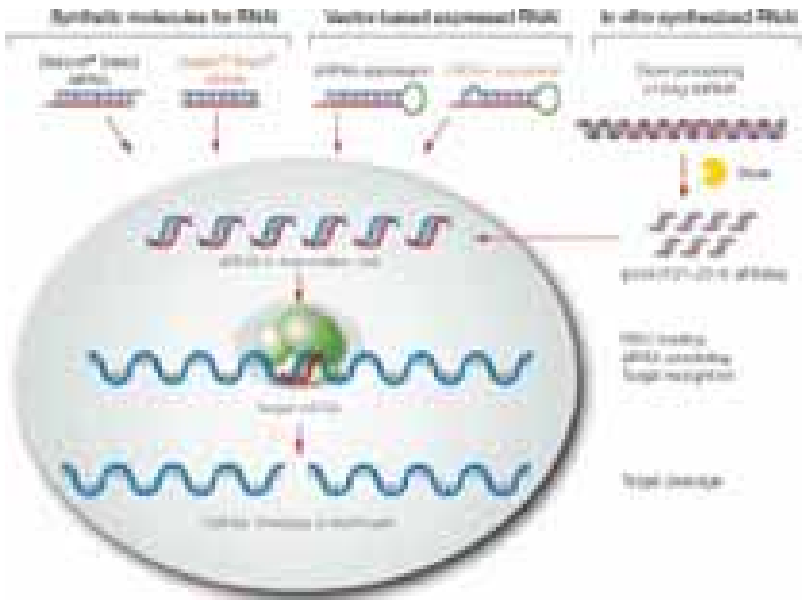

Figure 1.1. Methods of RNAi knockdown in mammalian cells.

Eight tips for a successful siRNA experiment

1. **Go to www.invitrogen.com/rnai and utilize the best-in-class design algorithms to design your siRNAs.** Do not attempt to design siRNAs on your own.
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. Ambion 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 media and serum-free media 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: Lipofectamine® RNAiMAX (Cat. No. 137780-75) is our best-performing transfection reagent for siRNA. See page 73 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 and to your experimental target siRNA. 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 and death. For maximum convenience, Invitrogen offers positive control siRNAs against a variety of gene targets.
7. **Use a negative control siRNA to distinguish nonspecific effects.** Negative controls should be designed by scrambling the nucleotide sequence of the most active siRNA. However, be sure to perform a homology search to ensure that your negative control sequence lacks homology to the genome of the organism being studied.
8. **Use labeled siRNAs for protocol optimization.** Fluorescently labeled siRNA can be used to analyze siRNA stability and transfection efficiency. Labeled siRNA is also useful to study siRNA subcellular localization and in double label experiments (with a labeled antibody) to visualize cells that receive siRNA during transfection and to correlate transfection with down-regulation of the target protein.

Considerations

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 & shRNA
Long-term stable knockdown		●
Inducible knockdown		●
Delivery to hard-to-transfect cells		●
Least hands-on time	●	
Most immediate effect	●	
Higher potency likely	●	
Design more efficient	●	

CHOOSINGsiRNA FOR TARGET OF INTEREST—IMPROVED SPECIFICITY

When choosing siRNA for a target of interest it is very important to use a state-of-the-art algorithm for choosing your siRNA. For example, *Silencer*® Select siRNA's five-step bioinformatic filtering process allows for elimination of siRNAs that are predicted to elicit off-target effects. www.invitrogen.com/silencerselect

With Stealth Select RNAi™ siRNA, the work of designing highly effective sequences has been done for you. The development of these molecules begins with identifying all human, rat, and mouse transcripts in the Unigene database. In four steps we select highly effective *in silico*-designed sequences. www.invitrogen.com/stealthrnai

See page 40 for *Silencer*® Select siRNA and page 44 for Stealth Select RNAi™ siRNA.

Interview with Gregory Hannon

Q. Research into RNA interference (RNAi) and its emerging use as a tool to explore gene function has taken the research community by storm. Can you tell us how you first became interested in RNAi?

Dr. Hannon: It's sort of interesting, actually. I was at a Pew Scholars Meeting; it was my first year.

Craig Mello was also a Pew Scholar, and he gave a small "chalk talk" at the meeting—although we were in the bowels of Mexico somewhere, so there was no chalk. He presented this really interesting phenomenon. It was either just before or just after the first RNAi paper was published. It really excited me and we chatted a couple times about it at the meeting. But we didn't really do anything about it, not being *C. elegans* biologists. His theory just sort of percolated for a year.

Then the next year, again at the Pew Scholars Meeting, Rich Carthew showed that RNAi worked in *Drosophila*. He was doing this by embryo injection. And that's what really pulled me in. Here was something that was not just a biological oddity of *C. elegans* (I was unaware of the plant work at that time so I hadn't thought about it very deeply). The notion that this phenomenon was going to be universal really captivated me. This was partly because I was spending a lot of effort trying to do forward genetics in cultured mammalian cells using things like retroviral libraries. I saw the RNAi phenomenon as, one day long down the road, something that could complement over expression approaches, and that would give us the loss-of-function tool that mammalian systems have always lacked.

Q. Wow, "…one day long down the road…", I bet you were surprised at how quickly this field has progressed.

Dr. Hannon: Well, that's what got us started in RNAi. We started thinking about the cell culture models from *Drosophila*. Our initial goal had been to try to use S2 cells as a model for studying gene function. And so I actually called the lab from that Pew Meeting and said, "…You know what? Get out those *Drosophila* cells and see if they do RNAi…" And they did.

Q. So how did your interest in using RNAi to study gene function morph into your work focusing on the mechanism of RNAi?

Dr. Hannon: We got hooked on this technique in part because I have a background in RNA processing—I worked on trans-splicing with Tim Nilsen as a graduate student—so there was the possibility that we would have just run with the whole notion of doing gene function in S2 cells. As it turns out, for most of the things that we were interested in—cell cycle control, and such—S2 cells are a terrible system. But we said, okay, since nobody understands much about the RNAi mechanism yet, let's play for a little while and see if we can generate an *in vitro* system from cultured cells that we can use to try to figure out how this all works. And we sort of got sucked down the mechanism path from there.

Q. Your lab, Mello's lab, and others published research indicating that Dicer is the nuclease that digests long dsRNA into siRNAs which in turn mediate RNAi. Can you describe some of the experiments that led you to this conclusion?

Dr. Hannon: We had approached this purely from a biochemical standpoint, where we had taken a "candidate gene approach". We tagged all of these proteins, did IPs (immunoprecipitations), and looked for activity. It led to a beautiful biochemical correlation between Dicer activity and siRNA. In other words, we had an enzyme that did basically what it was supposed to do. But any time you have a biochemical result, you have to be somewhat concerned about whether or not the story you've derived based on *in vitro* experiments has any basis in reality. To really know that something is involved in any kind of specific pathway you need genetics. Ultimately the proof is always in the →

genetics. And we had done a really crappy experiment, which was to use RNAi to knock out Dicer—that's sort of become de rigueur in the field—so I don't know if I should still be embarrassed about or not. But our primary motivation was just to verify the hypothesis that Dicer was the initiating enzyme.

Q. Now we know that Dicer is also important in a gene regulatory pathway involving short temporal RNA (stRNA) and your lab's work helped to demonstrate that. How did you tie Dicer to the stRNA pathway?

Dr. Hannon: Shortly after Emily left, Plasterk's lab finally found a *C. elegans* Dicer mutant. It was initially somewhat worrying that there weren't somatic RNAi defects. Ronald tried to be reassuring and talked about maternal effects. But ultimately when they looked at germ line transgenes, silencing of germ line transgenes was defective. And this was sort of a nice confirmation. I think at least now there's no doubt that Dicer really is the initiating enzyme for the process.

Q. What do you think will be the single greatest outcome of research into RNA silencing?

Dr. Hannon: It's really an impossible question to answer. One of the things that makes this field very hot at the moment is that in a way it's all things to all people. You have very interesting biology. For example, scientists are studying transposon silencing, the evolution of the relationship between repetitive and mobile genetic elements, DNA parasites and their hosts, and the interaction between viruses and their hosts. That group is interested because RNAi is involved, at least in plants. And there is also a definitive relationship in *C. elegans*. RNAi seems to be involved in somehow controlling these kinds of nucleic acid parasites. But there are also people looking at these microRNAs, at least for endogenous gene regulation, and saying, look, here's a whole new regulatory paradigm where there are hundreds of, what you might call orphan hairpins, running around out there. We don't know what they do, we don't know who they regulate, we don't know how prevalent this is, how general it is, or even really at what regulatory levels these different things act. We know about two that act at the level of protein synthesis, but there's nothing to say that others don't act at the level of message stability, or even at the level of directing the modification of chromosome structure. Here we may find that this is something that is as important as the discovery of enhancer sequences, in terms of controlling gene expression itself.

You've also got a group of people who are interested in the basic mechanics of RNAi and what it means. Everybody is intrigued about a mechanism where a worm can eat a piece of RNA and knock out a gene in its progeny. There's something intrinsically appealing about that—understanding the mechanism of that bit of biology. And there's a whole group of people who want to understand the biological ramifications of the system—that it may regulate development in plants, maybe stem cell identity, or maintenance of stem cell character in both plants and animals.

And then, a much broader group of people, who don't really care that much about mechanism, are just interested in harnessing this phenomenon as a tool. So, it's really impossible to predict the single greatest outcome of this research. And I think if you ask that question of ten different people, you'd get ten different answers. The reason I can't give you just one is because we're interested in all of them.

Q. Your lab and other labs have developed expression vectors that express siRNA long term. Do you see the use of siRNA expression vectors as a replacement to transfection of siRNAs for inducing RNAi, or are these techniques complementary?

Dr. Hannon: Oh, I think they're complementary. Very much so. It's still early in terms of trying to understand the power of each of these technologies. What seems to be true, at least this early on, is that siRNAs can get into cells at very low concentrations to provoke a very good effect. I think the jury is still out on whether it's easier to get an effect with an siRNA on a sort of per cell or whole population basis. We don't really have that much information on it. And I suspect eventually these things will run even because of advances in different transfection technologies. But in terms of ease of use, nothing beats typing in a sequence, having a couple oligos show up, and then dumping them onto cells, right? If you're looking for a quick answer, and you only have one or two genes that you want to look at, nothing is going to beat the idea that you can chemically synthesize these things, just in terms of ease.

Q. What are some of the advantages of expression vectors over chemically synthesized siRNAs?

Dr. Hannon: The expression constructs are going to be powerful for a different reason. And maybe more appropriate for specific sorts of experiments that involve much more long term analysis of phenotype, or biochemical studies that involve larger cell numbers that might be more difficult or more expensive to do by transfecting each cell that you want to analyze.

Q. Cell logistic problems, right?

Dr. Hannon: Right. There are a lot of phenotypes that you want to look at over long time scales or in mosaic animals. That's where the power of these kinds of expression constructs are going to be. Now, another advantage of the expression constructs is the fact →

that they are propagatable—you make one and validate it and you have it forever. You never have to remake it. You never have to reorder it—it sits in the freezer and you can trot it out and use it any time you want. We are finding that you can marry these sorts of modular cassettes with pretty much any gene transfer technology that you want to talk about—viruses, etc. They will be useful with model systems like tissue slices, where it might be more difficult to get siRNAs [inside cells] in good numbers.

Q. I'd like to ask you about your laboratory. How many people do you have working with you currently, and how are they split between postdocs, grad students, etc.?

Dr. Hannon: We are at around 16 at the moment; 6 graduate students, a couple of visitors, about 4 or 5 postdocs, me, a few technicians, and a research associate who is semi-independent who's also working with me.

Q. We know that you're the founder of the biotech company, Genetica. And you're obviously busy as a professor at the Cold Spring Harbor labs, too. Do you still find time to work at the bench? And, if so, do you think that's unusual for someone in your position?

Dr. Hannon: Yeah, oh, definitely. I try to work at the bench every day. I'm not sure that I do anything useful, but I try to work. I think that Cold Spring Harbor has, not necessarily a tradition, but a lot of the faculty here do work at the bench. And, if you think about it, it's sort of what got us involved in this whole RNAi work in the beginning. I find that it keeps me engaged much more in the day to day activities of the lab. It also keeps me grounded in the reality of doing experiments and makes me much more understanding about students' ligations failing occasionally, because mine fail right alongside theirs. And I think that the work moves faster because I'm there. I'm available. We talk about things more—everything from the biology of RNAi to the nitty gritty details of lab work, like, "Gee, this isn't working," and "Maybe I've seen that before…" That helps us troubleshoot and move things along a little bit more quickly. I like being in the lab, and I like interacting with the people in my lab.

Q. What's next for Gregory Hannon?

Dr. Hannon: I'm going to go do minipreps for one of my students, that's what's next.

About Dr. Hannon

Gregory J. Hannon received his PhD from Case Western Reserve University in 1992 and was a 1997 Pew Scholar. He is currently an Associate Professor at the Cold Spring Harbor Laboratory, where his research is focused in part on determining the mechanism of RNAi, as well as using RNAi as a tool in the study of cancer development and investigating the potential of siRNAs as cancer therapeutic agents.

Dr. Hannon and his colleagues have been at the forefront of many of the important discoveries in the RNAi area. In 2000 they identified the nuclease activity responsible for dsRNA-guided mRNA degradation, now known as the RNA-induced silencing complex (RISC). They identified one of the protein components of RISC, Argonaute2, as well as the enzyme ("Dicer") that begins the RNAi process by cutting long dsRNAs into siRNAs. Most recently they have shown the effectiveness of short hairpin RNAs (shRNAs) at gene silencing, providing a longer-lasting alternative to siRNAs for much-needed functional studies.

Dr. Hannon is one of the founders of Genetica, Inc., a biotechnology company using RNAi and other tools to link genetic data with biologic function via genetic manipulation of mammalian cells. Such genetic manipulations include RNAi-mediated stable silencing of gene expression for the elucidation of disease pathways such as cancer development and subsequent drug target validation.

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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). We have simplified control reactions by providing a selection of RNAi technologies designed to assist researchers in identifying and validating drug targets, generating publishable data, and submitting grants. Our RNAi control kits allow you to:

- → Determine which RNAi molecules deliver the best knockdown results
- → 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 set of reagents optimized for inhibition of p53 in human cells

Table 2.1. RNAi interference controls.

Type of control	Recommended use	Products
Transfection control	Calculate and monitor transfection efficiency with fluorescence	See page 80
Negative controls	Nonspecific or scrambled controls used to measure knockdown levels vs. background	See page 82, 83, and 86
Positive controls	RNAi reagents known to achieve high levels of knockdown used to measure delivery and optimize experimental conditions	See page 82, 84, and 87
Untransfected control	Measure normal gene expression level and phenotype	
Multiple RNAi sequences to the same target	Use to verify phenotypic change, control for off-target effects for generating publication quality results	
Titration of RNAi	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 the RNAi sequence will not affect	BLOCK-iT™ Pol II miR RNAi or BLOCK-iT™ shRNA vectors with inducible promoters (CMV/TO and H1/TO respectively) (see page 57)

Transfection controls

With overexpression experiments, even low transfection efficiencies can often yield 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 three transfection reagents for delivering *Silencer*® Select siRNA or Stealth RNAi™ siRNA, Lipofectamine® RNAiMAX, Lipofectamine® 2000, and Oligofectamine™ reagents. To monitor transfection efficiency using these reagents, Invitrogen offers two fluorescently labeled RNAi duplexes: the BLOCK-iT™ Alexa Fluor® Red Fluorescent Control for use with Lipofectamine® RNAiMAX, and the green BLOCK-iT™ Fluorescent Oligo for use with Lipofectamine® 2000 or Oligofectamine™ reagents (the BLOCK-iT™ Alexa Fluor® Red Fluorescent Control can also be used with Lipofectamine® 2000 or Oligofectamine™ reagents).

For more information on controls to use for optimizing transfection with synthetic RNAi or a table listing the contents for each of the transfection control kits, visit www.invitrogen.com/RNAicontrols.

Invitrogen also recommends two transfection reagents for delivering RNAi vectors to cells: Lipofectamine® 2000 and Lipofectamine® LTX reagents. We also recommend transfecting a vector that expresses a fluorescent protein to monitor cellular uptake of the RNAi vector (BLOCK-iT™ Pol II miR RNAi Expression Vector with EmGFP).

For more information on controls to use for optimizing transfection with RNAi vectors or a table listing the contents for each of the transfection control kits, visit www.invitrogen.com/RNAivectorcontrols.

Negative controls

Negative control siRNAs—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 *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 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, 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.

By using the BLOCK-iT™ RNAi Designer, you can choose scrambled controls for any Stealth RNAi™ siRNA sequence. 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 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
- → No homology to any known vertebrate gene
- → Tested sequences, which do not induce a stress response

We recommend using one or more negative 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 RNAi molecules that 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 the anticipated phenotype, carefully evaluate your experimental conditions and decide if some factors need to be adjusted. Examples of positive controls 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.

Downstream controls

Before transfecting cells and performing qRT-PCR and western blots to measure mRNA and protein levels, we recommend validating your downstream 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, IFIT-1 and 5'OAS 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 micro RNA (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 non-overlapping 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, then you cannot conclude that the gene of interest was successfully knocked down.

Titrate siRNA

Silencer® Select siRNA and Stealth RNAi™ siRNA can be very effective even at low concentrations. Titrating down the dose of the *Silencer*® Select siRNA and Stealth RNAi™ siRNA duplex 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 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 the 3'UTR and then transfecting the cells after knockdown with a vector expressing the the open reading frame (ORF) of the gene of interest. If the RNAi sequences were designed to 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

Delivering RNAi to cells—transfection and viral delivery

The two common approaches for RNAi delivery are lipid-mediated transfection and viral-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 *Silencer*® Select siRNAs or Stealth RNAi™ siRNA duplexes, 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. Another approach for determining the most favorable RNAi delivery conditions is to use Invitrogen's delivery optimization service—a scientific resource with extensive knowledge and expertise in viral vectors and nonviral delivery reagents for testing a matrix of delivery parameters.

Methods to achieve high transfection efficiency

Transfection efficiency describes the percentage of cells that have received the RNAi duplex or expression plasmid. Typically, researchers strive to achieve the highest levels of transfection efficiency possible. This objective is particularly important for RNAi applications because nontransfected cells will continue to express the gene targeted for knockdown, thus contributing to background expression levels.

For many disease models, the most desirable cell types are primary cultures. However, these cannot be transfected adequately with commercially available cationic lipid-mediated transfection reagents. A powerful alternative is viral delivery of vectors expressing RNAi sequences. This option is recommended for delivery to hard-to-transfect, primary, and nondividing cells. Viral delivery can also be used to create stable cell lines with inducible RNAi expression or to express RNAi sequences with tissue-specific promoters.

Importance of 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 with similar GC content to that of the target gene. 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.

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 *Silencer*® Select siRNA or Stealth RNAi™ 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 *Silencer*® Select siRNA or Stealth RNAi™ 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 (www.invitrogen.com/RNAiMAX), and for electroporation we recommend the Neon™ Transfection System (www.invitrogen.com/neon).

Significance of 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 down-regulation of genes due to the gene delivery procedure. 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 duplex 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 or modified Stealth RNAi™ siRNA duplexes, can help alleviate these concerns.

FEATURED PROTOCOL

Transfecting Stealth RNAi™ siRNA or *Silencer*® Select siRNA into A549 cells using Lipofectamine® RNAiMAX

Introduction

Lipofectamine® RNAiMAX Reagent is a proprietary formulation specifically developed for highly efficient delivery of Stealth RNAi™ siRNA or *Silencer*® Select siRNA to mammalian cells for RNAi analysis. This reference provides a recommended procedure to transfect Stealth RNAi™ siRNA or *Silencer*® Select siRNA into human A549 lung carcinoma cells (ATCC, Cat. No. CCL-185) using Lipofectamine® RNAiMAX (Cat. Nos. 13778-075, 13778-150). Lipofectamine® RNAiMAX 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 Stealth RNAi™ siRNA or *Silencer*® Select siRNA into A549 cells using Lipofectamine® RNAiMAX:

- Both *Reverse transfection* and *Forward transfection* protocols (page 12) can be used for transfecting A549 cells.
- To assess transfection efficiency, we recommend using a KIF11 Stealth Select RNAi™ siRNA, as described in *Assessing transfection efficiency* (page 12).
- We recommend using 10 nM of the siRNA duplex and indicated procedures. However, the efficacy of the RNAi sequence 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 RNAi concentration (1–50 nM can be used) and assay time (up to 72 hours) to establish optimal knockdown of your target gene.
- We recommend Opti-MEM® I Reduced Serum Medium (Cat. No. 31985-062) to dilute RNAi duplexes and Lipofectamine® RNAiMAX before complexing.
- **Do not** add antibiotics to media during transfection as this causes cell death.
- Test serum-free media for compatibility with Lipofectamine® RNAiMAX.
- Lipofectamine® RNAiMAX 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 13).

Materials needed

Have the following reagents on hand before beginning:

- A549 cells maintained in 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 siRNA or Stealth RNAi™ siRNA of interest
- Lipofectamine® RNAiMAX Reagent (store at +2–8°C until use)
- Opti-MEM® I Reduced Serum Medium
- Appropriate tissue culture plates and supplies

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FEATURED PROTOCOL, *CONTINUED*

Reverse transfection

Use this procedure to reverse-transfect Stealth RNAi™ siRNA or *Silencer*® Select siRNA into A549 cells in a 24-well format (for other formats, see *Scaling up or down transfections*, page 13). 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 RNAi duplex–Lipofectamine® RNAiMAX complexes as follows:
   a. Dilute 6 pmol RNAi duplex in 100 µL Opti-MEM® I Medium without serum in the well of the tissue culture plate. Mix gently.
      Note: If the volume of your RNAi duplex solution is too small to dispense accurately (less than 1 µL), and you cannot pool dilutions, predilute your RNAi duplex 10-fold in 1X RNA Annealing/Dilution Buffer (or the dilution buffer recommended by your RNAi duplex manufacturer), and dispense a 10-fold higher amount (should be at least 1 µL per well). For example, to get 6 pmol of RNAi duplex from a 20 µM RNAi duplex stock solution, dilute your RNAi duplex 10-fold to a concentration of 2 µM, and dispense 3 µL.
   b. Mix Lipofectamine® RNAiMAX gently before use, then add 1 µL Lipofectamine® RNAiMAX to each well containing the diluted RNAi molecules. 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 contains 30,000 cells (cell density should be 30–50% confluent 24 hours after plating).
3. To each well with RNAi duplex–Lipofectamine® RNAiMAX 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 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 Stealth RNAi™ siRNA or *Silencer*® Select siRNA into A549 cells in a 24-well format (for other formats, see *Scaling up or down transfections*, page 13). 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 RNAi duplex–Lipofectamine® RNAiMAX complexes as follows:
   a. Dilute 6 pmol RNAi duplex in 50 µL Opti-MEM® I Reduced Serum Medium without serum. Mix gently.
      Note: If the volume of your RNAi duplex solution is too small to dispense accurately (less than 1 µL), and you cannot pool dilutions, predilute your RNAi duplex 10-fold in 1X RNA Annealing/Dilution Buffer (or dilution buffer recommended by your RNAi duplex manufacturer), and dispense the proper higher amount (should be at least 1 µL per well). For example, to get 6 pmol of RNAi duplex from a 20 µM RNAi duplex stock solution, dilute your RNAi duplex 10-fold to a concentration of 2 µM, and dispense 3 µL.
   b. Mix Lipofectamine® RNAiMAX gently before use, then dilute 1 µL in 50 µL Opti-MEM® I Reduced Serum Medium. Mix gently.
   c. Combine the diluted RNAi duplex with the diluted Lipofectamine® RNAiMAX. Mix gently and incubate for 10–20 minutes at room temperature.
3. Add the RNAi duplex–Lipofectamine® RNAiMAX 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 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.

Assessing transfection efficiency

To qualitatively assess transfection efficiency, we recommend using a KIF11 Stealth Select RNAi™ siRNA (available through www.invitrogen.com/rnaiexpress; for human cells, oligo HSS105842 is a good choice). Adherent cells in which KIF11/Eg5 is knocked down exhibit a "rounded-up" phenotype after 24 hours due to a mitotic arrest [1]; slow growing cells may take up to 72 hours to display therounded phenotype. Alternatively, growth inhibition can be assayed after 48–72 hours.

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

FEATURED PROTOCOL, *CONTINUED*

Acceptable range for maximal activity

Due to the broad range of maximal activity exhibited by Lipofectamine® RNAiMAX, a range of cell densities and volumes of Lipofectamine® RNAiMAX can be used for transfection. For transfecting A549 cells in 24-well format, 0.5–1.25 µL Lipofectamine® RNAiMAX 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 RNAi duplex can be varied between 1 and 50 nM. A concentration of 10 nM RNAi duplex is suitable to knock down many target genes. However, the optimal concentration of RNAi duplex 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 Stealth RNAi™ siRNA or *Silencer*® Select siRNA, Lipofectamine® RNAiMAX, cells, and medium used in proportion to the relative surface area, as shown below.

Note: 20 µM Stealth RNAi™ siRNAor siRNA = 20 pmol/µL.

Recommended reagent amounts and volumes.

			Cells plated per well		Dilution medium		RNAi duplex amount		Final RNAi duplex concentration		Lipofectamine® RNAiMAX†	
Culture vessel	Relative surface area*	Volume of plating medium	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 is too small to dispense accurately, and you cannot pool dilutions, predilute Lipofectamine® RNAiMAX 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.

Want transfection protocols? Go to www.invitrogen.com/rnaitransfectionprotocol.

Cell health

In general, healthy cells take up nucleic acids better than poorly maintained cells. Many cells undergo expression profile changes that can adversely affect your experiments when they are stressed by culture conditions. 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* [2].

Culture conditions

Overly crowded or sparse cultures are not conducive for healthy cells. As a rule, cells should be replated before the medium becomes depleted. As cell cultures approach confluence, they typically contain some number of either 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 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 to cells in culture that can deleteriously effect experimental results. Mycloplasma are small, free-living prokaryotes that are not observable by light microscopy. Because they grow as filamentous or coccal froms 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.
- → **Optimization of 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 [3]. 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/50 µL cells (1.5 µM) of siRNA (range, 0.5–2.5 µg/50 µL 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 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, pre-plated 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 complex formation has occurred, 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 pre-plated 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

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CHAPTER 4

Vector-based RNAi technologies

Introduction to adenoviral and lentiviral RNAi vectors for delivery

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 Coxsackie 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	●		●			

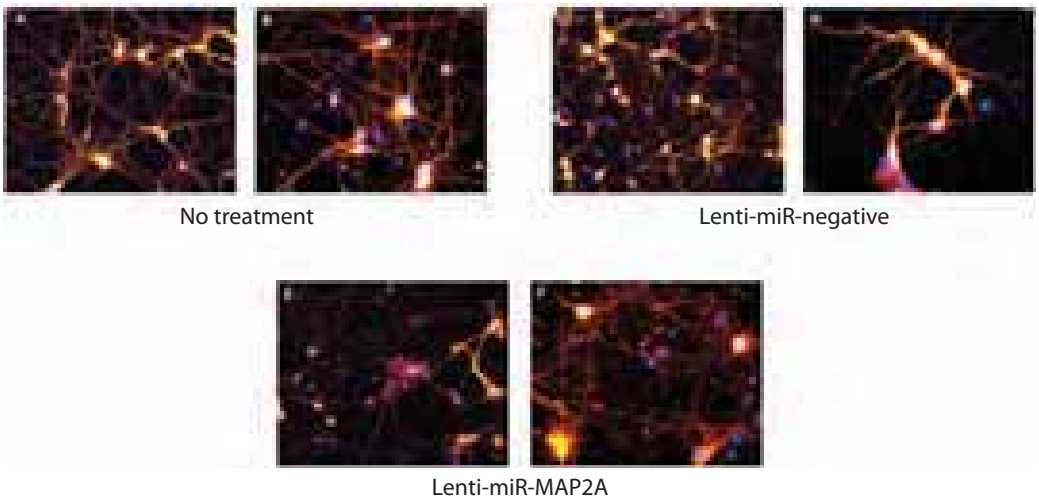


Figure 4.1. Lentiviral transduction of miR RNAi. Untreated (**A, B**) and transduced (**C–F**) samples were stained with MAP2 antibodies (orange) and DAPI (blue). E and F clearly show less target protein expression compared to the untreated neurons and those transduced with the Lenti-miR-negative control.

Lentiviral and adenoviral RNAi vectors—choose any cell type for RNAi

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/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 quantitative real-time PCR (qRT-PCR) or western blot analysis, the background of mRNA or protein in nontransfected 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).

The procedure for using both RNAi viral systems (Figure 4.2):

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 (use 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 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 non-dividing 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)
BLOCK-iT™ RNAi Adenoviral 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

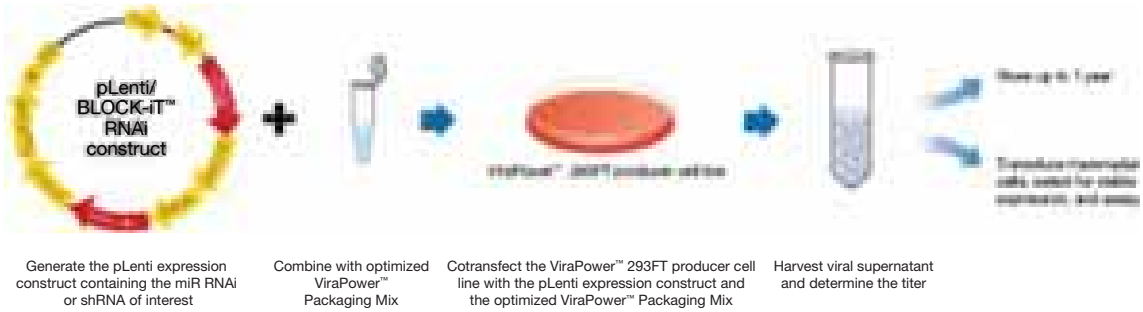


Figure 4.2. How the BLOCK-iT™ lentiviral RNAi systems work.

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. They are easy to design, synthesize, and use. siRNA can be rapidly identified and multiple genes can be targeted at the same time. RNAi vectors offer steadier expression because the vectors can target nondividing stem cells, lymphocytes, and neurons. Additionally, there is the possiblity of stable integration of the siRNA plasmid into the genome of the targeted cell. 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 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*. Stealth RNAi™ siRNAs are chemically modified RNA duplexes are 25 base pairs in length with blunt ends. They offer higher stability in serum for longer-lasting knockdown effects in cells (Figure 5.1). Modifications on the sense strand ensure that only the antisense strand is utilized by the RNA-induced silencing complex (RISC) to inhibit a target

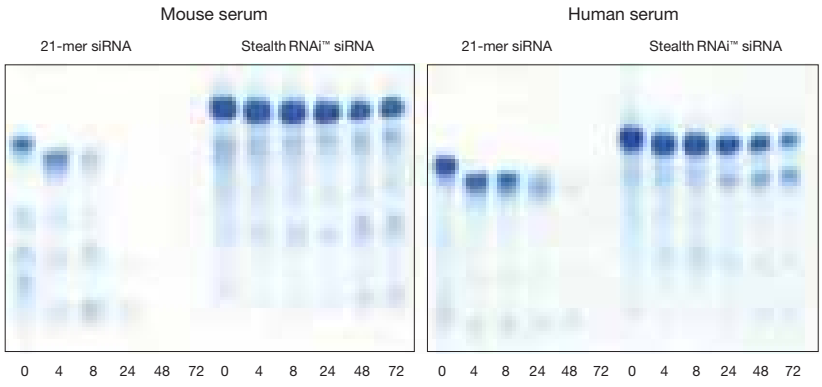


Figure 5.1. Chemical modification makes Stealth RNAi™ siRNA ideal for *in vivo* applications. Stealth RNAi™ siRNA duplexes are chemically modified to enhance stability against nucleases in serum. Unmodified 21-mer dsRNA sequences and corresponding Stealth RNAi™ siRNA sequences were analyzed at 0, 4,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

RNA. This decreases the potential for off-target effects since the sense strand cannot cleave unintended targets. Additonally, siRNA has the potential to activate the innate immune response, setting off defense systems usually used to combat viruses. The presence of the chemical modifications in Stealth RNAi™ siRNAs abolish the immunostimulatory response (Figure 5.2) observed with some sequences.

A. Protocol

6 hr → Sacrifice and collect serum → Luminex® assays

3 mice per treatment:
→ No treatment
→ CpG in PBS (positive control)
→ 37 bp dsRNA
→ Stealth RNAi™ siRNA in PBS

19 markers

IL-1α	G-CSF
IL-1β	GM-CSF
IL-2	FGF-basic
IL-4	VEGF
IL-5	PDGF-BB
IL-6	MIP-1α
IL-10	MIP-1β
IL-12p40	MIP-3β
IL-12p70	KC
IL-13	IP-10
IL-17	MCP-1
TNF-α	MIG
IFN-γ	RANTES

Multiplex panels

Cytokine 10-plex
Th1/Th2 6-plex
Inflammatory 4-plex
Chemokine 5-plex
Growth factor 4-plex
Cytokine 20-plex

B. Results

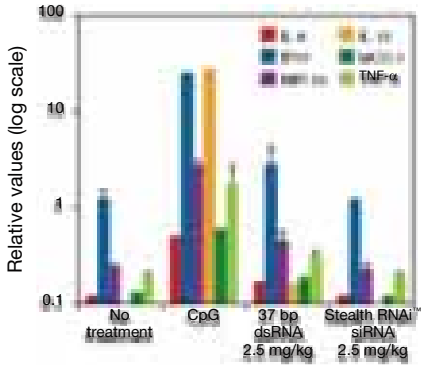
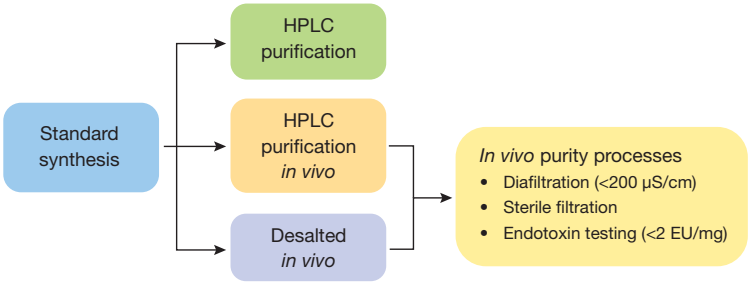


Figure 5.2. Stealth RNAi™ siRNA avoids stress response. (**A**) Tail veins of Balb/c mice were injected with 50 µg of either ssDNA with a CpG motif, a 37 bp standard siRNA, or Stealth RNAi™ siRNA duplex. (**B**) After 6 hr, serum was collected and processed to measure IFN markers using Luminex® multiplex assays.

Choosing RNAi purity

Invitrogen's production of *in vivo* RNAi duplexes begins with standard synthesis of RNAi oligos using high-quality starting materials (Figure 5.3). All RNAi oligos are then duplexed and desalted. At this point the researcher can also request HPLC purification. 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 RNAi oligo synthesis followed by HPLC purification
   Advantage: Can be conjugated with dyes
   Disadvantage: Does not include extra salt and solvent removal
2. Desalted, *in vivo* purity—standard RNAi oligo synthesis followed by diafiltration to remove salts and solvents to a level of <200 µS and sterile filtration
   Advantages: Available at high scale (without custom order), offers important salt and solvent removal, most cost-effective
   Disadvantage: Cannot be modified with dyes
3. HPLC, *in vivo* purity—standard RNAi 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



Purity	Modifications
• HPLC	• Alexa Fluor® 488
• Desalted *in vivo*	• Alexa Fluor® 546
• HPLC *in vivo*	• Alexa Fluor® 555
	• Alexa Fluor® 647
	• Alexa Fluor® 680
	• Alexa Fluor® 750
	• Biotin

Figure 5.3. Invitrogen produces high-quality *in vivo* RNAi duplexes in three purity selections supporting Alexa Fluor® dye and biotin modifications.

Tracking delivered duplexes

We have demonstrated that labeling Stealth RNAi™ siRNA duplexes does not hamper their knockdown potency (Figure 5.4). An alternative approach is to mix unlabeled duplexes with labeled control duplexes (Figure 5.5); this method is more commonly used when performing *in vivo* RNAi and allows the progression to clinical research to proceed unhindered by questions about the possible effects of a dye label. Tracking options are:

→ Stealth RNAi™ siRNA can be combined with bright BLOCK-iT™ fluorescent controls (available in different scales, and colors shown in Figure 5.3) without loss of activity

→ Stealth RNAi™ siRNA can be directly labeled with Alexa Fluor® dyes, without loss of activity

→ Stealth RNAi™ siRNA can be biotinylated and combined with either streptavidin-Alexa Fluor® or streptavidin-Qdot® labels

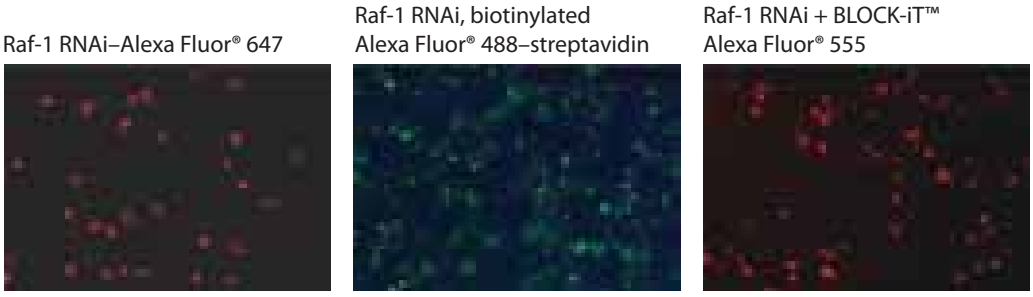


Figure 5.4. Labeled Stealth RNAi™ siRNA exhibits potent knockdown. MDA-MB-435 cells were transfected with 25 nmol unlabeled Stealth RNAi™ siRNA duplexes, or Stealth RNAi™ siRNA duplexes labeled with an Alexa Fluor® dye or biotin, in each case the duplexes targeting Raf-1. Knockdown was assessed at >90% by qPCR; delivery is demonstrated through fluorescence imaging.

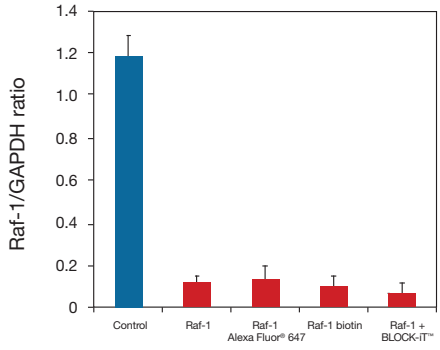


Figure 5.5. An alternate method to show knockdown. Mixing unlabeled duplex with labeled control duplexes.

Table 5.1. Overview of products for *in vivo* applications. Explore our full line of *in vivo* RNAi resources at www.invitrogen.com/invivornai.

RNA	Purification	Scale	Alexa Fluor® dye	Biotin
BLOCK-iT™ siRNA and Stealth RNAi™ siRNA	Desalted + *in vivo* purity	25 nmol, 100 nmol, 2 µmol	NA	NA
	HPLC	5, 20, or 500 nmol	488, 546, 555, 647, 680, 750	Yes
	HPLC + *in vivo* purity	5, 20, or 500 nmol	488, 546, 555, 647, 680, 750	Yes
BLOCK-iT™ Fluorescent Oligo Control	HPLC	5, 20, or 500 nmol	488, 546, 555, 647, 680, 750	Yes
	HPLC + *in vivo* purity	5, 20, or 500 nmol	488, 546, 555, 647, 680, 750	Yes

IN VIVO RNAi WITH VECTORS

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.

RNAi vector 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

For protocols and further recommendations, see www.invitrogen.com/invivornai.

In vivo RNAi protocols

Refer to the following supplemental protocols for special consideration for *in vivo* experiments. For questions about a protocol, please contact Invitrogen Technical Support to have a representative guide you through the procedure.

Protocols for successful *in vivo* RNAi experiments:

- → Resuspension of Stealth RNAi™ siRNA for *in vivo* applications
- → Measuring RNA concentration
- → Tissue harvest/RNA extraction
- → Tissue sectioning
- → Protein extraction from tissues
- → Tissue preparation for flow cytometry analysis

In vivo–purity Stealth RNAi™ siRNA duplexes are specifically formulated for use in animals. Resuspend the RNA duplex in 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. Table 5.2 and Table 5.3 specify the recommended resuspension volume for *in vivo*–purity Stealth RNAi™ siRNA and BLOCK-iT™ siRNA.

NOTE: The recommended resuspension volumes are for an RNAi molecule with 50% GC content. The molecular weight of RNAi molecules varies slightly depending on the GC content, but these differences are negligible for *in vivo* RNAi experiments.

Table 5.2. Desalted *in vivo* purity: recommended resuspension volume for 5 mg/mL final concentration.

Desalted *in vivo* quantity	Stealth RNAi™ siRNA resuspension volume—*in vivo* purity	siRNA resuspension volume—*in vivo* purity
25 nmol	80 µL	67 µL
100 nmol	320 µL	260 µL
2 µmol	6.4 mL	5.4 mL

Table 5.3. HPLC purity: recommended resuspension volume for 5 mg/mL final concentration.

HPLC-purified delivered quantity	Stealth RNAi™ siRNA resuspension volume—*in vivo* purity	siRNA resuspension volume—*in vivo* purity
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, 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

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 siRNA are slightly different due to chemical and size differences.

Harvesting tissue—RNA extraction from tissue

1. Homogenize 50–100 mg of tissue in 1 mL TRIzol® Plus Reagent using lysing matrix D on the FastPrep®-24 Instrument (MP Biomedical) at 4°C. A tissue homogenizer or rotor-stator can also be used.
2. For harder tissues (tumors, lungs), perform 3 cycles of 60 seconds each at 6 m/s using a tissue homogenizer or rotor stator. For softer tissues (brain, liver), 1 cycle of 60 sec at 4.5 m/s 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 PureLink™ RNA Mini Kit Purification System manual.
3. Determine the quantity and purity of the purified RNA using UV absorbance at 260 nm or Quant-iT™ RNA Assay Kit (Cat. No. Q33140). To determine RNA quality, electrophorese on an E-Gel® agarose gel or analyze on the Agilent 2100 Bioanalyzer.
4. After quantification, use 750 ng of total RNA for first-strand synthesis using the SuperScript® VILO™ Kit and qPCR analysis performed using TaqMan® Gene Expression Assays to measure knockdown.

Sectioning tissue

Slide preparation

If you are preparing your own slides, precoat slides with HistoGrip™ Concentrate (Invitrogen Cat. No. 008050) or 0.1% poly-L-lysine in water, then air-dry. Commercially available precoated glass slides are available and can be used to mount frozen or formalin-fixed, paraffin-embedded tissue sections.

Frozen tissue

A protocol for preparing frozen tissue samples is described below, but this is only a example. If your laboratory already has an optimized protocol for your sample type, we recommend that you use that protocol.

1. Snap freeze fresh tissues in cryomolds containing OCT® (Optimal Cutting Temperature) compound (a solution of glycols and resins which provides an inert matrix for sectioning). Store frozen tissue blocks at –70°C until you are ready for tissue sectioning.
2. For sectioning, allow the frozen tissue block to equilibrate to the cryostat temperature, cut 4–20 µm cryostat sections, and mount on coated glass slides.
3. Dry tissue sections at room temperature for 30 minutes.
4. If desired, store slides at –70°C before fixing. If slides are stored at –70°C, warm the slides to room temperature before the fixing step.
5. Place the slides in 100% acetone at 4°C for 10 minutes to fix the sections. Remove the slides from acetone and air dry for 10–30 minutes.
6. Circle each tissue section using the Mini PAP Pen. Store at –70°C until ready to use.
7. The slides should be washed in PBS for 10 minutes prior to staining or mounting.

Paraffin-embedded sections—deparaffinization and rehydration

To use the formalin-fixed, paraffin-embedded sections for immunohistochemical staining, deparaffinize slides with xylene and then rehydrate in a graded series of alcohol.

1. To prepare sections, dry slides containing 4 µm formalin-fixed, paraffin-embedded sections in a 55°C oven for 2 hours or overnight (do not allow the temperature to exceed 60°C) and store the slides at room temperature until needed.
2. Place the slides in xylene for 5 minutes at room temperature to deparafinize.
3. Remove slides and place in a second xylene wash for 5 minutes.
4. Remove the slides and place in two successive 100% ethanol washes for 5 minutes each.
5. Next, place the slides in 95% ethanol for 5 minutes and then transfer to 80% ethanol for 5 minutes.
6. Remove the slides from the 80% ethanol and place in PBS for 10 minutes.
7. Drain any excess reagent by tapping the edge of the slide on paper towels and wipe the area near the tissue sections with a laboratory wipe.
8. Circle each tissue section using the Mini PAP Pen. The slides are now ready to use.

Protein extraction from tissues

Sample preparation

Proper sample preparation is key to the success of a western blot analysis experiment. Various factors affect the design of the sample preparation protocol. Due to the large variety of proteins present in different cells and tissues, it is not possible to have a single sample preparation protocol that is suitable for all proteins. Based on the starting material and goal of the experiment, the sample preparation protocol needs to be determined empirically. The sample preparation conditions may also be optimized based on your initial results. General guidelines are provided below to prepare samples from various sources, and example procedures are provided.

Mammalian tissue samples

The protocol below describes the preparation of a mammalian tissue lysate from 100 mg tissue using 1 mL Cell Extraction Buffer. The following protocol is suitable for use with a variety of tissue types, although some optimization may be necessary for some tissue types.

1. If needed, cut the tissue into smaller pieces and place ~100 mg tissue in a microcentrifuge tube.
2. Add 1 mL Cell Extraction Buffer containing Protease Inhibitor Cocktail.
3. Homogenize the tissue using a pestle that fits snugly into the microcentrifuge tube.
4. Incubate the samples on ice for 10 minutes with intermittent vortexing.
5. Centrifuge the lysate at 10,000 x *g* for 5–10 minutes to remove any particulate material.
6. Transfer the supernatant to sterile microcentrifuge tubes and aliquot into single use tubes.
7. Proceed to Preparing Samples for SDS-PAGE after protein estimation, or store aliquots at –80°C. For more detailed information, follow protocol described in the kit (Cat. No. WB7401).

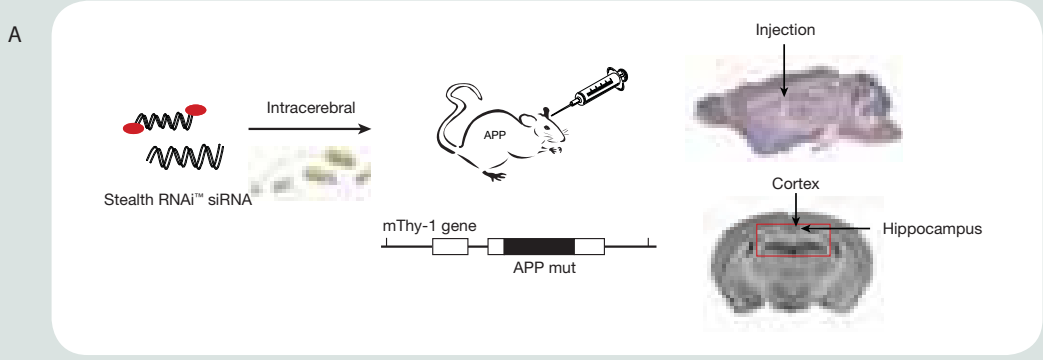
Preparing tissue for flow cytometry analysis

1. Following euthanasia, collect the tissue of interest and place it in cold PBS.
2. Mince the tissue into ~1 mm cubes using a razor blade in a 35 mm Petri dish containing 1–2 mL of Liver Digest Medium (17703-034).
3. Incubate 15 to 45 minutes, depending on the degree of connective tissue, and pipette up and down every 15 minutes using a 5 mL pipettor to mix.
4. Following incubation, add 4–5 mL of PBS, and use a 100 µm strainer to gently force cells through the strainer into a 10 mL conical tube.
5. Wash the isolated cells by adding 5 mL PBS and centrifuging at 1,000 x *g* for 5 minutes.
6. Discard the supernatant and repeat 1–2x for a total of 2–3 washes.
7. If the pellet is red, indicating a high number of red blood cells (RBCs), wash the pellet with Erythrocytes Lysis Buffer (L5) from the PureLink™ Total RNA Blood Kit (K1560-01).
8. If you need to remove RBCs, add 500 µL of L5 and incubate for 10 minutes on ice.
9. Gently vortex the tube 2–3 times during the incubation step to promote complete lysis of erythrocytes. The solution should become translucent.
10. Centrifuge the tube in a 4°C centrifuge at 400 x *g* for 10 minutes (to make sure cell debris is not retained supernatant). Carefully remove the supernatant and discard it.
11. Gently resuspend the cell pellet in PBS and proceed with the wash. The cell pellet should be white with no traces of red.
12. Resuspend the pellet into 0.5% paraformaldehyde and force cells through strainer (50 µm) before flow cytometry analysis.

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 Invitrogen 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 (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. 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 LSCM as previously described. 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

Looking for an answer to your question? Browse the following topics for an answer. For answers to additional questions, please refer to the Invitrogen's technical support FAQ database or contact Invitrogen Technical Support to have a representative assist you.

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

A. Chemically modified Stealth RNAi™ siRNA duplexes have a number of advantages over standard RNAi duplexes, including minimization of off-target effects, enhanced stability, and reduced toxicity. For these reasons, Stealth RNAi™ chemically modified RNAi duplexes are recommended for *in vivo* RNAi experiments. For some pilot experiments, where the goal is to determine biodistribution, siRNA duplexes and fluorescent controls are useful and more cost-effective.

Q. Should I use labeled or unlabeled duplexes for my in vivo RNAi experiments?

A. We have demonstrated that labeling Stealth RNAi™ siRNA duplexes does not hamper their knockdown potency. An alternative approach is to mix unlabeled duplexes with labeled control duplexes; this method is more commonly used with *in vivo* RNAi, and eliminates possible effects of the dye on the target, which is useful should you wish to use the data as a model for future clinical studies.

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

A. Invitrogen's production of *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.

Q. Should I use siRNA or vectors for in vivo RNAi?

A. 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 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].

Q. How should I deliver my in vivo RNAi molecules?

A. Several different approaches have been used for siRNA delivery, including various local delivery techniques and systemic delivery.

Q. How many micrograms or milligrams are delivered of the nanomole quantities of synthetic duplexes offered by Invitrogen for in vivo RNAi?

A. Table 5.4 provides nanomole quantities in micrograms (µg) and milligrams (mg).

Table 5.4. Quantities delivered, in micrograms (µg) and milligrams (mg).

			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

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

A. Please contact rnairesearcher@invitrogen.com regarding large-scale *in vivo* RNAi

Q. Do I need endotoxin testing for my in vivo RNAi duplexes?

A. Please check with the appropriate regulatory agency for guidance.

Q. How can I order in vivo RNAi duplexes?

A. RNAi duplexes for *in vivo* experiments can be ordered using the simple BLOCK-iT™ RNAi Express.

Q. How much is the price per duplex of the in vivo RNAi duplex I wish to purchase?

A. Pricing is calculated in the following manner:

Duplex price = (purity price) + (price per base for chosen scale x quantity of bases) + (optional 5′ sense strand label) + (optional endotoxin testing)

NOTE: siRNA has 42 bases. Stealth RNAi™ siRNA has 50 bases.

Chapter references

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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 prior to initial library purchase, including the quality of the reagents, formatting needs, validation strategy, and support. Recently, we interviewed scientists from the top 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. Ambion® *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).

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 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.

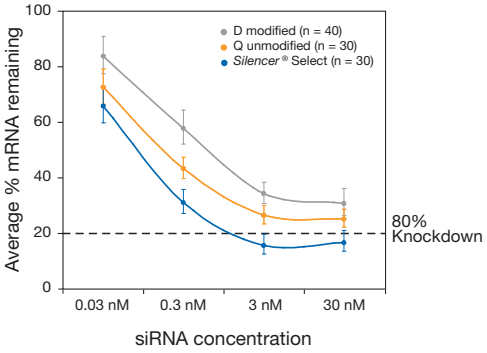

Figure 6.1. *Silencer*® Select siRNAs provide up to 100x higher potency compared to other siRNAs. *Silencer*® Select siRNAs to 10 different targets and siRNAs from two other suppliers to the same 10 different targets were individually transfected into HeLa cells in triplicate at the indicated siRNA concentrations. 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.

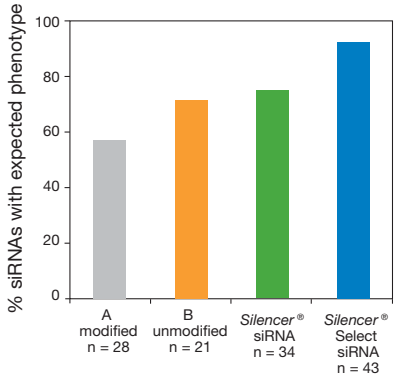

Figure 6.2. *Silencer*® Select siRNAs elicit expected phenotype at a higher rate than other siRNAs. siRNAs to 7 gene targets with well-understood RNAi-induced phenotypes were individually transfected at 3 nM and phenotypes measured 48 hr later. Each bar represents the percent of siRNAs that gave the 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.

Key consideration: siRNA quality

siRNA design and structure is important; however, siRNA manufacturing quality is equally critical. Dr. Hakim Djaballah, Director of the High Throughput Screening Core Facility at Memorial Sloan Kettering Cancer Center, said, "Cellular viability is a major concern in our screening experiments. We need to know that the contaminants that result from the production of siRNA are at extremely low levels. Often we dilute our reagents and use them on a small scale with a small number of cells, and we cannot afford impurities to be present." Ambion® *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 spec, 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 Ambion® *Silencer*® Select siRNA algorithm has been used to generate siRNAs to the human, rat, and mouse genomes, and an siRNA library for 21,585 unique genes within the human genome is available for immediate shipment. Other species are covered on a custom basis. Predesigned siRNAs sets are available for a number of important human gene families that include kinases, phosphatases, GPCRs, and several others. An extended druggable genome set is available that targets 10,415 genes of potential therapeutic interest. Although prepared sets are very useful, more and more researchers prefer to screen their own subsets of targets. To that end, Ambion can provide a custom preparation of *Silencer*® Select siRNAs targeting your list of favorite genes. Finally, our bioinformatics team is standing by to help you generate a list of genes within a functional class or biological pathway for your custom siRNA library.

Key consideration: customization

siRNA screens are quite varied in nature. "Every experiment has different requirements. What is the scale of siRNA desired in each well? How are the duplexes oriented? Can the supplier provide custom aliquotting? And most importantly, what are the turnaround times for ordered libraries?" said Dr. Djaballah. All are critical factors in selecting an siRNA library vendor, both for the initial siRNA library purchase and for hit confirmation follow-up studies.

Ambion® *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. "We don't think simply using another supplier's siRNA is a good enough method for validation if you are relying on the differences between the two suppliers' algorithms. We prefer qRT-PCR followed by protein level measurements. We sometimes look at rescue experiments using an expression vector, but these can be tedious and there can be toxicity concerns," said Dr. Djaballah. Dr. Ganesan remarked, "We validate hits by several different strategies. We utilize multiple siRNAs to eliminate off targets. We do quantitative qRT-PCR to ensure that the siRNA impacts the expression of the target gene, and we examine the impact of these siRNAs in several different cell lines. More recently, we have also been validating our hits with shRNAs." Dr. Ganesan went on to say, "The major cost of screens is usually not from the screen itself, but from the follow-ups." 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. Since 2002, Ambion has consulted with and helped thousands of scientists using siRNAs. Custom sets of *Silencer*® Select siRNAs are available with as little as 0.1 nmol siRNA per well—arranged in plates any way you like. Of course, following up on large numbers of false positive results can easily drain your hit validation budget. By performing our 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. Dr. Djaballah, who has directed many siRNA screening experiments, stated that "there must be access to open discussion" when it comes to planning a screen. We certainly understand this need. To that end, we encourage open communication through siRNA library user group meetings, we support major RNAi symposia, and 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 Ambion® *Silencer*® Select siRNA libraries, please contact your local Invitrogen sales representative or visit www.invitrogen.com/rnai.

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 hundreds of genes for their role in cellular processes. siRNA libraries can be obtained in a variety of formats, including single-transfection, ready-to-use versions, making genome-wide or smaller RNAi screens possible in almost any laboratory.

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 are not confirmed by other distinct siRNAs targeting the same gene or by secondary assays. The anomalous assay result from a false positive 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

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

Are caused by suboptimal screening reagents, conditions, or assay design. They can also result from experimental variability. 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.

Challenges

While many laboratories are performing RNAi screens with siRNA libraries to determine gene function in mammalian cultured cells, the technology is just now being broadly applied. In the past, three factors have limited the adoption of this technology: (1) the perceived cost of siRNA libraries, (2) the belief that expensive robotic liquid handling systems are required, and (3) the anticipated difficulties in performing transfections reproducibly in 96- or 384-well plates.

Tools are now available that address all of these issues, making siRNA library screening feasible for more labs than ever before. siRNA libraries are available in formats suitable for a single transfection, and up to thousands of transfections. There are now extensive sets of positive and negative controls and delivery optimization reagents for developing optimal screening protocols. High-throughput delivery reagents, instruments, and protocols yield easy and reproducible siRNA delivery in multiwell plates using either manual or robotic means.

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, on the protein product's activity and half-life within the cell, and on redundancy of 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.

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 (biological false negatives cannot be avoided; see Definitions on page 31). 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 (30–100 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 numerous 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(s)
- → 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 to 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 (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 screen assay background. Finally, in screens that include a treatment of some sort to induce the phenotypic effect, untreated controls serve as a baseline for the screening assay.

Step 2. Optimize the siRNA delivery method

Screening siRNAs that target hundreds to thousands of genes requires conditions for high-throughput siRNA delivery. Furthermore, efficient, reproducible siRNA delivery is critical for effective siRNA library screens. 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 controls 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 31) 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 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 that 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 intital 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 candiate 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 inerest. It would not be uncommon for the second screen to eliminate up to 90% of candiate 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 driect measure of target gene silcencing (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) designed to help independently confirm data and expand upon results found in the published literature.

Toxicity

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 any gene silencing effects caused by siRNAs on nontarget mRNAs 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 the case of limited homology cause translation inhibition (act as miRNA). The SVM algorithm used to design our siRNAs selects only the sequences that have no or little 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 the 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 signalling 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. 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 [6], 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 your prioritize follow up to first 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 [7]. To confirm that a hit is associated with a specific pathway, additional phenotypes (e.g., up- or down-regulation of other genes, increase or decrease in protein modification), that are known to occur when the pathway of interest is perturbed, can be assayed after siRNA treatment [8,9]. 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 [6,10].

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 a silent mutation(s) 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 recognizes 5′ or 3′ untranslated sequences can be rescued by expressing a cDNA of the target gene that contains only coding sequences [11]. Recently, bacterial artificial chromosome transgenesis in cultured cells has been used to create RNAi-resistant transgenes [12].

Measure mRNA and protein levels

As mentioned above, confirmation of positive hits involves verifying that the observed phenotype is specifically due to reducing the expression of the target gene. Whenever possible, determining siRNA-induced reduction in 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 that of samples treated with nontargeting negative control siRNAs.

If target mRNA levels are not appreciably reduced, yet the siRNA induces a positive result in the assay, it is possible that the observed phenotype is caused by an off-target effect. However, 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 knockdown is not observed at either the mRNA or protein level, yet the siRNA induces a distinct phenotype, 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 in 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, it might be possible to monitor the effects of siRNA treatment at shorter timepoints 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, 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 rapidity; however, replicates and normalization controls need to be carefully assessed to avoid missing slight changes in levels of gene expression.

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 is based 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 among proteins and may vary with cell cycle. As described in protein based assays on page 91, 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 with relatively low expression or without available antibodies.

For example, cotransfection of siRNA with plasmids expressing the target transcript may be helpful for studying low abudance mRNA targets [11]. 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., Green Fluorescent Protein or Red Fluorescent Protein) [12,3] 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 [7]. Alternatviely, 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 reporter genes, another approach involves fusing epitope tages (e.g., glutathione-*S*-transferase, Myc, hemagglutinin, 6-histidine, or FLAG® epitope tags) to the target protein to enable detection with well-characterized commercially available antibodies [7,11,14].

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Section II—Products for RNA Interference

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CHAPTER 7

siRNA technologies

Introduction to nonvector methods for transient RNAi

For transient knockdown experiments, synthetic, nonvector approaches offer significant advantages over vector-based methods for RNAi delivery. In particular, nonvector experiments are typically easier to design and perform and can result in higher levels of transient knockdown. In addition, recent improvements in RNAi design have increased the likelihood of achieving high-level knockdown after testing only a few RNAi molecules. Consequently, using synthetically generated 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]. While these studies have shown that duplexes with partial homology to a target can regulate gene expression, the rules for designing duplexes to eliminate off-target effects are not completely understood.

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. Recently, 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.

Ambion® *Silencer*® Select siRNA and Stealth RNAi™ siRNA

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* and *in vivo* analyses. Invitrogen now offers Ambion® products. As a result, you will find the two best siRNA technologies available in one place. *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. Stealth RNAi™ siRNAs are the gold standard for *in vivo* applications, and have been proven effective in helping to meet the demands of *in vivo* experiments. Together, these tools allow your experiments to yield accurate data and conclusions—from hypothesis to demonstrated phenotype.

Silencer® Select siRNAs

The best siRNAs for *in vitro* applications

→ Classical 21-mer siRNAs with enhanced chemical modifications and improved algorithm

→ Specific knockdown—novel chemical modifications reduce off-target effects by up to 90%

- → Effective knockdown—designed with an all-new 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
- → Guaranteed 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. Current 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 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.

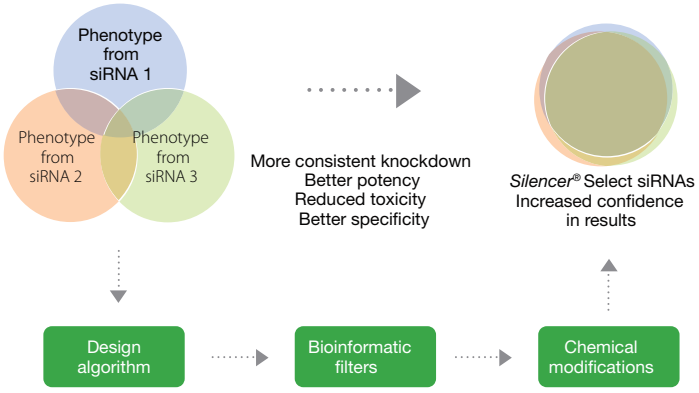

Figure 7.1 The concept behind *Silencer*® Select siRNAs.

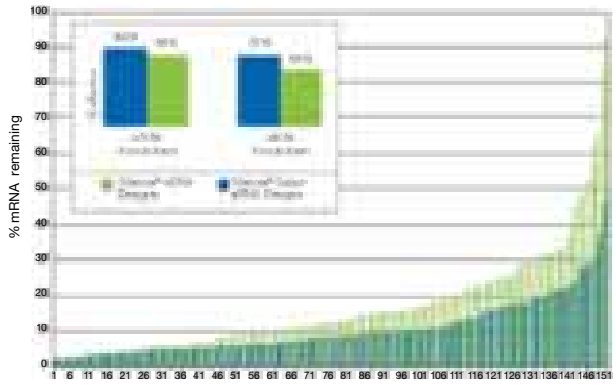

Figure 7.2. *Silencer*® Select siRNA design algorithm significantly improves effective siRNA prediction accuracy. The *Silencer*® Select siRNA design algorithm was used to design 155 siRNAs to 40 different targets. These siRNAs were tested side by side with siRNAs designed using the previous algorithm at 5 nM in HeLa cells. mRNA knockdown was measured 48 hr posttransfection via qRT-PCR using TaqMan® Gene Expression Assays. Results are expressed as percent of mRNA remaining compared to *Silencer*® Negative Control #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

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

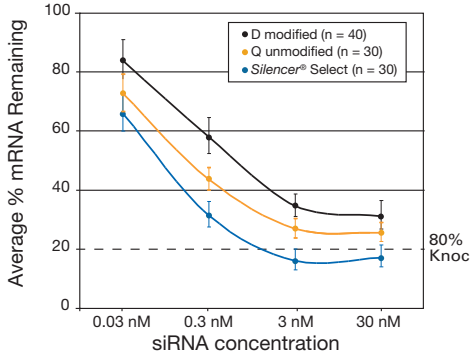

Figure 7.3. *Silencer*® Select siRNAs provide up to 100x higher potency compared to other siRNAs. *Silencer*® Select siRNAs to 10 different targets and siRNAs from two other suppliers to the same 10 different targets were individually transfected into HeLa cells in triplicate at the indicated siRNA concentration. 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.

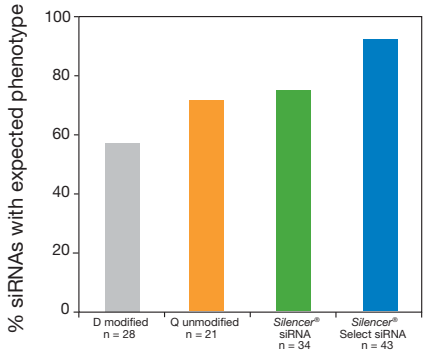

Figure 7.4. *Silencer*® Select siRNAs elicit expected phenotype at a higher rate than other siRNAs. siRNAs to 7 gene targets with well-understood RNAi-induced phenotypes were individually transfected at 3 nM and phenotypes measured 48 hr later. Each bar represents the percent of siRNAs that gave the 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 Pre-designed siRNAs to a target; both are guaranteed to knock down target gene expression by ≥70%
- → Buy 3 *Silencer*® Select Pre-designed siRNAs to a target; 2 of 3 are guaranteed to knock down by ≥80%
- → *Silencer*® Select Validated siRNAs are guaranteed to knock down by ≥80%

For details, visit www.invitrogen.com/rnai.

Bioinformatic filtering improves siRNA specificity. Although using siRNAs at low concentrations decreases off-target effects, further specificity gains can be made 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 *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

Chemical modifications enhance guide strand bias. Strong guide strand bias, where the guide strand of the siRNA is preferentially taken up into the RISC over the 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 siRNAs:

- → 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

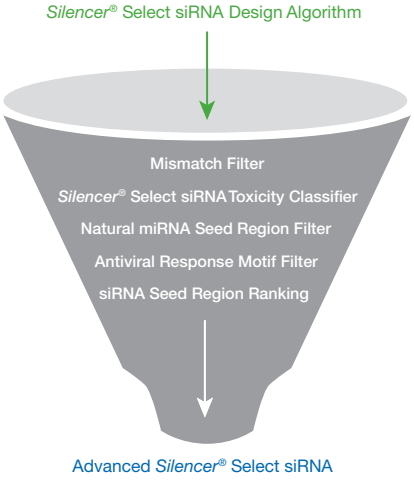

Figure 7.5. Five-step bioinformatic filtering process that eliminates siRNAs predicted to elicit off-target effects.

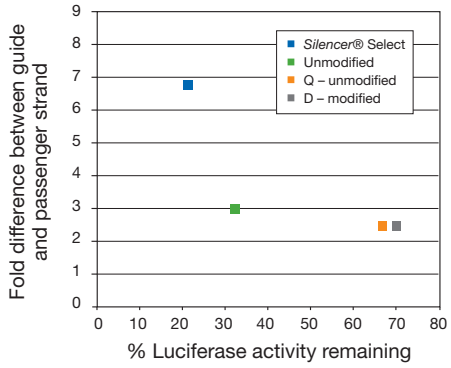

Figure 7.6. *Silencer*® Select siRNAs show enhanced guide strand bias. Luciferase reporter gene constructs with siRNA targets cloned in either the sense (guide strand target) or antisense (passenger strand target) orientation were cotransfected with the corresponding siRNA and a β-galactosidase–encoding 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 to 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 new 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

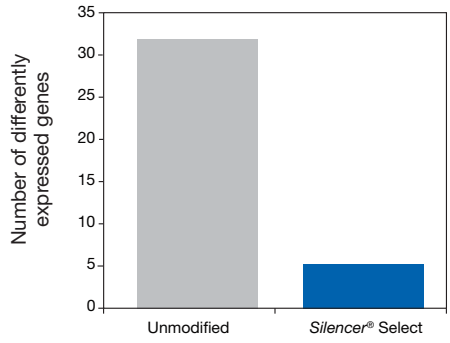

Figure 7.7. *Silencer*® Select siRNA modifications reduce the number of off-target, differentially expressed genes. Three negative control siRNAs with and without the *Silencer*® Select modifications were individually transfected in quadruplicate into HeLa cells at 30 nM. RNA was extracted and analyzed on an Affymetrix 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.

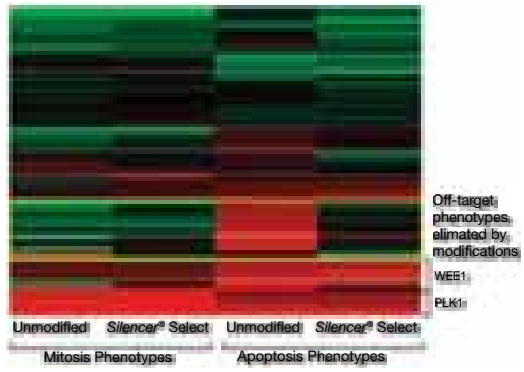

Figure 7.8. *Silencer*® Select siRNA modifications reduce off-target effects and yield more reliable phenotypic data. 53 different siRNAs, including older designs previously noted to elicit off-target phenotypes, were transfected into U2OS cells at 30 nM in both unmodified and *Silencer*® Select modified formats. Mitosis and apoptosis were measured 48 hours later. Data are expressed relative to negative control siRNA–transfected cells. Black = similar mitosis/apoptosis levels as control. Green = down-regulation. Red = up-regulation. Note that the expected mitotosis 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 modifications.

Stealth RNAi™ siRNA

The gold standard for *in vivo** RNAi applications

- → Award-winning design algorithm—allows the highest rates of knockdown success
- → 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 organs targeted in the subject animal.

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

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. Stealth RNAi™ siRNA duplexes are designed with Invitrogen's award-winning BLOCK-iT™ RNAi Designer (www.invitrogen.com/rnaidesigner), which uses Smith-Waterman alignment to compare the RNA duplex region to the entire genome, including untranslated regions. This design step minimizes the potential for off-target effects and allows a very high percentage of experimentally successful duplexes (Figures 7.9 and 7.10).

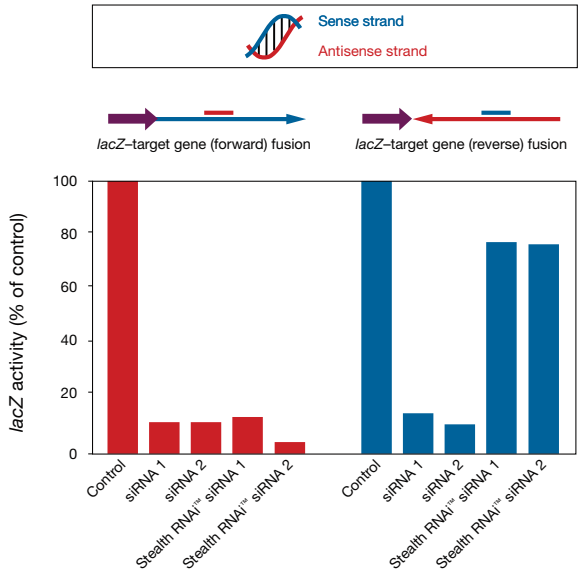

Figure 7.9. Stealth RNAi™ siRNA exhibits increased target specificity. Sequences 1 and 2 were designed as either siRNA or Stealth RNAi™ siRNA duplexes against a target gene. Only the antisense strand of a Stealth RNAi™ siRNA duplex can enter the RISC for specific targeting of mRNA, whereas either strand of siRNA can enter the RISC, increasing the possibility of nonspecific silencing.

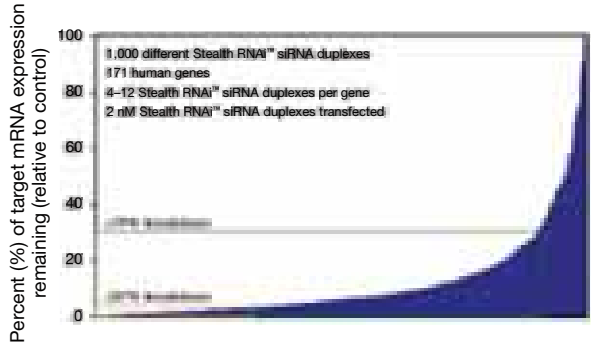

Figure 7.10. Success rate of Stealth RNAi™ siRNA design exceeds 90%. Invitrogen has been generating data to evaluate the efficacy of a large number of Stealth RNAi™ siRNA duplexes since initiation of its extensive Validated Stealth RNAi™ siRNA program in 2004. This data set was derived from 1,000 Stealth RNAi™ siRNA duplexes targeted to 171 genes generated using the BLOCK-iT™ RNAi Designer from 2004. Knockdown was measured using the RNAi target screening system, which assesses knockdown at the protein level. Over 90% of these duplexes (bounded by green box) resulted in ≥70% knockdown, and 30% (bounded by yellow box) resulted in ≥97% knockdown. These results were obtained using 2 nM Stealth RNAi™ siRNA duplexes. Typically, 10 to 100 nM is used for these types of experiments; the higher end of this range can lead to off-target effects. The data were analyzed at each position to determine the differences between "good" and "bad" Stealth RNAi™ siRNA sequences. This information was then used to train the algorithm and has been incorporated into the latest version of the BLOCK-iT™ RNAi Designer (www.invitrogen.com/rnaidesigner).

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™ siRNA duplexes have proprietary chemical modifications that confer remarkable stability for *in vivo* studies (Figure 7.11); standard siRNAs lack such modifications, making them more susceptible to degradation by nucleases.

No induction of the interferon response. The interferon response is a powerful immune reaction that can make analysis of knockdown 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™ siRNA molecules abolish the immunostimulatory response observed with some sequences. Stealth RNAi™ siRNA complexes do not induce the interferon response (Figure 7.12), 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 necessary to ensure that the delivered material has targeted the desired tissues. Invitrogen uses the industry-leading Alexa Fluor® fluorescent dyes to label Stealth RNAi™ siRNA 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™ siRNA 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™ siRNA duplex (Figure 7.13). Direct labeling of Stealth RNAi™ siRNA duplexes does not affect their potency.

Together, the *Silencer*® Select siRNA and the Stealth RNAi™ siRNA technologies provide a superior, complete, and robust solution for your RNAi studies both *in vitro* and *in vivo*. For more information on these and other products for RNAi studies, visit www.invitrogen.com/rnai.

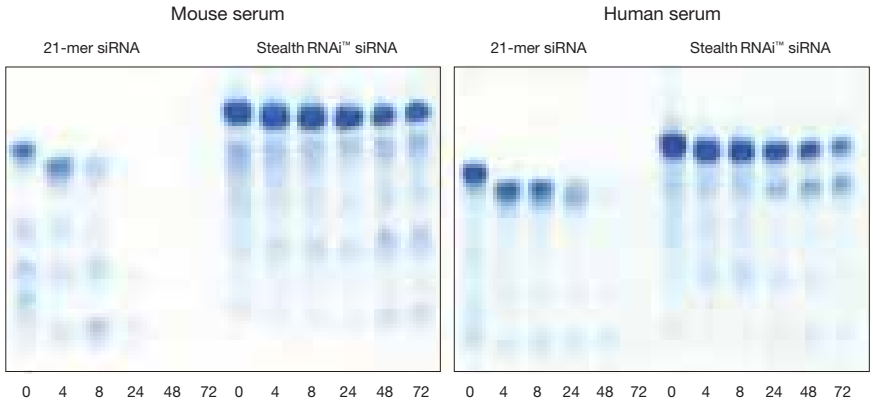

Figure 7.11. Chemical modification makes Stealth RNAi™ siRNA more stable for *in vivo* applications. Stealth RNAi™ siRNA duplexes are chemically modified to enhance stability against nucleases in serum. Unmodified 21-mer dsRNA sequences and corresponding Stealth RNAi™ siRNA sequences were analyzed at 0, 4, 8, 24, 48, and 72 hr following incubation in either 10% mouse or human serum. Samples were separated on a Novex® 15% TBE-urea polyacrylamide precast gel and stained with methylene blue. These data demonstrate that Stealth RNAi™ siRNA duplexes remain intact, whereas unmodified 21-mer dsRNA sequences were susceptible to pronounced degradation.

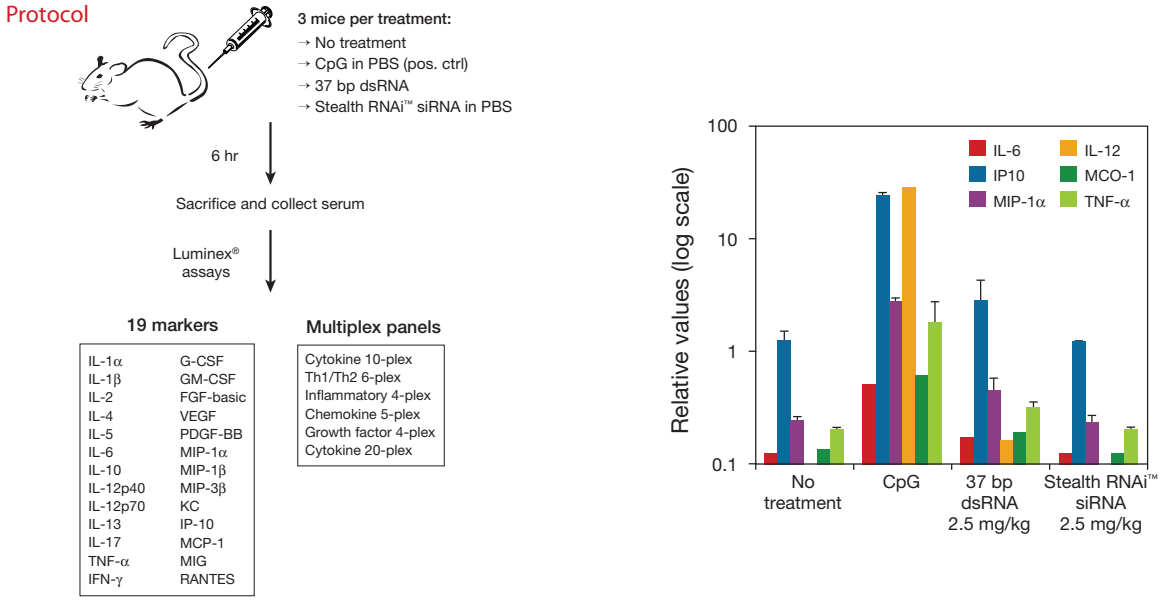

Figure 7.12. Stealth RNAi™ siRNA avoids the interferon stress response so that data can be confidently correlated with knockdown activity. Tail veins of Balb/c mice (three mice per treatment) were injected at 5 mg/kg and 10 mg/kg of Stealth RNAi™ siRNA duplexes. After 6 hr, serum was collected and processed to measure interferon markers using the Invitrogen Cytokine Mouse 20-Plex Panel for the Luminex® instrument (Cat. No. LMC0006).

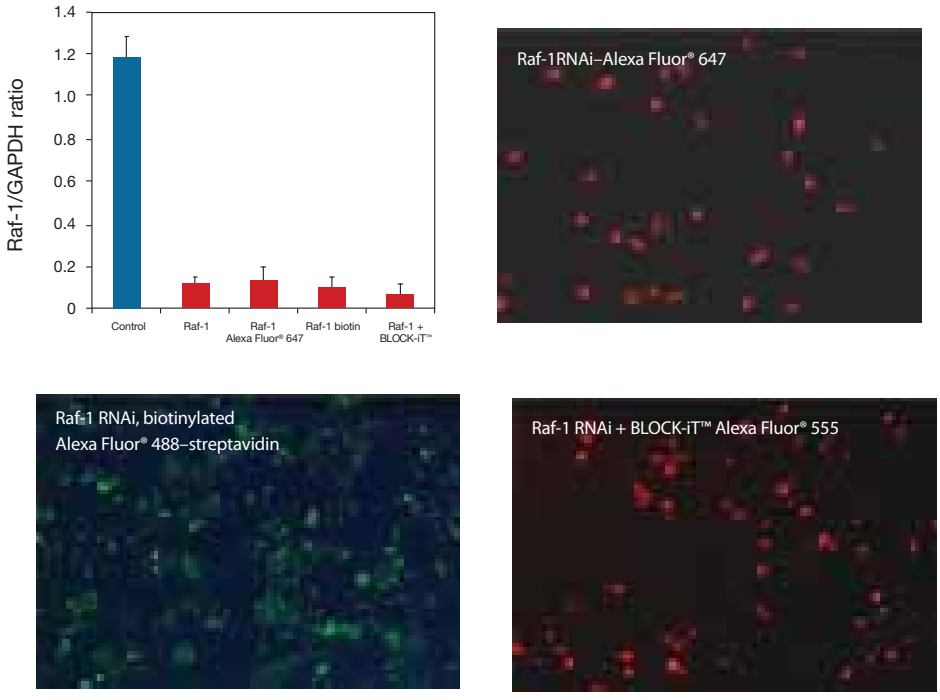

Figure 7.13. Labeled Stealth RNAi™ siRNA exhibits potent knockdown. MDA-MB-435 cells were transfected with 25 nmol unlabeled Stealth RNAi™ siRNA duplexes, or Stealth RNAi™ duplexes labeled with an Alexa Fluor® dye or biotin. In each case the duplexes targeted Raf-1. Knockdown was assessed at >90% by real-time PCR; delivery is demonstrated through fluorescence imaging. Regardless of the labeling strategy, Stealth RNAi™ siRNA knockdown results were consistent.

siRNA selection guide

siRNA type	Description	Technology	Recommended use
Pre-designed/Validated (tubes)			
Stealth Select RNAi™ siRNA and Validated Stealth RNAi™ siRNA	• Modified for serum stability and improved specificity • Pre-designed 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® Select Pre-designed and Validated siRNA	• Improved potency and guaranteed knockdown • Modified for improved specificity • Pre-designed 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
Silencer® Pre-designed and Validated siRNA	• Pre-designed 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 all applications
Pre-made sets/collections			
Made-to-Stock *Silencer*® Select siRNA Libraries	• Pre-defined 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 pre-defined *Silencer*® Select siRNA sets such as human kinases, GPCRs, druggable and whole genome
Made-to-Stock *Silencer*® siRNA Libraries	• Pre-defined 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
Plated			
Made-to-Order *Silencer*® Select siRNA Libraries	• Custom sets of *Silencer*® Select siRNAs at 0.1 to 5 nmol • Priced by quote and ordered offline	LNA® modified 21-mer	• For screening 36 to >10,000 *Silencer*® Select siRNAs • Submit a gene list or choose one of our functional sets • 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
PlateSelect™ RNAi w/ Stealth Select RNAi™ siRNA	• Stealth Select RNAi™ siRNA in plated format at 1 nmol size • Configure plates with Pre-designed siRNA or siRNAs designed online with the BLOCK-iT™ RNAi Designer	Modified 25-mer	• For Stealth Select RNAi™ siRNA experiments in plates with 1 to >96 siRNAs • Specify a set siRNAs using online interface • Made to order
PlateSelect™ RNAi w/ BLOCK-iT™ siRNA	• BLOCK-iT™ siRNA in plated format at 1 nmol size • Configure plates with siRNAs designed online with the BLOCK-iT™ RNAi Designer	Unmodified 21-mer	• For unmodified siRNA experiments in plates with 1 to >96 siRNAs at 1 nmol • Specify a set of siRNAs using online interface • Made to order
Design-your-own siRNAs			
Stealth RNAi™ siRNA	• Chemically modified siRNA designed using the BLOCK-iT™ RNAi Designer	Modified 25-mer	• For genes that fall outside of our Pre-designed siRNA collection • Suitable for all applications • Particularly good for *in vivo* applications
Block-iT™ siRNA	• Traditional, unmodified siRNA designed using the BLOCK-iT™ RNAi Designer	Unmodified 21-mer	• For genes that fall outside of our Pre-designed siRNA collection • Lower cost alternative, but without some of the performance benefits of Stealth RNAi™ siRNAs

siRNA type	Description	Technology	Recommended use
Custom (you provide the sequence)			
Stealth RNAi™ siRNA	• Modified for serum stability and improved specificity Modified 25-mer	Modified 25-mer	• When you have an siRNA sequence and need it manufactured
Block-iT™ siRNA	• Traditional siRNA • Available with a selection of fluorescent labels and modifications	Choice of 21–25-mers	• When you have an siRNA sequence and need it manufactured—with or without a label • Lower-cost alternative, but without some of the performance benefits of Stealth RNAi™ siRNAs
Controls			
Stealth RNAi™ siRNA Positive & Negative Controls	• siRNA controls for use with Stealth RNAi™ siRNA • Fluorescent labels available with some	Modified 25-mer	• Any time you are using a Stealth RNAi™ siRNA
Silencer® Select Positive & 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 & 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
Custom Scrambled Negative Control siRNA	• siRNA controls for BLOCK-iT™ siRNAs • Match your scrambled negative control to the GC content of your unmodified 21-mer siRNA	Unmodified 21-mer	• Any time you are using a BLOCK-iT™ siRNA

Silencer® Select Pre-designed siRNAs

Silencer® Select Pre-designed siRNAs come with a *100% guarantee to silence* their intended target, and are available for >98% of genes in the human, mouse, and rat genomes in a purified, ready-to-use format. siRNA sequence information is always provided.

Silencer® Select Validated siRNAs

Silencer® Select Validated siRNAs are individual siRNA duplexes that have 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*® Select Pre-designed siRNAs. However, each *Silencer*® Select Validated siRNA has also been functionally confirmed and is guaranteed to reduce target gene expression by at least 80% when measured 48 hours posttransfection.

Custom Designed *Silencer*® Select siRNAs

The *Silencer*® Select siRNA design algorithm yields an extremely high percentage of effective siRNA sequences. *Silencer*® Select Pre-designed siRNAs are immediately available for all human, mouse, and rat genes listed in the public RefSeq database maintained by NCBI. However, if your needs extend beyond our current selection of Pre-designed siRNAs, we offer *Silencer*® Select Custom Designed siRNAs, which are designed using the same algorithm and with the same modifications as our other *Silencer*® Select Pre-designed siRNAs.

Order *Silencer*® Select siRNAs at www.invitrogen.com/RNAi.

Silencer® Select siRNA libraries

Pre-defined siRNA libraries plated to order

Pre-defined, made-to-order *Silencer*® Select siRNA Libraries are available for human:

- → Genome
- → Druggable genome
- → Extended druggable genome
- → Kinases
- → Phosphatases
- → GPCRs (non-olfactory)
- → Ion channels
- → Nuclear hormone receptors
- → Proteases

Human Genome siRNA Library

(Consists of Part Numbers: 4397922 + 4397924 + 4397923)

Increasingly, researchers are performing genome-scale surveys of gene function. The *Silencer*® Select Human Genome siRNA Library V4 is ideal for this purpose. Three unique, non-overlapping siRNAs are provided for each of 21,585 human targets. The siRNAs targeting the "druggable" portion of this library are arranged by gene functional class to enable easy screening of important gene subsets such as kinase, phosphatase, GPCR, etc. 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 *Silencer*® Select Human Druggable Genome siRNA Library V4 and *Silencer*® Select Human Extended Druggable Genome siRNA Library V4 are popular choices for many researchers. The druggable genome set targets 9,032 genes, whereas the extended druggable genome collection targets the same 9,032 genes plus the "Extension Set" for a total of 10,415 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.

Human Kinase siRNA Library

Because of their importance in cell signaling and many biological pathways, kinases are key drug targets and subjects of intense scrutiny. The *Silencer*® Select Human Kinase siRNA Library V4 targets 710 human kinases with three individual *Silencer*® Select siRNAs per gene. This siRNA library enables systematic yet cost-effective RNAi studies of these key cell regulators. Validated siRNAs are included where available. The *Silencer*® Select Human Kinase siRNA Library V4 is provided in 96-well plates.

Human Phosphatase siRNA Library

Along with kinases, phosphatases are important regulators of biological pathways and cell signaling cascades. The *Silencer*® Select Human Phosphatase siRNA Library V4 targets 298 human phosphatases with three individual siRNAs per gene supplied in 96-well plates. This library is the ideal companion to the *Silencer*® Select Kinase siRNA Library V4, enabling more detailed studies of biological pathways.

Human GPCR siRNA Library

G-protein–coupled receptors, or GPCRs, are key components of many signal transduction pathways and also represent an important class of druggable genes. The *Silencer*® Select Human GPCR siRNA Library V4 targets 380 non-olfactory human GPCRs with three individual *Silencer*® Select siRNAs per target supplied in 96-well plates.

Human Protease, Ion Channel, and Nuclear Hormone Receptor siRNA Libraries

Focused siRNA sets to human proteases, ion channels, and nuclear hormone receptors provide a cost effective means of studying these important regulators of cellular function. These siRNA libraries are each supplied in 96-well plates. The *Silencer*® Select Human Protease siRNA Library V4 features 3 siRNAs per target for 494 human proteases. The *Silencer*® Select Human Ion Channel siRNA Library V4 features 3 siRNAs per target for 338 human ion channels. The *Silencer*® Select Human Nuclear Hormone Receptor siRNA Library V4 includes 3 siRNAs per target for 47 nuclear hormone receptors.

To create these siRNA libraries, the Applied Biosystems bioinformatics team uses PANTHER (www.pantherdb.org) and Gene Ontology™ annotation information to create up-to-date target gene sets. siRNAs targeting the collection are then plated to order (minimum 0.25 nmol), giving you not only the most up-to-date siRNA libraries, but also the freedom to customize your library so that it is most convenient for your particular experimental setup. Want to add siRNAs to your collection? No problem! We can also provide siRNAs in 96-well or 384-well plates, deliver multiple aliquots of each plate, arrange siRNAs in custom configurations, and provide siRNAs individually and/or in pools.

(Our most popular collections are also available as pre-plated, ready-to-ship libraries with 0.25 nmol of each siRNA.)

Custom siRNA libraries—the ultimate in flexibility

Do you have a favorite list of human genes you would like to target with a collection of siRNAs? We can prepare a custom *Silencer*® Select siRNA Library to any set of human genes. The minimum order is only 36 siRNAs. All you need to supply is a list of genes or transcripts identified by NCBI Entrez Gene ID or RefSeq mRNA accession number. With this user-friendly option, you can specify the siRNA layout on either 96- or 384-well plates. For a 96-well plate, our standard format includes your choice of 0.1, 0.25, 1, 2, or 5 nmol of each siRNA, with three individual siRNAs provided per target. For a 384-well plate, our standard format includes your choice of 1, 2, or 5 nmol of each siRNA, with three individual siRNA provided per target. Multiple aliquots, pooling of siRNAs, and even custom siRNA design requests can all be accomodated.

Information to accelerate discovery. All *Silencer*® Select siRNA Libraries, whether custom, pre-defined, or pre-plated, are supplied with full siRNA sequence information, and when available, with siRNA validation data. Also provided on the CD accompanying the siRNA library are gene annotation information, which includes gene symbol, gene name, aliases, NCBI Entrez Gene ID, associated RefSeq mRNA accession numbers, and other 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.

Silencer® Select siRNA Libraries contain 0.25 nmol of each siRNA, sufficient for 500 transfections (at 5 nM siRNA, 100 µL transfection volume). Preloaded 96-well plates are supplied with lyophilized siRNAs. One column of each 96-well plate is left empty to allow for the addition of controls at the time of use. The libraries are delivered with full siRNA sequence information, as well as the exon targeted and each targeted gene's symbol, full name, aliases, RefSeq number, and Entrez Gene ID, plus results from any validation experiments done using the included siRNAs.

Silencer® Select siRNA Libraries to these or any other list of human genes can also be provided with 1, 2, or 5 nmol of each siRNA. Custom formatting is available with these larger sizes.

If you would like a gene list, additional information, or would like to discuss your research with one of our technical experts, contact your local Invitrogen sales representative or e-mail us at rnairesearcher@invitrogen.com.

Silencer® Pre-designed 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

Silencer® Pre-designed siRNAs—chemically synthesized, unmodified siRNAs available for human, mouse and rat genes—are designed with a rigorously tested siRNA design algorithm (see *Silencer*® Select Pre-designed siRNAs on page 40 for chemically modified siRNAs designed with an even more effective algorithm). Invitrogen guarantees that when three *Silencer*® Pre-designed siRNAs are obtained to the same target, at least two will reduce target mRNA levels by 70% or more.

Silencer® Validated 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*® Pre-designed siRNAs. However, each one has also been functionally proven and is guaranteed to reduce target gene expression by at least 70% 48 hours posttransfection.

High-quality synthesis

Invitrogen synthesizes and purifies 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 20 for a description of siRNA purity grades.

Silencer® siRNAs are easy to obtain

Invitrogens' searchable online siRNA database makes it easy to obtain effective, guaranteed-to-work siRNAs. Simply visit www.invitrogen.com/siRNA to find siRNAs for your human, mouse, or rat gene of interest. Interested in other organisms? Invitrogen provides Custom Designed *Silencer*® and *Silencer*® Select siRNAs (see page 49).

Order *Silencer*® siRNAs at www.invitrogen.com/RNAi.

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CHAPTER 8

Vector-based RNAi technologies

RNAi vectors for increased RNAi experimental options

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 vectors limit knockdown to specific tissues or cells. There is a suitable BLOCK-iT™ RNAi vector for every RNAi application.

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

The powerful new 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 new 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
Gateway® vector compatibility with most 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								
pcDNA™6.2-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)	57
pcDNA™6.2-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)	57
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)	58
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)	58
pLenti6.4/R4R2/V5-DEST	miR RNAi	Transient and stable	Blasticidin	Vital 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)	58
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)	66
pENTR™/H1/TO	shRNA	Transient and stable	Zeocin™	Transfection	Inducible or constitutive	None	BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit (also included in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System)	66
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	69
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	70
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	69
pBLOCK-iT™6-DEST	shRNA	Transient and stable	Blasticidin	Transfection	Constitutive	None	pBLOCK-iT™6-DEST	69
pBLOCK-iT™3-DEST	shRNA	Transient and stable	Geneticin®	Transfection	Inducible or constitutive	None	pBLOCK-iT™3-DEST	71

Continued on next page

Table 8.2. BLOCK-iT™ vector technologies, continued.

Vector	RNAi vector technology	Transient or stable expression	Selection marker	Delivery method	Constitutive or inducible expression	Reporter	Kits	Page
Selected Gateway® DEST vectors compatible with miR RNAi vector technology*								
pT-REx™-DEST30	miR RNAi	Transient and stable	Geneticin®	Transfection	Inducible (CMV)	Cocistronic EmGFP	No kit available. Vector Cat. No. 12301-016. Needs to be used in a T-REx™ cell line or in cells that express the Tet repressor protein.	62
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.	62
pEF-DEST51	miR RNAi	Transient and stable	Blasticidin	Transfection	Constitutive (EF-1α)	Cocistronic EmGFP or none †	No kit available. Vector Cat. No. 12285-011	62
pDEST™ R4-R3	miR RNAi	Transient	None	Transfection	Your choice	Cocistronic EmGFP or none †	MultiSite Gateway® Three-Fragment Vector Construction Kit	62
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	62
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	62
pcDNA™6/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	62
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	64
Ready-to-use vectors: cloned and sequence verified	miR RNAi or shRNA	Transient and stable	Blasticidin, Zeocin™, or Geneticin®	Transfection or viral transduction	Inducible or constituitive		Custom RNAi Services	93

* Additional components, such as 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.

How vector-mediated RNAi works

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 8.1).

BLOCK-iT™ Pol II miR RNAi expression vector kits utilize the endogenous miRNA pathway. Artificial miRNAs expressed from the pcDNA™ 6.2-GW/EmGFP-miR Expression Vector are transcribed by RNA Polymerase II, which enables cocistronic expression of Emerald Green Fluorescent Protein (EmGFP) and multiple miRNA hairpins on the same transcript. The primary miRNA (pri-miRNA) transcript contains the EmGFP sequence on the 5′ end, followed by 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 this 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] Exportin-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].

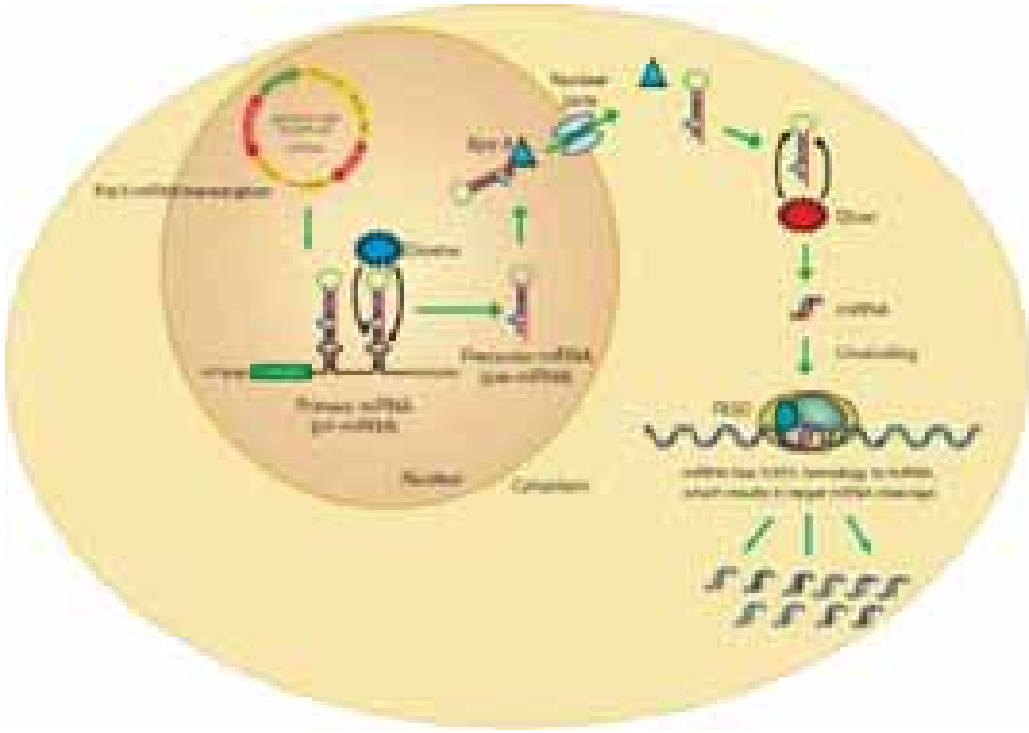

Figure 8.1. Expression of miR RNAi sequences using the BLOCK-iT™ Pol II miR RNAi expression vectors.

BLOCK-iT™ Pol II miR RNAi expression vectors—versatile Pol II vector-based RNAi technology

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 pcDNA™6.2-GW/miR and pcDNA™6.2-GW/EmGFP-miR vectors (Figure 8.2), 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 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
- → BLOCK-iT™ miR RNAi Select—target the majority of annotated human, mouse, and rat genes with predesigned BLOCK-iT™ miR RNAi Select hairpins, ready to anneal and clone into either of the BLOCK-iT™ Pol II miR RNAi expression vectors

Figure 8.2. The BLOCK-iT™ Pol II miR RNAi expression vectors. The pcDNA™6.2-GW/miR vector is driven by the CMV promoter, has the blasticidin resistance-marker, and is available with or without cocistronic EmGFP expression as a reporter.

NOTE: Find information on miR RNAi expression vectors online at www.invitrogen.com/mir.

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

BLOCK-iT™ and 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 ViraPower™ Lentiviral Expression Vectors (Figure 8.3), 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 8.4). These vectors deliver miRNA sequences into HT1080 and HeLa cells to knock down lamin A/C or *lacZ* (Figure 8.5), 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 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 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

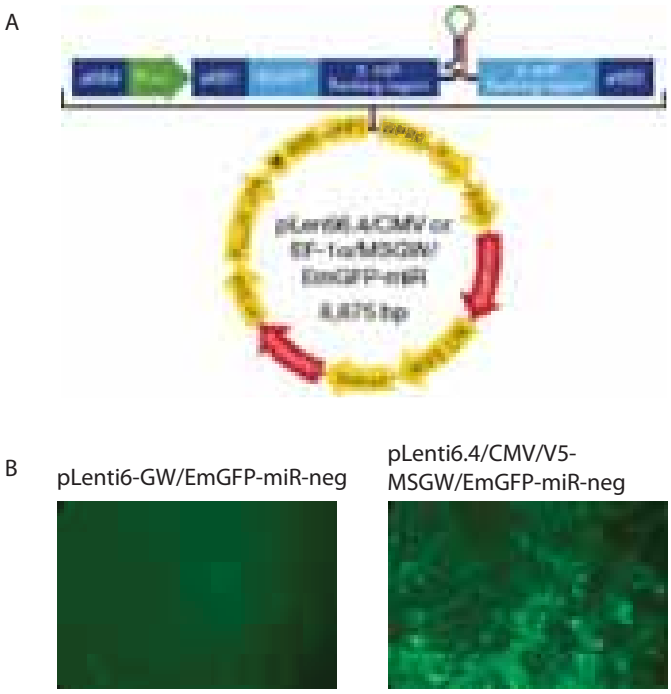

Figure 8.3. pLenti6/V5-DEST vector. A. The BLOCK-iT™ Lentiviral Pol II miR RNAi Expression Systems utilize the pLenti6/V5-DEST vector for constitutive expression from the CMV immediate-early promoter, and the blasticidin selection marker for compatibility with a tetracycline-regulated inducible system. B. Images taken 4 days following transduction of GripTite™ 293 MSR cells at an MOI of 3 with lentiviral particles generated using the indicated vectors.

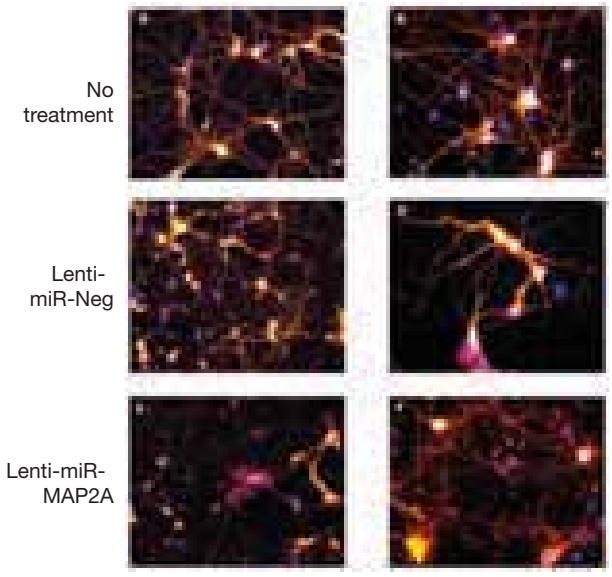

Figure 8.4. Target gene knockdown in primary neurons. Unlike standard retroviruses, lentiviruses can transduce nondividing cells, such as these rat primary cortical neurons. Here, microtubule-associated protein 2 (MAP2A) was targeted, and its expression was dramatically decreased. No treatment (A and B), Lenti-miR-Neg (C and D), and Lenti-miR-MAP2 (E and F) were stained for anti-MAP2 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.

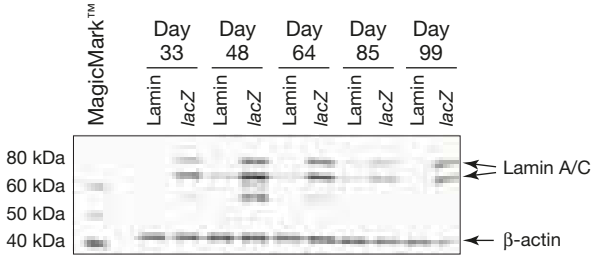

Figure 8.5. Stable lentiviral transduction of miRNA expression cassettes. Western blot analysis of HeLa cell populations stably transduced (MOI = 20) for 33 to 99 days with lamin or *lacZ* miRNA–expressing lentiviral particles. The blot was probed with anti–lamin A/C (top half) or anti–β-actin (bottom 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 pcDNA™6.2-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 correlates nearly 100% with the expression of the miRNA (Figure 8.6), allowing miRNA expression to be tracked in any cell type.

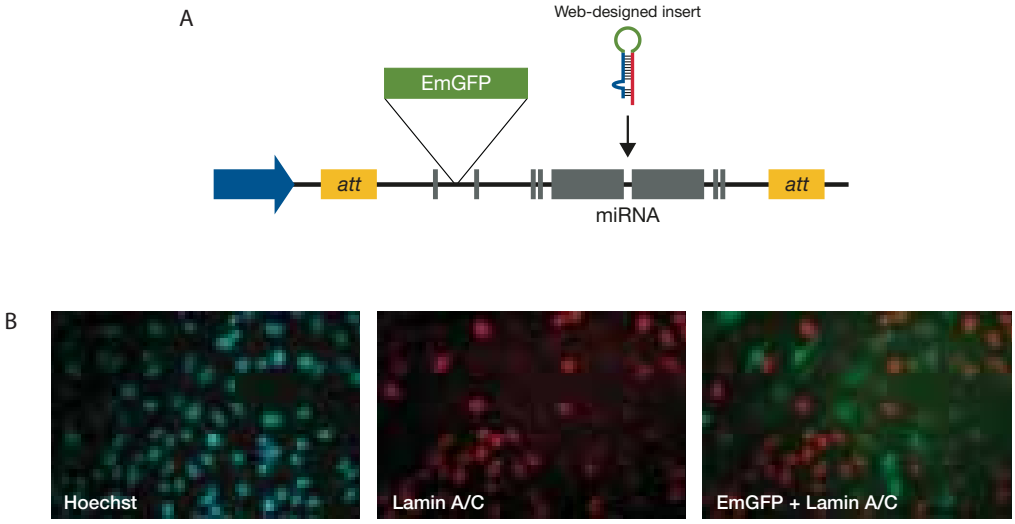

Figure 8.6. 100% correlation of EmGFP and miRNA expression. (**A**) Map of the BLOCK-iT™ Pol II miR RNAi expression cassette shows EmGFP between DraI restriction sites for easy removal. (**B**) Cells were transfected with pcDNA™6.2-GW/EmGFP-miR (directed against lamin) and Lipofectamine® 2000 Transfection Reagent, at an expected 50% efficiency, to demonstrate the 100% tracking of Emerald Green Fluorescent Protein (EmGFP) and miRNA expression. After 48 hr 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 of the miRNA that greatly reduces lamin expression.

Knockdown of multiple targets for experimental flexibility

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.

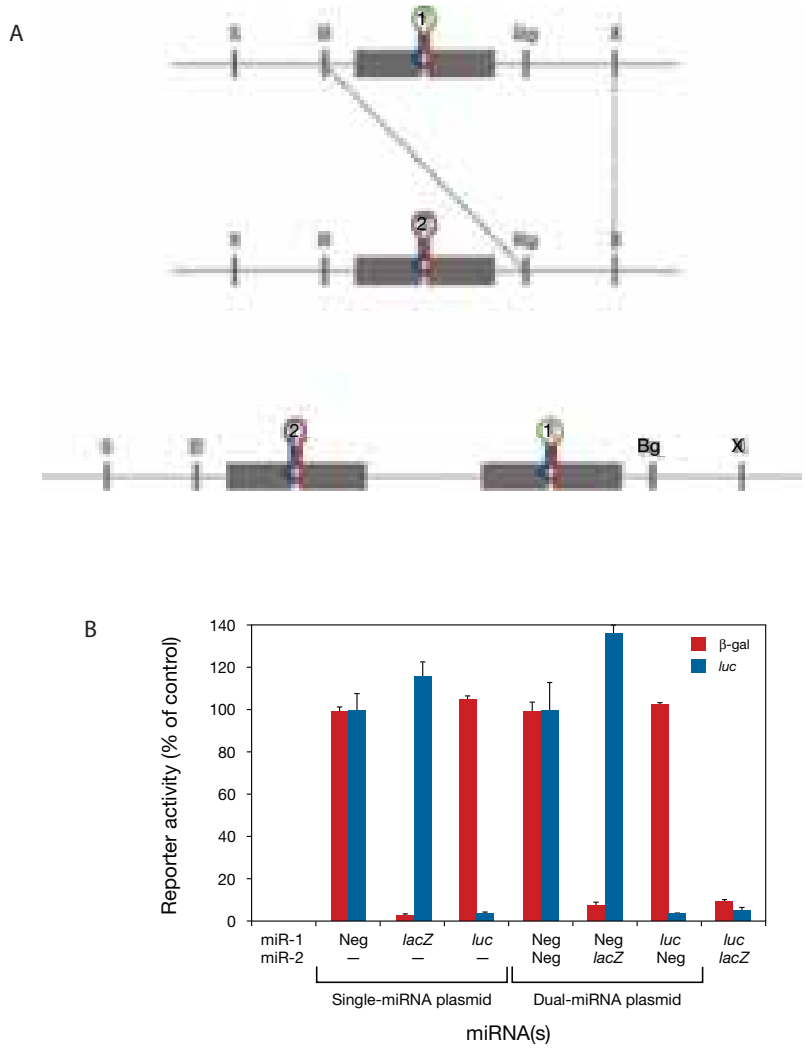

Figure 8.7. Knock down multiple targets with BLOCK-iT™ Pol II miR RNAi expression vectors. (**A**) Example of restriction digestion/ligation scheme for combining miRNAs from different vectors. SalI (S), BamHI (B), BglII (Bg), and XhoI sites (X) around the miRNA flanking regions (bars) are indicated. By cloning the BamHI–XhoI fragment containing miRNA 1 into the BglI–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**) 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® destination (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, as opposed to Gateway® entry vectors. As a result, there are two key differences between the pcDNA™6.2-GW/miR and pcDNA™6.2-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 pcDNA™6.2-GW/EmGFP-miR). For expression in different DEST vectors, the inserts must first be transferred to a pDONR™ (donor) vector and then to a DEST vector of choice by a dual 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 pcDNA™6.2/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	12537-023

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 8.8), providing a flexible system to regulate knockdown or to use promoters that are active in specific tissues for *in vivo* studies.

Inducible expression for control over your experiment

The new 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 8.9). 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.

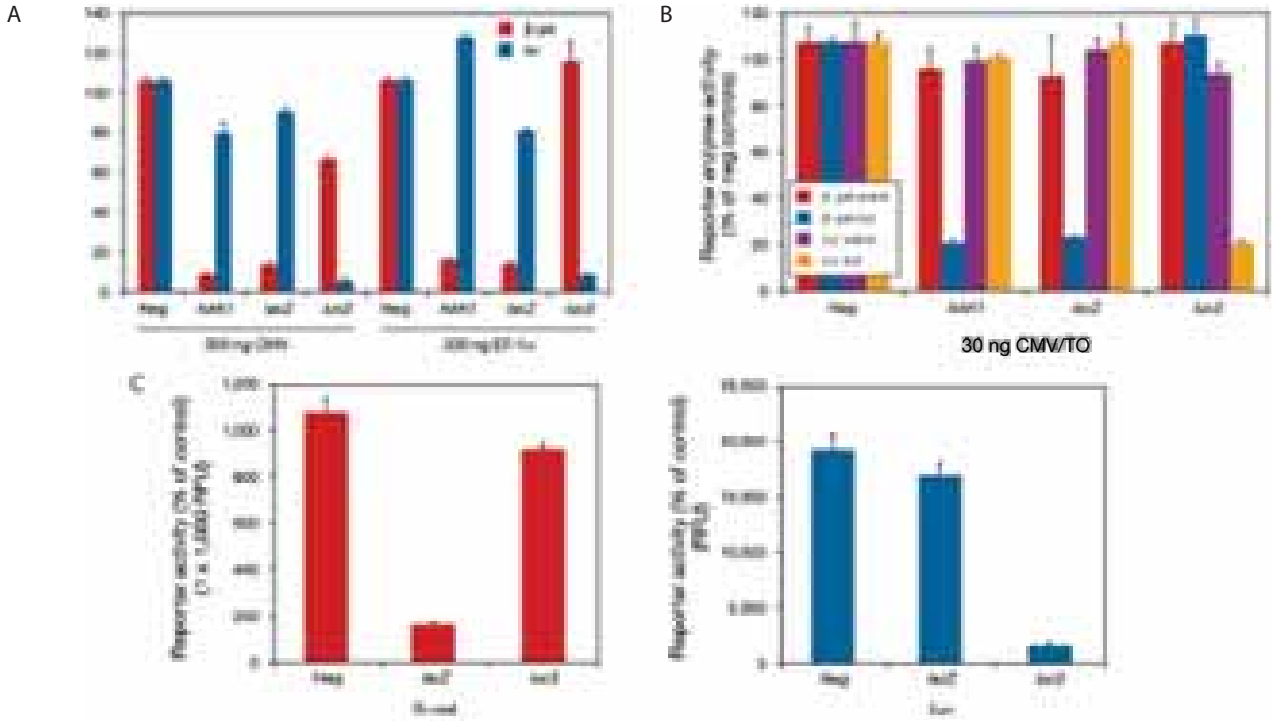

Figure 8.8. BLOCK-iT™ Pol II miR RNAi Expression Vectors are compatible with multiple promoters. (**A**) Normalized reporter activities from lysates of GripTite™ 293 cells cotransfected with 100 ng each of luciferase and *lacZ* reporter plasmids and 300 ng/well of either pcDNA™6.2-GW/EmGFP-miR (CMV) 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 reportergenes 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.

Figure 8.9. 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™ miR RNAi Select

Now it is easier than ever to harness the power of the BLOCK-iT™ Pol II miR RNAi Expression Vectors with predesigned BLOCK-iT™ miR RNAi Select hairpins, which target the majority of the human, rat, and mouse genes in the RefSeq database. The BLOCK-iT™ miR RNAi hairpins, designed *in silico* using the award-winning BLOCK-iT™ RNAi Designer, are provided as DNA oligos that are ready to anneal and clone into one of the BLOCK-iT™ Pol II miR RNAi Expression Vectors. Ready-to-order BLOCK-iT™ miR RNAi Select hairpins provide significant advantages:

- → Easy access to BLOCK-iT™ Pol II miR RNAi Expression Vector technology, which combines the high design success rate of artificial miRNAs with tissue-specific, inducible, multiple gene–targeting, and GFP reporter options
- → Effortless ordering using the BLOCK-iT™ RNAi Express search engine
- → Allows maximum reduction of t due to combined power of BLAST and the advanced Smith-Waterman alignment specificity search
- → Four BLOCK-iT™ miR RNAi Select hairpin designs directed to nonoverlapping regions of the target mRNA sequence.

With BLOCK-iT™ miR RNAi Select 4 Sets, you can avoid the work of designing RNAi hairpin sequences in-house.

Product	Quantity	Cat. No.
miR RNAi vector kits		
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit	20 rxns	K4935-00
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit With EmGFP	20 rxns	K4936-00
BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System	20 rxns	K4937-00
BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System With EmGFP	20 rxns	K4938-00
BLOCK-iT™ Pol II miR-*lacZ* Validated miRNA Control Vector	10 µg	V49350-00
BLOCK-iT™ Pol II miR-*luc* Validated miRNA Control Vector	10 µg	V49351-00
BLOCK-iT™ Pol II miR-LMNA Validated miRNA Control Vector	10 µg	V49352-00
BLOCK-iT™ miR RNAi Select	1 hairpin	†
BLOCK-iT™ miR RNAi Select 4 Sets*	4 hairpins	†
BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System With EmGFP	20 rxns	K4934-00
BLOCK-iT™ Inducible Pol II miR RNAi Expression Vector Kit With EmGFP	20 rxns	K4939-00
Recommended destination vector kits		
Gateway® pT-REx™-DEST30 Vector	6 µg	12301-016
ViraPower™ T-REx™ Lentiviral Expression System	20 rxns	K4965-00
Gateway® pEF-DEST51 Vector	6 µg	12285-011
MultiSite Gateway® Three-Fragment Vector Construction Kit	20 rxns	12537-023
ViraPower™ Promoterless Lentiviral Gateway® Expression System with MultiSite Gateway® Technology	20 rxns	K5910-00

* Go to www.invitrogen.com/rnaicentral for details on the 100% performance guarantee.

† Visit www.invitrogen.com/rnaiexpress for ordering information on the latest BLOCK-iT™ miR RNAi Select, BLOCK-iT™ miR RNAi Select 4 Sets, and BLOCK-iT™ Pol II miR Validated miRNA Vector DuoPaks that are available.

Guaranteed results with BLOCK-iT™ miR RNAi Select 4 Sets

The purchase of a BLOCK-iT™ miR RNAi Select 4 Set includes 100% guaranteed results: two of the four designs will result in at least 70% transcript knockdown, given a transfection efficiency of at least 80%. If, under these conditions, three or more of the designs fail to knock down the target mRNA by at least 70%, Invitrogen will design and ship two additional target-specific BLOCK-iT™ miR RNAi hairpins at no expense.*

* Please contact Invitrogen Technical Services to take advantage of this guarantee. Be prepared to fax or email your order reference number, BLOCK-iT™ miR RNAi Select sequences, and data showing transfection efficiency and level of knockdown. This guarantee can be used only once per target gene.

With BLOCK-iT™ miR RNAi Select 4 Sets, you can avoid the work of designing RNAi hairpin sequences yourself. The development of these molecules begins with identification of the entire human, rat, and mouse transcripts in the Unigene database. Invitrogen then selects exceptionally effective *in silico*–designed sequences by a four-step process:

- → BLAST search to remove sequences within the target mRNA that are homologous to other mRNA sequences within the target species
- → BLOCK-iT™ miR RNAi Select hairpin design using the BLOCK-iT™ RNAi Designer, which includes both standard and proprietary rules based on testing thousands of RNA duplex and RNAi vector sequences
- → Smith-Waterman mismatch alignment to rigorously screen sequences for potential off-target effects
- → Selection of the top four artificial miRNA hairpin designs targeting nonoverlapping regions in all major splice variants found in RefSeq for the target mRNA sequence; these designs are offered independently as BLOCK-iT™ miR RNAi Select hairpins or as a complete BLOCK-iT™ miR RNAi Select 4 Set

BLOCK-iT™ RNAi Entry Vector Kits

pENTR™ vectors for fast cloning and expression of shRNA cassettes

The 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 found at RNAi Central (www.invitrogen.com/rnai).

Clone the annealed shRNA oligo sequence into the pENTR™ vector with a convenient 5-minute cloning reaction (Figure 8.10). 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.

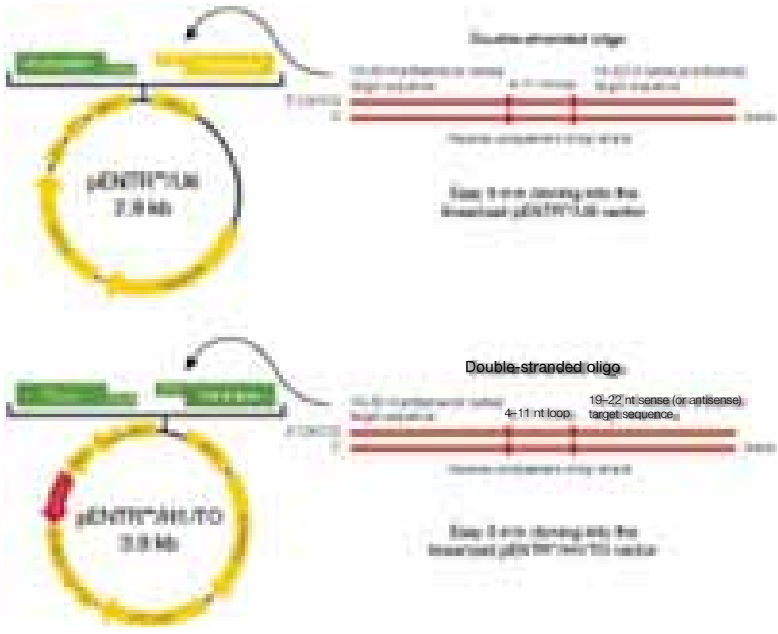

Figure 8.10. Easy shRNA cloning into inducible and constitutive BLOCK-iT™ entry vectors. A vector expressing the shRNA is generated by designing a short double-stranded DNA oligo containing a sense-loop-antisense sequence against the target. The sequence has 4-nucleotide overhangs on the ends that can 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.

NOTE : Design your shRNA oligos online at www.invitrogen.com/rnai.

Inducibility with shRNA vectors

The BLOCK-iT™ Inducible H1 Entry Vector Kit confers the ability for tightly regulated inducible RNAi in any mammalian cell type. The pENTR™/H1/TO vector, included in the kit, carries an H1/TO Pol III promoter that exhibits superior regulation compared to precursor versions, which contained only one $TetO_2$ site in either position. Upon addition of tetracycline, shRNA is expressed, leading to effective knockdown of the target gene (Figure 8.11). Tight control over shRNA expression permits a variety of experimental conditions to be examined:

- → Observe changes over time by controlling the initiation of the RNAi response
- → Study loss of function in a stably transfected cell line, even if the gene of interest is required for cell viability or proliferation
- → Terminate induction of shRNA expression to observe phenotypic changes during recovery of the function of the target gene

NOTE: Find information on shRNA vectors online at www.invitrogen.com/shrna.

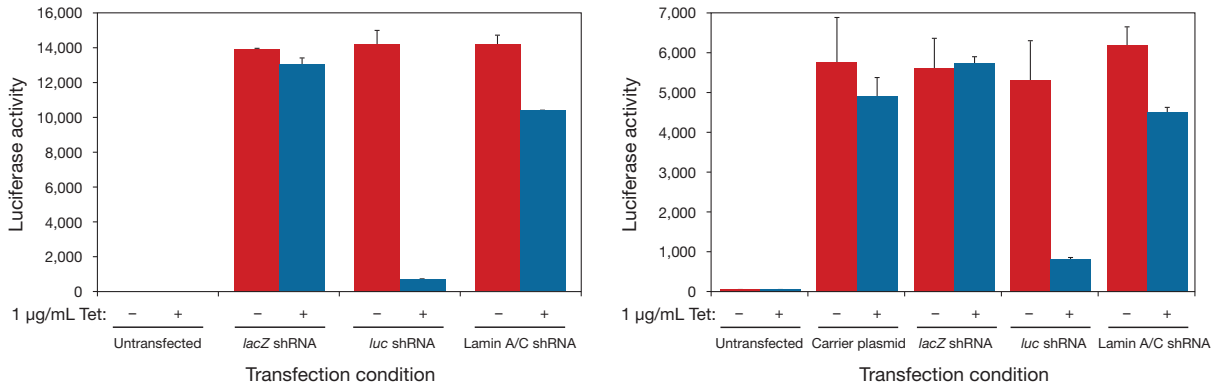

Figure 8.11. Tightly regulated induction of gene knockdown from the pENTR™/H1/TO vector. T-REx™-CHO cells (left panel) or a polyclonal population of HeLa cells stably transduced with lentiviral particles expressing the TR protein (right panel) were cotransfected with *lacZ* and the luciferase (*luc*) reporter gene plus H1/TO entry clones inducibly expressing *lacZ*-, *luc*-, or lamin A/C-directed shRNAs. For the HeLa cells, a nonspecific carrier plasmid was included as a control. After 3 hr, medium was replaced with fresh medium, with or without 1 µg/mL tetracycline (Tet) as indicated. Cells were lysed 48 hr posttransfection and assayed for luciferase activity.

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 quantitative real-time PCR (qRT-PCR) or western blot analysis, the background of mRNA or protein in nontransfected 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 (Figure 8.12):

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 kit-provided packaging cells (use 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 non-dividing 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)
BLOCK-iT™ RNAi Adenoviral 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 8.12. How the BLOCK-iT™ lentiviral system works.

Powerful shRNA delivery with BLOCK-iT™ viral vectors

The viral delivery systems include these vectors, which are also available separately (Figure 8.13):

- → pLenti4/BLOCK-iT™-DEST—lentiviral vector with the 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

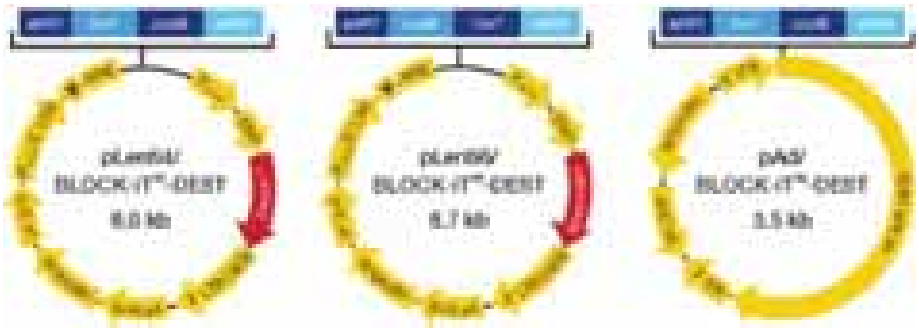

Figure 8.13. BLOCK-iT™-DEST viral vectors. The BLOCK-iT™ Inducible H1 Lentiviral RNAi System can generate long-term inducible knockdown results whether a clonal population is used or individual clones are selected for analysis. The promoter configuration of the H1/TO vector ensures tight regulation; 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 rxns	K4937-00
BLOCK-iT™ Lentiviral Pol II miR RNAi Z′-factor Expression System with EmGFP	20 rxns	K4938-00
BLOCK-iT™ Inducible H1 Lentiviral RNAi System	20 rxns	K4925-00
BLOCK-iT™ Lentiviral RNAi Expression System	20 rxns	K4944-00
BLOCK-iT™ Adenoviral RNAi Expression System	20 rxns	K4941-00

BLOCK-iT™ Lentiviral RNAi System

Stable RNAi expression in nondividing mammalian cells

The RNAi cassette from the 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 System. Then, prepare a lentiviral stock, transduce cells, and assay for the RNAi response (Figures 8.14 and 8.15). 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 8.14. Generating BLOCK-iT™ lentiviral RNAi stock.

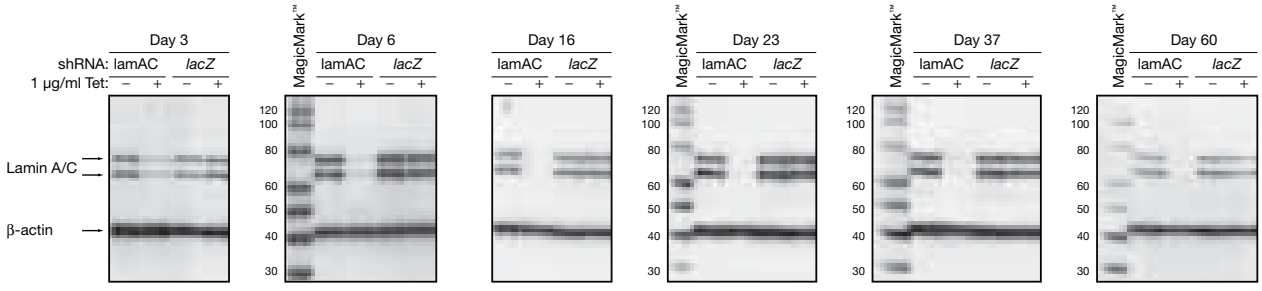

Figure 8.15. Long-term inducible knockdown in stably transduced T-REx™ HeLa cells. T-REx™ HeLa cells were transduced with lentiviral particles expressing lamin A/C (lamAC)-directed or control *lacZ*-directed shRNA from the tetracycline (Tet)-inducible H1/TO promoter at an effective MOI of <1. Stable 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 western blotting and probed with antibodies against lamin A/C and β-actin as a 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 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 Coxsackie 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 8.16). Gateway® recombination provides a way 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.

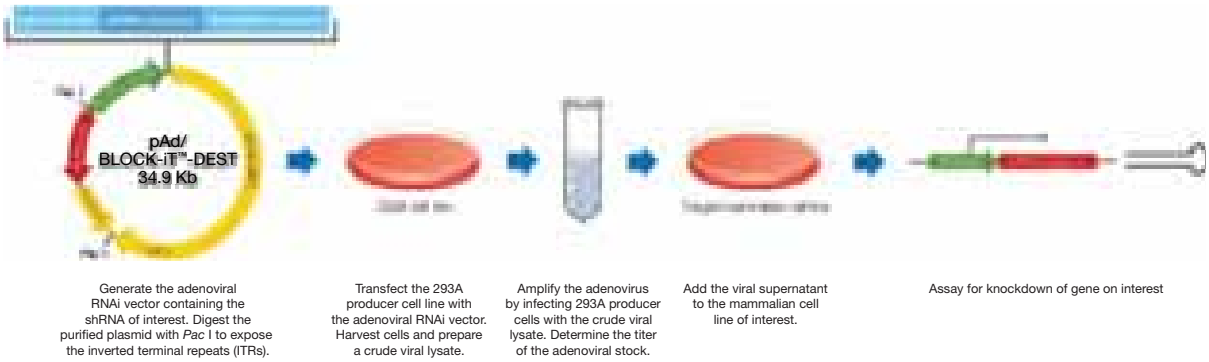

Figure 8.16. How the BLOCK-iT™ adenoviral RNAi system works.

Product	Quantity	Cat. No.
BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit	20 constructions	K4920-00
BLOCK-iT™ U6 RNAi Entry Vector Kit	20 constructions	K4945-00
T-REx™-Jurkat Cell Line	3 x 10^6 cells	R722-07
T-REx™-CHO Cell Line	3 x 10^6 cells	R718-07
T-REx™-HeLa Cell Line	3 x 10^6 cells	R714-07
T-REx™-293 Cell Line	3 x 10^6 cells	R710-07
pLenti6/TR Vector Kit	1 kit	V480-20
pcDNA™6/TR	20 µg	V1025-20
BLOCK-iT™ Adenoviral RNAi Expression System	20 rxns	K4941-00
pAd/BLOCK-iT™-DEST RNAi Gateway® Vector	6 µg	V492-20
293A Cell Line	3 x 10^6 cells	R705-07

Chapter references

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CHAPTER 9

RNAi delivery

Introduction to RNAi delivery

The two most common approaches for RNAi delivery are lipid-mediated transfection and virus-mediated transduction. Determining which of these approaches to use depends on the cell type being studied and whether transient or stable knockdown is desired (Table 9.1). The most popular application, transient transfection of unmodified siRNAs, modified siRNA duplexes, or RNAi vectors, utilizes cationic lipid–based reagents because they are suitable for delivering these molecules across a diverse range of commonly used cell lines. For cell types not amenable to lipid-mediated transfection, viral vectors are often employed. Adenoviral vectors work well for transient delivery in many cell types; however, for stable delivery in dividing and nondividing cells, lentiviral vectors are best. To determine the most favorable RNAi delivery conditions, Invitrogen offers you our delivery optimization service (see Chapter 12), a resource backed by extensive knowledge and expertise in viral vectors and nonviral delivery reagents for testing a matrix of delivery parameters.

Methods to achieve high transfection efficiency

Transfection efficiency is expressed as the percentage of cells that have received the RNAi duplex or expression plasmid. Typically, researchers strive to achieve the highest efficiencies possible. This objective is particularly important for RNAi applications, because nontransfected cells will continue to express normal levels of the targeted gene, which will result in background expression levels. With the cationic Lipofectamine® RNAiMAX Transfection Reagent, transfection efficiency can be optimized and verified easily using the BLOCK-iT™ Alexa Fluor® Red Fluorescent Control. In the case of Lipofectamine® 2000 Transfection Reagent, either the BLOCK-iT™ Alexa Fluor® Red Fluorescent Control or the original green BLOCK-iT™ Fluorescent Oligo can be used.

For some disease models, primary cultures or nondividing cells, which are often the most appropriate cell types to use, cannot be transfected adequately with commercially available transfection reagents. A powerful alternative to cationic lipid–mediated transfection is viral delivery of vectors containing RNAi cassettes. This option is best for delivery to hard-to-transfect, primary, and nondividing cells. Viral delivery can also be used to create stable cell lines with inducible RNAi or to express RNAi sequences with tissue-specific promoters. See Chapter 4 for more information about using viral vectors in RNAi experiments.

Importance of minimizing transfection-mediated cytotoxicity

The delivery of siRNAs, or the method of delivery, can result in cytotoxicity in gene silencing experiments. Minimizing transfection-mediated cytotoxicity is essential for proper interpretation of the outcome of any RNAi experiment. Cytotoxic effects can be difficult to distinguish from the expected phenotype resulting from target gene knockdown. Cytotoxicity due to the delivery method is often seen as apparent knockdown

Table 9.1. Selecting transfection reagents and viral delivery methods.

Product	Key advantages
Lipofectamine® RNAiMAX Transfection Reagent	• Designed and manufactured for siRNA delivery • Superior efficiencies, allowing low concentrations of siRNA to be used • Wide range of compatibility with diverse cell lines • Optimized protocols available for many common cell lines
Lipofectamine® 2000 Transfection Reagent	• Designed for optimal expression when delivering plasmids, including shRNA and miR RNAi vectors • Robust cotransfection of vectors and synthetics (siRNA or Stealth RNAi™ siRNA duplexes)
Lipofectamine® LTX Reagent	• Designed for improved transfection performance in hard-to-transfect and primary cells • High transfection efficiency and significantly lower toxicity levels for a wide range of cell lines
Oligofectamine™ Transfection Reagent	• Expressly formulated for delivery of antisense oligos • Dependable delivery of siRNA
BLOCK-iT™ Adenoviral RNAi Expression System	• Ideal system for long-term transient expression of RNAi vectors in a wide range of cell lines
BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System with EmGFP BLOCK-iT™ Lentiviral RNAi Expression System BLOCK-iT™ Inducible H1 Lentiviral RNAi System	• Stable expression of RNAi vectors in difficult-to-transfect cell lines • Suitable methods for *in vivo* applications • Inducible systems available

in cells transfected with several negative controls, relative to no treatment. The easiest way to combat transfection-mediated cytotoxicity is to choose a transfection reagent designed for either double-stranded RNA or plasmid-based RNAi transfections. Most often, these reagents have been formulated to maximize knockdown efficiency while minimizing cytotoxicity. Optimization experiments, when using published or suppliers' protocols as guidelines, can help to determine the concentration of transfection reagent that works best for the cell line of interest. Using the lowest amount of transfection reagent needed to give the highest level of knockdown is recommended.

Significance of reducing off-target effects

As well as being a source of cytotoxicity, a suboptimal transfection reagent or excess reagent can result in apparent off-target effects. One cause of off-target effects is the up- or down-regulation of genes due to the gene delivery procedure. However, with appropriate controls, these effects can be identified and diminished (see Chapter 2 for a discussion of controls for RNAi experiments). Again, the lowest amount of transfection reagent that provides the best gene silencing activity is advised.

The potential also exists for off-target effects caused by the siRNA duplex itself. To determine the most favorable conditions for transfection, the concentration of siRNA should be varied while holding the concentration of transfection reagent constant, using the lowest concentration (identified previously) to limit cytotoxicity. Keep in mind that the specificity of the siRNA will impact potential off-target effects. The use of exceptionally specific reagents, such as modified Stealth RNAi™ siRNA duplexes, can help alleviate these concerns (see Chapter 7 for detailed information about the specificity advantage of Stealth RNAi™ siRNA).

Lipofectamine® RNAiMAX Transfection Reagent—unmatched gene silencing with reduced cytotoxicity for siRNA experiments

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 a minimum of 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

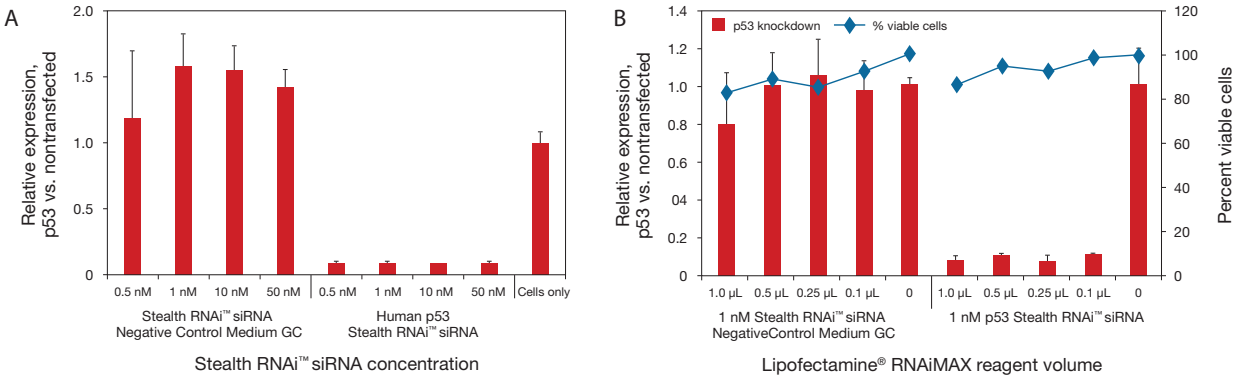

Figure 9.1. Optimal gene silencing and minimal cytotoxicity with Lipofectamine® RNAiMAX Transfection Reagent. (**A**) Superior knockdown levels were observed with as little as 0.5 nM Stealth RNAi™ siRNA with Lipofectamine® RNAiMAX Transfection Reagent. Transfection complexes containing 0.3 µL of Lipofectamine® 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 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 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 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 www.invitrogen.com/rnaimax.

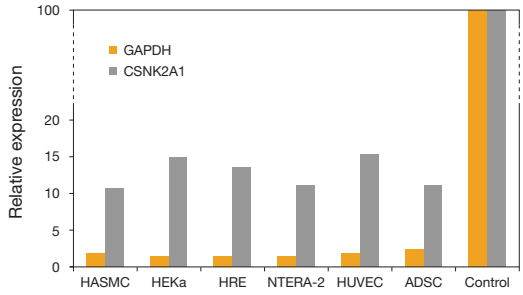

Figure 9.2. Superior knockdown of *Silencer*® Select siRNA with Lipofectamine® RNAiMAX Transfection Reagent. (Email rnairesearcher@invitrogen.com for protocol details).

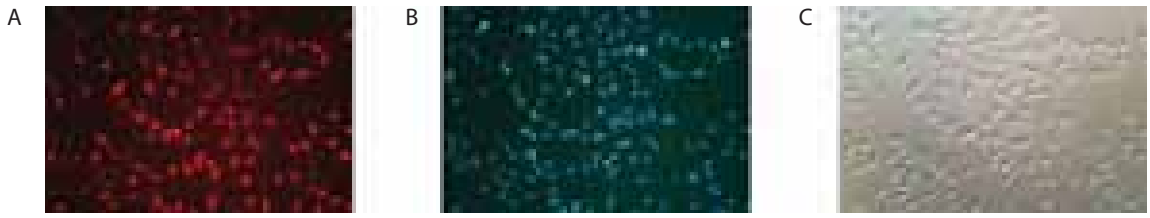

Figure 9.3. Assessing transfection efficiency of the Lipofectamine® RNAiMAX Transfection Reagent with the BLOCK-iT™ Alexa Fluor® Red Fluorescent Control. Lipofectamine® RNAiMAX Transfection Reagent was used to transfect HeLa cells with the BLOCK-iT™ Alexa Fluor® Red Fluorescent Control (50 nM) (**A**). Twenty-four hours after transfection, growth medium was removed and replaced with PBS containing 10 mg/mL Hoechst 33342 for visualization of cell nuclei (**B**). Nuclear localization of the red-fluorescent control oligo is seen (**A**). The bright-field image (**C**) 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 duplexes, and not for transfecting vectors expressing RNAi sequences.

Lipofectamine® 2000 and LTX Transfection Reagents—ideal for delivery of shRNA and miR RNAi vectors

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. Lipofectamine® LTX Reagent, used in combination with PLUS™ Reagent, is the choice reagent for 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 cotransfections of synthetics and vectors
- → Easy-to-follow protocols; media changes not required
- → Convenient optimization of transfection conditions and efficiency with the BLOCK-iT™ Fluorescent Oligo
- → Excellent performance in a wide variety of cell types

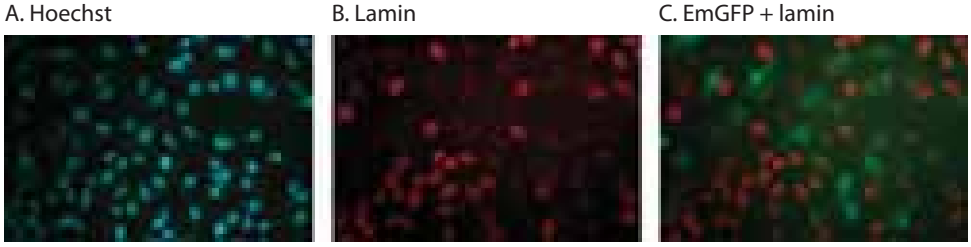

Figure 9.4. Delivery of BLOCK-iT™ Pol II miR RNAi expression vectors. Cells were transfected with pcDNA™6.2-GW/EmGFP-miR (directed against lamin) and Lipofectamine® 2000 Transfection Reagent, at an expected 50% efficiency, to demonstrate the 100% tracking of Emerald Green Fluorescent Protein (EmGFP) and miRNA expression. After 48 hr, cells were stained with Hoechst nuclear stain, which stains all cells (**A**), and a red lamin stain (**B**), and monitored for GFP expression. Approximately half of the cells highly expressed the lamin protein, but red-stained cells were not expressing EmGFP, and EmGFP-expressing cells were not expressing 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 1.5 mL	11668-027 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 Lipofectamine® 2000 Transfection Reagent and does not work well with Lipofectamine® RNAiMAX Transfection Reagent.

Oligofectamine™ Transfection Reagent—potent internalization of RNA oligos

Oligofectamine™ Transfection Reagent is designed to deliver antisense oligos and is also useful for transfecting RNAi reagents into eukaryotic cells. To optimize transfection conditions, Oligofectamine™ Transfection Reagent can be used in conjunction with the BLOCK-iT™ Fluorescent Oligo or the BLOCK-iT™ RNAi Basic Control Kit. In the first published demonstration of siRNA-mediated knockdown in a mammalian cell line, Elbashir et al. reported their use of Oligofectamine™ Transfection Reagent to transfect HeLa cells with siRNAs [1]. This work was continued by Harborth et al., who used a similar transfection protocol with Oligofectamine™ Transfection Reagent to demonstrate the knockdown of 20 additional essential and nonessential target genes [2]. These early studies established the general applicability of using siRNA for RNAi.

Product	Quantity	Cat. No.
Oligofectamine™ Transfection Reagent	1.0 mL	12252-011

The Neon™ Transfection System

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

The company that continues to bring you transfection reagents on the cutting edge now introduces the Neon™ Transfection System.

- → **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 employs an electroporation technology using 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 as few as 2 x 10^4 or as many as 8 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 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 www.invitrogen.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	98.5
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 www.invitrogen.com/neon.

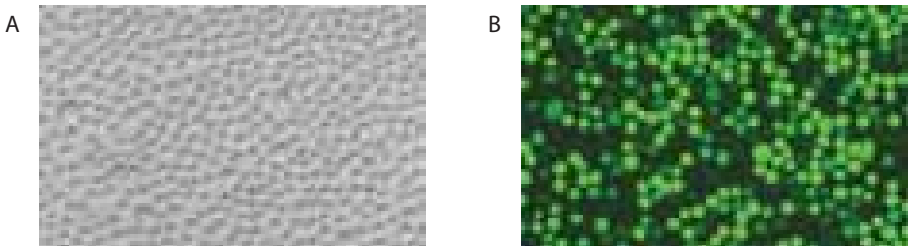

Figure 9.5. High transfection efficiency of Jurkat cells with the Neon™ Transfection System. Intracelluar uptake of reporter vector encoded with EGFP at 24 hr following transfection of Jurkat cells using the Neon™ Transfection System. **B** is the corresponding fluorescence image of **A**.

Figure 9.6. Standard electroporation cuvette compared to Neon™ system pipette tip. 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 couldn't 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 rxns	MPK10096
Neon™ Transfection System 10 µL Kit	192 rxns	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 SM et al. (2001) *Nature* 411:494–498.
2. Harborth J et al. (2001) *J Cell Sci* 114:4557–4565.
3. Kim JA, Cho K, Shin MS et al. (2008) *Biosens Bioelectron* 23(9):1353–1360.

CHAPTER 10

RNA interference controls

Controls for RNAi experiments

Overview of 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 Invitrogen's 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 inhibition of positive control genes in human cells
- → Using negative controls that match the GC content of the target molecule

Table 10.1. Control kits ensure successful gene inhibition experiments.

	Components						
Products/kits	Fluorescent oligo	Transfection reagent	Dead-cell stain	Nuclear stain	RNAi positive controls	RNAi negative controls	qRT-PCR primers
BLOCK-iT™ Alexa Fluor® Red Fluorescent Control	●						
BLOCK-iT™ Fluorescent Oligo	●						
BLOCK-iT™ Transfection Kit	●	●					
Silencer® Select Positive Control siRNA					●		
Silencer® Select Negative Control siRNA						●	
Stealth RNAi™ siRNA Negative Control						●	
Stealth RNAi™ siRNA Positive Control (actin, GAPDH, cyclophilin B)					●		
Stealth RNAi™ siRNA Reporter Control					●		
BLOCK-iT™ Transfection Optimization Kit (Human)	●		●		●	●	
BLOCK-iT™ Pol II miR Validated miRNA Control Vector					●	●	
Applications	Track transfection				Monitor experimental variation	Measure the effect of experimental knock-down vs. background	Assess knockdown
	Optimize transfection conditions						
			Assess cell viability				

BLOCK-iT™ Alexa Fluor® Red Fluorescent Control and green BLOCK-iT™ Fluorescent Oligo

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

- → Strong, easily detectable signal that indicates transfection efficiency (Figure 10.1)
- → Clear, persistent signal that exceeds 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 green BLOCK-iT™ Fluorescent Oligo and the BLOCK-iT™ Alexa Fluor® Red Fluorescent Control strongly correlate with transfection efficiency with Stealth RNAi™, *Silencer*® Select, and *Silencer*® siRNAs, as well as Ambion® Pre-miR™ miRNA mimics and Ambion® Anti-miR™ miRNA inhibitors.

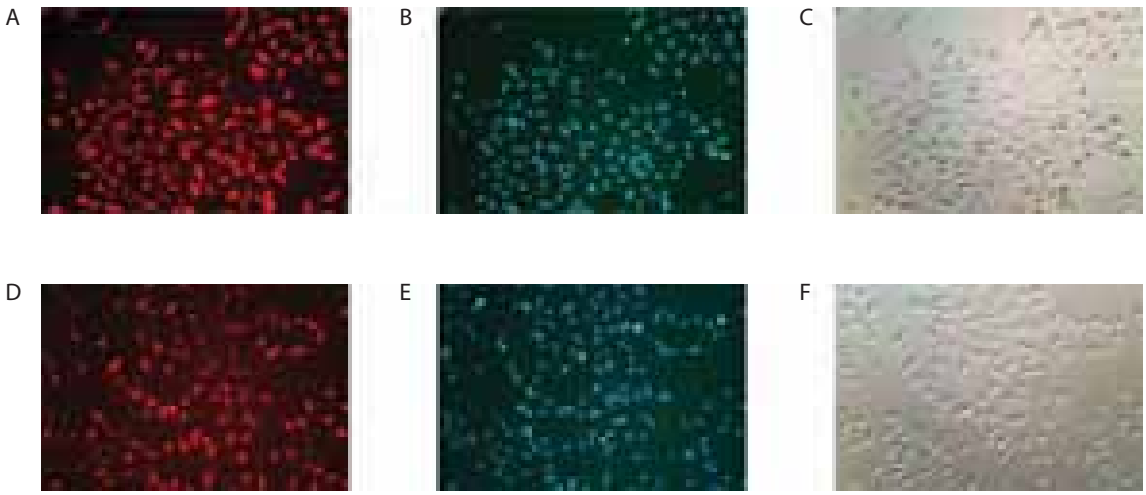

Figure 10.1. BLOCK-iT™ Alexa Fluor® Red Fluorescent Control for clear visualization of transfection results. HeLa cells were transfected with the BLOCK-iT™ Alexa Fluor® Red Fluorescent Control using either Lipofectamine® 2000 (**A**–**C**) or Lipofectamine® RNAiMAX Transfection Reagent (**D**–**F**). 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** and **E**). Nuclear localization of the control is seen with both transfection reagents (**A** and **D**). Almost 100% of the cells take up the control, and cells retain a normal morphology, as seen in the bright-field images (**C** and **F**).

BLOCK-iT™ Transfection Kit—simple optimization of Lipofectamine® 2000 transfection reagent conditions

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 green 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.

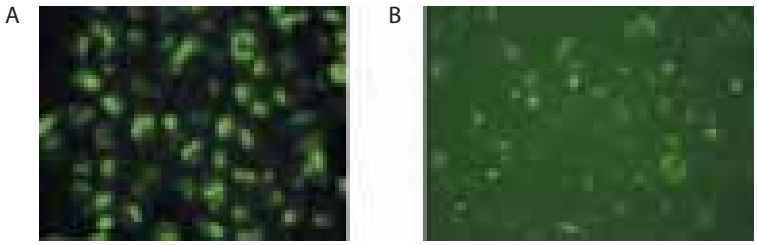

Figure 10.2. Strong fluorescence signal to optimize transfection. A549 cells were transfected with green BLOCK-iT™ Fluorescent Oligo or standard fluorescently labeled RNA, using Lipofectamine® 2000 Transfection Reagent. Twenty-four hours later, the fluorescent signal had persisted in the cells transfected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

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 0.75 mL	13778-150 13778-075
Lipofectamine® 2000 Transfection Reagent	1.5 mL 0.75 mL	11668-019 11668-027

Silencer® Select positive and negative control siRNAs

- → Validated siRNA controls for optimizing siRNA experiments
- → GAPDH Positive Control functionally tested in several common cell lines
- → Negative Controls 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 Ambion® KDalert™ GAPDH Assay Kit. The *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—siRNAs with sequences that do not target 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 *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—ideal negative controls for every experiment

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. Multiple sequences in each GC range give even more certainty of results.
- → 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

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 anything 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

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. These reagents, used together, provide the means to optimize transfection conditions with Lipofectamine® 2000 Transfection Reagent, and a functional positive control for every RNAi experiment.

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

Optimize RNAi experiments with the BLOCK-iT™ Fluorescent Oligo

Saving time and reagents by not requiring separate assays to be performed in different wells, the BLOCK-iT™ Fluorescent Oligo allows you to obtain a clear visual appraisal of RNAi transfection efficiency. The dead-cell stain, nuclear stain, and BLOCK-iT™ Fluorescent Oligo emit signals at different wavelengths, permitting them to be detected together in a single well. The nuclear stain (Hoechst dye) and dead-cell reagent (ethidium homodimer-1) allow assessment of cell death and quantitation of cell number, making sample-to-sample and experiment-to-experiment comparisons more accurate. The simple addition of two dyes to an extra cell culture sample shows the physiological state of cells. The BLOCK-iT™ Fluorescent Oligo for transfection optimization and monitoring, used in conjunction with two cell-staining dyes, helps achieve the best RNAi results possible (Figure 10.3).

Transfection efficiencies of at least 70% can be confirmed by this procedure:

1. Transfect human cell line with target-specific Stealth RNAi™ siRNA or siRNA duplexes, positive and negative controls, and BLOCK-iT™ Fluorescent Oligo.
2. Incubate a portion of the cells with dead-cell stain and nuclear stain.
3. Visualize the BLOCK-iT™ Fluorescent Oligo and stained cells to assess transfection efficiency and cell health; if more than 70% of the cells are transfected (green) and cell death is less than 5% to 10%, proceed to step 4.
4. Measure RNAi-mediated inhibition of targeted genes using TaqMan® Gene Expression Assays (see Chapter 11)

Product	Size	Cat. No.
BLOCK-iT™ Fluorescent Oligo for Lipid Transfection	2 x 125 µL	2013
BLOCK-iT™ Fluorescent Oligo for Electroporation	2 x 125 µL	13750062

Positive controls for BLOCK-iT™ Pol II miR RNAi vectors

The BLOCK-iT™ Pol II miR Validated miRNA Control Vectors are valuable positive controls for initial optimization and validation of experiments that use the BLOCK-iT™ Pol II miR RNAi vectors (see Chapter 8 for more information on these vectors). Three validated positive controls are available, and each control targets a different commonly used reporter: *lacZ*, luciferase, or lamin A/C. These controls are cloned into the BLOCK-iT™ miR RNAi vectors with Emerald GFP (EmGFP) and are ready to use for the following applications:

→ Optimizing transfection conditions and monitoring experimental variation for robust results
→ Identifying the most potent reagent for optimal RNAi knockdown
→ Validating miR RNAi expression in commonly used reporter systems

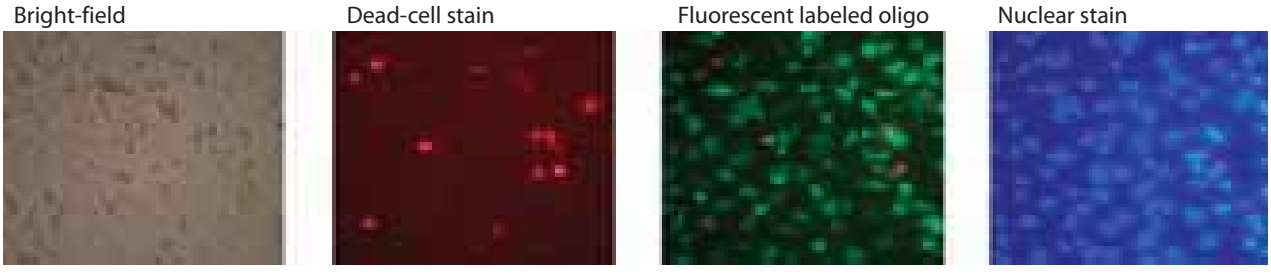

Figure 10.3. Early cell visualization to assess quality of RNAi experiments. HeLa cells were transfected with 100 nM BLOCK-iT™ Fluorescent Oligo (green) and 2.5 mg/mL Lipofectamine® 2000 Transfection Reagent. Twenty-four hours posttransfection, cells were stained with dead-cell stain (ethidium homodimer-1, red) or nuclear stain (Hoechst 33342, blue). Cells were evaluated at 40x magnification by bright-field microscopy or with appropriate filters. The blue Hoechst dye stains the nuclei of all cells, while the red ethidium homodimer-1 stains only dead or dying cells. Transfection efficiency is determined by comparing the BLOCK-iT™ Fluorescent Oligo transfection control and nuclear-stained cells; cell viability is determined by comparing the dead cells and nuclear-stained cells. If cell viability is low, further transfection optimization may be required to reduce cell death.

Silencer® negative control siRNAs

- → These nontargeting siRNAs 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. 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

Our "*In Vivo* Ready" *Silencer*® Negative Control #1 siRNA is subjected to anextra level of purification and testing required for the introduction of siRNAs into animals. After HPLC purification and annealing, each siRNA is further purified utilizing a process that removes excess salt via a semipermeable membrane. 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+}.

Silencer® siRNA Screening Control Panel

The *Silencer*® siRNA Screening Control Panel includes all seven negative control siRNAs and is intended for laboratories performing siRNA screens that employ hundreds or thousands of siRNAs per experiment. Because of the importance of such screens and the fact that some negative control siRNAs may have unintended effects in a particular assay, most researchers carefully test their negative control siRNAs before choosing 2–6 control siRNAs for inclusion in their siRNA library screen. The *Silencer*® siRNA Screening Control Panel simplifies this process. As an added benefit, a positive control siRNA to human, mouse, and rat KIF11 (Eg5) is included in the panel. Knockdown of KIF11, which encodes a kinesin family motor protein, leads to mitotic arrest, so effective delivery of KIF11 siRNA can be assessed visually; it can also be measured by several cell-based assays.

The *Silencer*® siRNA Screening Control Panel contains 1 nmol of each of the seven negative control siRNAs plus the KIF11 siRNA in a dried format. Interested in larger amounts of any Negative Control siRNA? Contact us at custom_services@ambion.com for a quote.

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® siRNA Screening Control Panel (includes negative control siRNAs #1–7, plus KIF11 siRNA)	8 x 1 nmol	AM4640

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 scrambled 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" *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 utilizing a process that removes excess salt via a semipermeable membrane. 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
Silencer® Renilla Luciferase siRNA	5 nmol + 2 nmol Neg Control (50 µM)	AM4630

Chapter references

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, at least 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 TaqMan® probe and primer sets for quantitative gene expression studies in human, mouse, rat, and 16 other species
- → Convenient single-tube format and 20X formulation
- → Universal thermal cycling conditions

TaqMan® Gene Expression Assays (Figure 11.1) are a comprehensive collection of over 1.2 million pre-designed primer and TaqMan® probe sets designed to quickly and easily perform quantitative gene expression studies on human, mouse, rat, and 16 other species. Each gene expression assay consists of a FAM™ dye–labeled TaqMan® MGB probe and a pair of PCR primers designed to amplify the target of interest from cDNA. Assays are supplied in a preformulated 20X mix 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 assay selection with a streamlined approach to provide you with a convenient, standardized process for quantitative gene expression.

Approximately 60,000 TaqMan® Gene Expression Assays for human, mouse, rat, and seven other species are available as inventoried, off-the-shelf products. This collection includes an average of 1 assay for each RefSeq mRNA transcript. Approximately 1.1 million additional assays are available on a made-to-order basis, and cover most exon-exon junctions of mRNA transcripts for human, mouse, rat, *C. elegans*, *Drosophila*, *Arabidopsis*, and 13 other species.

Our extensive online catalog of TaqMan® Gene Expression Assays can be accessed at www.appliedbiosystems.com. You can 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.). For a complete list of TaqMan® Gene Expression Assays in single-tube format, or preloaded in 96- and 384-well plates and micro fluidic cards, visit www.allgenes.com.

Figure 11.1. TaqMan® Gene Expression Assays are delivered with a compact disc containing an electronic assay information file.

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 (www.appliedbiosystems.com/cadt) to format and submit your target sequence, or to search for specific sequences or pre-designed 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 identify 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 Applied Biosystems' 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	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: see pages 90, 107
Primer-limited			VIC® dye	Yes		
Custom TaqMan® Probes			FAM™ dye VIC® dye TET™ dye NED™ dye	No	4–7 days	Various: see page 90

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, Applied Biosystems can synthesize them for you (Table 11.1). As the market leader in real-time PCR, our high-quality custom products can be used in all your real-time and end-point applications. These products offer you the ideal in flexibility if you prefer to optimize your own reaction formulation or if you simply prefer to buy in bulk.

Table 11.1. TaqMan® probe usage chart for gene expression. For gene expression, the minimum number 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 reaction volumes.

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.
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.			
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 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 instrument systems 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-Ct™ family of kits

Ready to perform. Right out of the box.

- → Complete, pre-optimized expression workflows in a single box are robust and reliable
- → Prepare samples at room temperature in 10 minutes or less, including DNase treatment
- → Results equivalent to purified RNA with validated accuracy, reproducibility, and sensitivity
- → Validated with hundreds of primers sets and TaqMan® Gene Expression Assays

Cells-to-Ct™ Kits enable you to quickly and easily transform cultured cells into real-time 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. Cells-to-Ct™ Kits, however, don't stop at sample preparation. The lysis technology is integrated into a complete workflow that includes reverse transcription reagents and high-performance TaqMan®- or SYBR® Green–based PCR master mixes (Figure 11.2).

For more information, visit www.invitrogen.com/cellstoct.

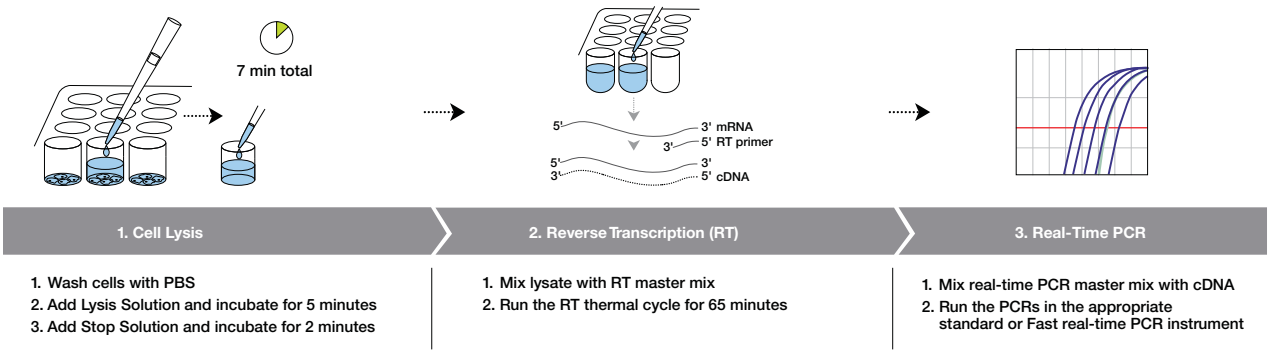

Figure 11.2. Cells-to-Ct™ Kits provide a complete workflow from cells to qRT-PCR. Cells-to-Ct™ Kits require 10 min or less to release nucleic acids into a cell lysate solution at room temperature that is compatible with the included reverse transcriptase and real-time PCR reagents.

Protein separation and western blotting

To verify how knockdown of a gene results in phenotypic differences, analyze protein from RNAi-treated cells by protein separation and western blotting techniques. Invitrogen supplies kits, reagents, and services for protein separation, stains, standards, blotting, and detection methods. Popular 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 www.invitrogen.com/proteomics.

Antibodies and immunodetection

Invitrogen's immunodetection offerings include research and pathology antibodies and flow cytometry, cytokine and signaling, and labeling and detection products. Find high-quality antibodies, assays, and kits for immunodetection at www.invitrogen.com/antibodies.

Nucleic acid purification and quantification

Invitrogen's 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 TRIzol® line of products and PureLink™ DNA and RNA purification systems. For quantifying DNA, RNA, and proteins, Quant-iT™ technology is the most accurate means. Quant-iT™ technology uses sensitive dyes that become highly fluorescent upon binding to their targets. Unlike 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 www.invitrogen.com/nap for a complete description of nucleic acid purification and quantification technologies.

Gene expression microarray analysis

DNA microarrays are the most commonly used method for detecting global changes in gene expression across the transcriptome. Invitrogen supplies a complete line of sample labeling, microarray content, quality control, and hybridization products. Extensively cited products include the SuperScript® Plus Direct and Indirect cDNA Labeling Systems, the SuperScript® RNA Amplification Systems, and the SuperScript® One-Cycle cDNA Kit (for Affymetrix® One-Cycle assays). For more information, visit www.invitrogen.com/microarray.

CHAPTER 12

RNAi services

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 designed to provide you with the guidelines and protocols for successful *in vivo* RNAi experiments.

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, cloning and viral production, or development of cell-based or biochemical assays. Allowing researchers to focus their efforts on determining the best targets and compounds to pursue, Invitrogen's scientists provide the expertise required to take a project from beginning to end in a timely, professional manner.

With a broad portfolio of products and high-quality services, Invitrogen is the industry leader in the powerful new technology of RNAi. Invitrogen's Custom RNAi Services utilizes synthetic and vector RNAi technologies and downstream assays to help customers achieve robust knockdown and to further the overall breadth of knowledge of complex biological systems. RNAi Services offers many advantages:

- → State-of-the-art technologies, equipment, and high-throughput capabilities
- → Quality RNAi reagents with proven performance—Stealth RNAi™ siRNA, BLOCK-iT™ siRNA, BLOCK-iT™ shRNA vectors, and BLOCK-iT™ Pol II miR RNAi vectors
- → An expert 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 (Figure 12.1). Whether you need assistance in designing RNAi reagents or you desire a more expansive service such as high-throughput cloning, screening, or lentiviral production, Invitrogen has the resources to accelerate research.

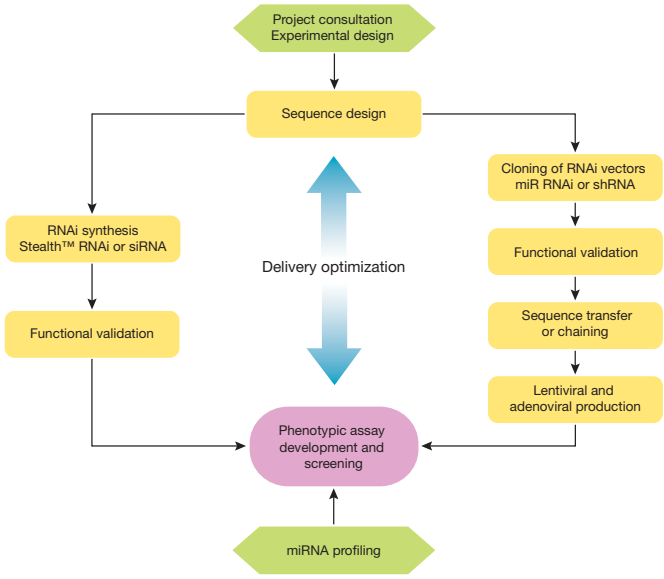

Figure 12.1. Invitrogen RNAi Services workflow.

RNAi design services

Successful RNAi experiments start with well-designed molecules. Invitrogen's RNAi Design Services uses a proprietary algorithm to generate highly effective Stealth RNAi™ siRNA, BLOCK-iT™ siRNA, BLOCK-iT™ Pol II miR RNAi, and BLOCK-iT™ shRNA sequences. 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
- → Conversion of siRNA to Stealth RNAi™ siRNA sequences, and of Stealth RNAi™ siRNA to BLOCK-iT™ Pol II miR RNAi sequences
- → Design of sequences for creating reporter vectors
- → Design of LUX™ Fluorogenic Primers for evaluating RNAi reagents by quantitative reverse transcription PCR (qRT-PCR)

Stealth RNAi™, *Silencer*® Select, and *Silencer*® siRNA custom synthesis

Synthetically generated RNAi molecules such as Stealth RNAi™, *Silencer*® Select, and *Silencer*® siRNA are the most popular RNAi molecules used in experiments. These duplexes are easily synthesized within just a few days. We offer Custom RNAi Synthesis Services for Stealth RNAi™, *Silencer*® Select, and *Silencer*® siRNA molecules, but we recommend the use of chemically modified, blunt-ended 25-mer Stealth RNAi™ double-stranded duplexes. Stealth RNAi™ siRNA reduces off-target effects, avoids induction of stress response pathways, and has enhanced nuclease stability without a loss in potency.

Vector-based RNAi services

DNA-based or vector-mediated RNAi is often used for long-term expression, hard-to-transfect cell lines, tissue-specific RNAi expression, and inducible RNAi. The DNA vectors express RNA sequences that are processed by Dicer, an endogenous enzyme, into functional RNAi duplexes that lead to knockdown of the target message. Vector technologies help accomplish these goals:

- → Achieve transient or stable target knockdown
- → Perform RNAi in any cell type—even hard-to-transfect, primary, and nondividing cells
- → Regulate gene inhibition with inducible RNAi
- → Enable study of long-term gene knockdown
- → Track expression of RNAi sequences with Emerald Green Fluorescent Protein (EmGFP)
- → Target multiple genes for knockdown using one vector

Invitrogen's RNAi vector technologies take advantage of the Gateway® system for easy cloning, and include both BLOCK-iT™ Pol II miR RNAi vectors and BLOCK-iT™ shRNA vectors. Chapter 8 contains more details on the features and benefits of the two vector technologies and the Gateway® system.

Cloning of BLOCK-iT™ Pol II miR RNAi vectors

BLOCK-iT™ Pol II miR RNAi vectors combine the benefits of traditional shRNA vectors—stable expression and the ability to use viral delivery—with capabilities for tissue-specific expression and multiple-target knockdown from the same transcript. These BLOCK-iT™ Pol II miR RNAi sequences can be designed, synthesized, and then cloned into one of the following miR RNAi entry vectors: pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR (see Chapter 8 for more information on these vectors). Each miR RNAi entry vector has a Pol II cytomegalovirus (CMV) promoter to drive expression, *attB* sites for compatibility with Gateway® technology and simplified cloning, and the ability to be excised from a long Pol II transcript for expression of more than one miR RNAi knockdown sequence from the same vector. In addition, the pcDNA™6.2-GW/EmGFP-miR vector features cocistronic expression of the EmGFP reporter, so that EmGFP expression can be correlated with the knockdown activity of the miR RNAi vector expression cassette. High-quality sequenced BLOCK-iT™ Pol II miR RNAi vectors are delivered as glycerol stocks and are ready for transfection.

Chaining BLOCK-iT™ Pol II miR RNAi sequences

The BLOCK-iT™ Pol II miR RNAi vectors are capable of expressing more than one miR RNAi sequence on the same transcript, allowing for the knockdown of multiple genes simultaneously. Using our restriction enzyme procedure, we can link, in any order, two or more cloned miR RNAi sequences from the pcDNA™6.2-GW/miR or pcDNA™6.2-GW/EmGFP-miR entry vector. We deliver the final vectors containing the chained miR RNAi sequences as glycerol stocks that can be prepared for transfecting cells or subcloning into a destination vector for a variety of downstream applications.

Subcloning BLOCK-iT™ Pol II miR RNAi vector sequences

Through Gateway® recombination, experimental possibilities are greatly increased. The miR RNAi cassette, which contains EmGFP (pcDNA™6.2-GW/EmGFP-miR vector only), miR flanking regions, and miR RNAi sequence homologous to the target of interest, can be transferred to many destination vectors. Available destination options include the following:

- → Lentiviral vectors for stable transduction of dividing and nondividing cells
- → Vectors with tissue-specific or regulated promoters
- → Vectors with alternative reporter fusion constructs
- → Flp-In™ system for single-site chromosomal integration

The BLOCK-iT™ Pol II miR RNAi Subcloning Service includes the Gateway® BP and subsequent LR recombination reactions to move the miR RNAi cassette from the miR RNAi entry vector into the Gateway® destination vector of choice. The resulting vectors are delivered as glycerol stocks that can be prepared for transfection or used to produce lentiviral particles. For a list of Invitrogen's destination vectors, please visit www.invitrogen.com/gateway.

Cloning BLOCK-iT™ shRNA vectors

It is important to begin knockdown experiments with high-quality, error-free shRNA vectors. BLOCK-iT™ shRNA sequences can be designed, synthesized, and cloned into one of two shRNA entry vectors: pENTR™/U6 or ENTR™/H1/TO (see Chapter 8 for more information on these vectors). Each shRNA entry vector has a Pol III promoter to drive expression and *attL* sites for compatibility with Gateway® technology and simplified cloning. We can also move the RNAi expression cassette to a number of other vector backbones for expanded experimental choices. The sequenced, verified BLOCK-iT™ shRNA vectors are delivered as glycerol stocks ready for transfection or adenoviral or lentiviral production.

Subcloning BLOCK-iT™ shRNA sequences

For increased experimental flexibility, the shRNA cassette containing the Pol III promoter and an shRNA sequence homologous to the target of interest can be transferred to many BLOCK-iT™ destination vectors through a Gateway® LR recombination reaction. Currently, five BLOCK-iT™ DEST vectors, including lentiviral and adenoviral vectors, are available. The resulting vectors are delivered as glycerol stocks that can be prepared for transfection or used to produce lentiviral or adenoviral particles.

Delivery optimization services

Achieving robust gene inhibition begins with efficient delivery of RNAi reagents while minimizing 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 over a decade. For easy-to-transfect cell lines, we typically use cationic lipid transfection reagents. For difficult-to-transfect cell lines, we recommend viral delivery. In the Delivery Optimization Services, we use our knowledge of and expertise with viral vectors and nonviral reagents to test a matrix of delivery parameters and find the optimal delivery conditions for the cell line of interest.

Viral production

Many mammalian cell types and experimental situations create challenges for standard transfection experiments. Lentiviral and adenoviral systems allow viral delivery of BLOCK-iT™ Pol II miR RNAi vectors and BLOCK-iT™ shRNA vectors to virtually any cell type:

- → Postmitotic and nondividing cells
- → Primary cells
- → Stem cells
- → Growth-arrested cells

BLOCK-iT™ lentiviral production services

Lentiviral particles containing BLOCK-iT™ Pol II miR RNAi or BLOCK-iT™ shRNA sequences are actively imported into the nuclei of cells by *cis*-acting elements. Expression of RNAi sequences of interest can occur transiently, but after integration into the genome, expression of sequences is stable, resulting in continuous high yields of expression. Invitrogen's Viral Production Services creates replication-incompetent lentiviral particles that efficiently transduce nearly any dividing or nondividing cell type. Crude lentiviral titers typically range from 10^4 to 10^6 transducing units per milliliter (TU/mL) as measured by a blasticidin resistance assay, and the working lentiviral stocks are available in two scales to meet individual experimental needs: 50 or 100 mL. Larger sizes may be available. The resulting titered lentiviral stocks are shipped ready for use in RNAi knockdown experiments.

When experiments require lentiviral stocks with titers above 10^4–10^6 TU/mL, Invitrogen can concentrate 50–100 mL of crude lentivirus down to 1–2 mL of concentrated viral stock. This process will increase the concentration of the stock 25- to 100-fold. The final lentiviral stocks are shipped ready for use in RNAi knockdown experiments.

BLOCK-iT™ adenoviral production services

Adenoviral systems are popular platforms for reliable delivery and high-level transient expression of BLOCK-iT™ Pol II miR RNAi or BLOCK-iT™ shRNA sequences in any mammalian cell type. Invitrogen uses a 293A producer cell line to generate high titers of replication-incompetent (E1- and E3-deleted) adenoviral particles that deliver and express RNAi sequences. Crude adenoviral titers typically range from 10^8 to 10^9 plaque-forming units per milliliter (PFU/mL), and the working adenovirus stocks are available in three scales: 10, 50, or 100 mL. Larger sizes may be available. The resulting titered adenoviral stocks are shipped ready for use in shRNA knockdown experiments. For some applications, it might be necessary to start with adenoviral titers above the normal 10^8 to 10^9 PFU/mL that are typically achieved with the BLOCK-iT™ Adenoviral Production Services. With the BLOCK-iT™ Adenoviral Concentration Services, 10–100 mL of crude adenovirus can be concentrated to achieve titers in the 10^{10}–10^{11} PFU/mL range. The final adenoviral stocks are shipped ready for use in RNAi knockdown experiments.

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.

Inducible RNAi—screen with RNAi expression "on" and "off"

Both BLOCK-iT™ Pol II miR RNAi and BLOCK-iT™ shRNA vectors can be cloned for inducible RNAi with tight tetracycline regulation.

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, Invitrogen 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

Custom stable cell line generation

Invitrogen Custom Services can transform a cell line to stably express an RNAi knockdown sequence or gene of interest at a constant level, indefinitely. The homogeneous cell population expressing the knockdown sequence or target gene can be used for many experiments, including differentiation studies, inducible expression studies, and *in vivo* transfer. Invitrogen's scientists will assist you in creating a stable cell line, and perform quality control testing to ensure that the cell line meets requirements. These are some of the advantages of having Invitrogen create your stable cell line:

- Skilled scientists with specialized training in creating stable cell lines, along with expert customer support and consulting
- Wide range of quality control testing assays
- BLOCK-iT™ Pol II miR RNAi and BLOCK-iT™ shRNA vector technologies and services
- Generation of T-REx™ host cell lines for expression that can be regulated
- Gateway® cloning services for a gene of interest

Invitrogen can create cell lines by transfection of a DNA plasmid and subsequent selection, or by using lentiviral particles. Lentiviral particles can integrate DNA efficiently into the recipient cell genome and can reduce the timeline for creating the stable cell line. The gene of interest or RNAi sequence will be randomly integrated into the cell's genome. To find at least one clone that meets your expression requirements, Invitrogen will expand and test many stable clones. The final stable cell line will be tested for mycoplasmas and supplied ready for further experiments.

RNAi custom collaborative research

Using a fully collaborative approach, Invitrogen 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, Invitrogen has the history, experience, and technology to help you succeed. Give us a call today to discuss your vision.

NOTE: Find information on RNAi services online at www.invitrogen.com/customservices.

CHAPTER 13

Overview of miRNAs

As the complexity of a genome increases, so does the ratio of non-coding to coding RNAs [1]. Non-coding 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 non-coding RNAs, microRNAs (miRNAs) are the most thoroughly characterized. These single-stranded 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]. Recent experimental evidence suggests that the number of unique miRNAs in humans could exceed 1,000 [3], though several groups have hypothesized that there may be up to 20,000 [4,5] non-coding RNAs that contribute to eukaryotic complexity.

miRNA function and regulation

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 base pairs 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, and 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 near-exact complementary base pairing and thereby direct cleavage and destruction of the mRNA [6,7]. 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 [8]. 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 [9]. Although hundreds of miRNAs have been discovered in a variety of organisms, little is known about their cellular function. 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.

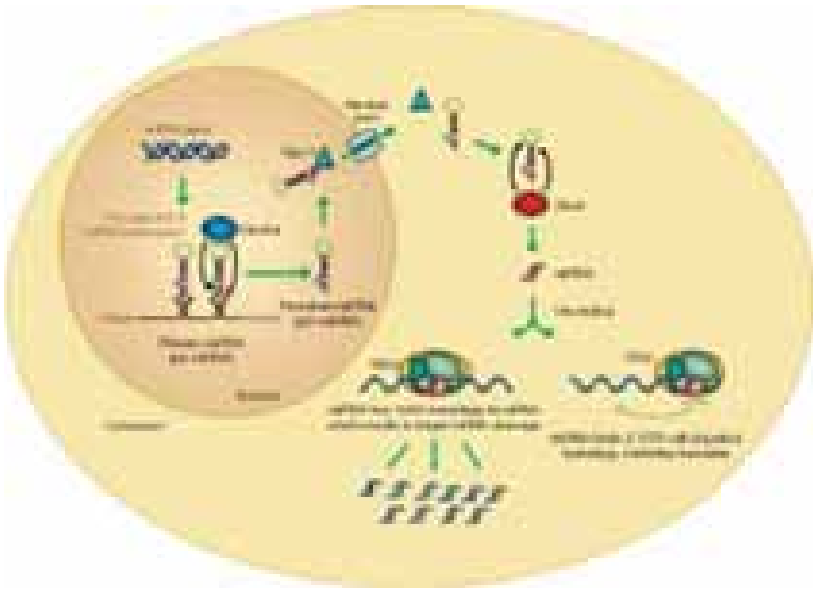

Figure 13.1. Biogenesis and function of miRNA. MicroRNA transcripts, generated by RNA polymerases II and III, are processed by the RNase III enzymes Drosha (nuclear) and Dicer (cytoplasmic), yielding 19–22 nucleotide miRNA duplexes. One of the two strands of the duplex is incorporated into the RISC complex,which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

Which miRNA profiling or validation system should I use?

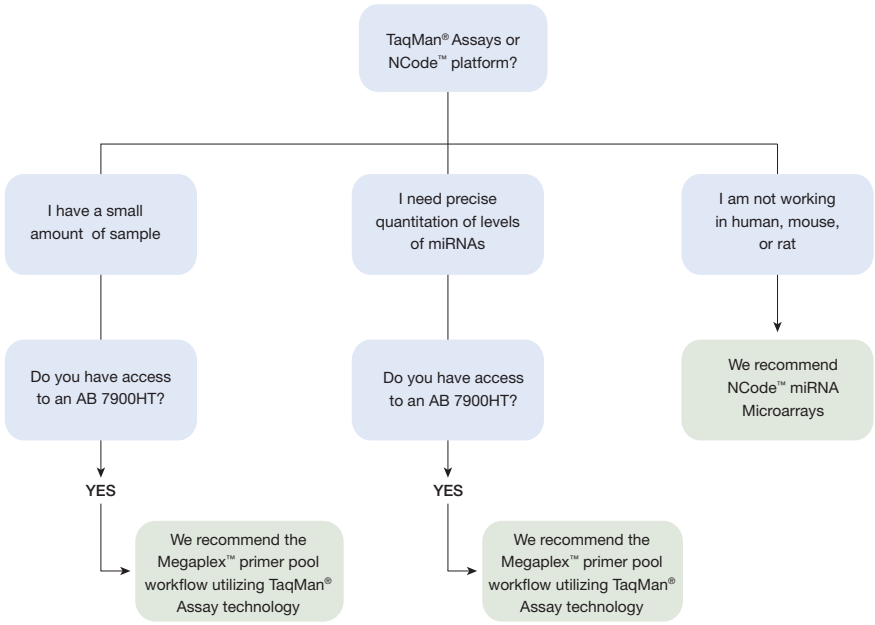

Figure 13.2. Which miRNA profiling system should I use?

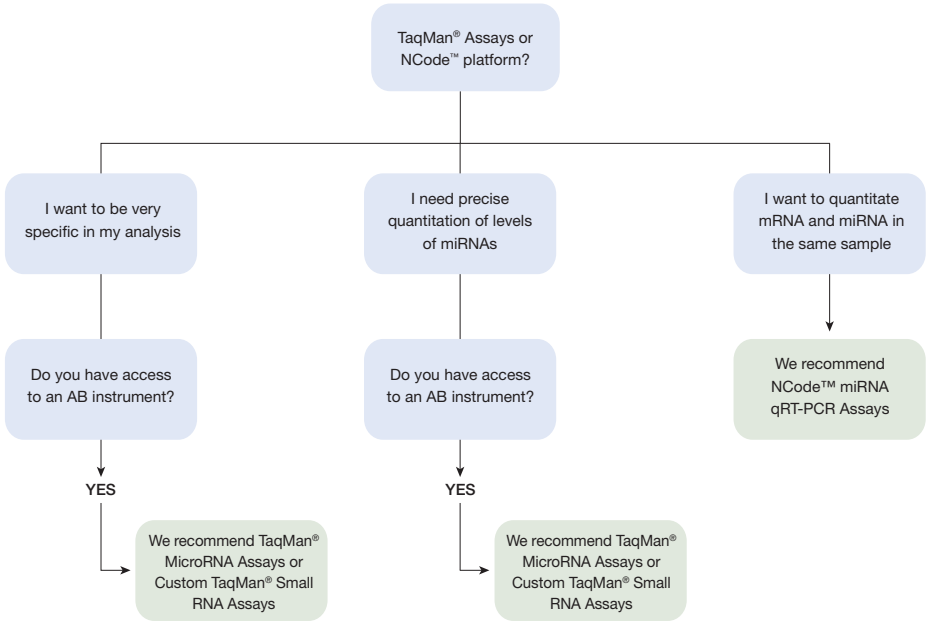

Figure 13.3. Which miRNA validation system should I use?

Genome-wide small RNA discovery and profiling

Use the SOLiD™ System to discover novel small RNAs and simultaneously determine which miRNAs are expressed, and where and when they are expressed.

MicroRNA Profiling Using TaqMan® Chemistry-Based Technology

Use Megaplex™ Primer Pools and TaqMan® MicroRNA Arrays to find out which miRNAs are expressed, and where and when they are expressed.

Targeted MicroRNA Quantitation

Use individual TaqMan® MicroRNA Assays to get specific information about where and when miRNAs are expressed.

MicroRNA Functional Analysis

Use synthetic miRNA mimics and inhibitors for gain- and loss-of-function studies to learn which cell processes are regulated by miRNAs. Further investigate miRNA targets and effects on protein expression to determine which genes miRNAs control.

MicroRNA: a new frontier in biology

MicroRNAs (miRNAs) are small, non-coding RNA molecules that direct posttranscriptional suppression of gene expression. The mechanisms by which miRNAs exert their regulatory effects are still being investigated, but it is well established that they interact with messenger RNAs (mRNA) based on sequence homology and ultimately affect translation and/or stability of the targeted mRNA. Each miRNA may regulate multiple genes, and it is likely that more than one third of all human genes may be regulated by miRNA molecules [10]. First referred to as the "biological equivalent of dark matter" [11], miRNAs have been shown to act as key regulators in many basic biological processes such as development, cell proliferation, differentiation, and the cell cycle. Emerging evidence also implicates miRNAs in the pathogenesis of human diseases such as cancers, metabolic diseases, neurological disorders, infectious diseases, and other illnesses [12].

Fundamental questions in microRNA research

Researchers have only just begun to understand how miRNAs work and what roles they play in biology. Thus, even fundamental questions about miRNAs have yet to be answered. Laboratories around the world are examining the effects of miRNAs in various experimental systems. Initial inquiries typically investigate which miRNAs are present in a particular cell type and the differences in miRNA expression between different tissues, developmental stages, or disease states. Additionally, many efforts to discover previously unreported miRNAs are underway. Once miRNAs of interest are identified, subsequent studies may be designed to artificially perturb miRNA expression levels in cultured cells while measuring resulting mRNA, protein, or phenotypic changes. Ultimately, the goal of many projects is to identify the effects of miRNAs on cellular processes, and as a result, assess their biological impact and significance.

Invitrogen and Applied Biosystems, both part of Life Technologies, lead the way in developing microRNA research tools

Active, mature miRNAs are typically 17–24 nucleotide, single-stranded RNA molecules that are excised from larger precursors. Because of their small size, the study of mature miRNA and other classes of small RNA requires specialized techniques. Invitrogen and Applied Biosystems provide innovative tools developed specifically for miRNA research, from isolation through discovery, profiling, quantitation, validation, and functional analysis. These tools enable scientists to not only address the fundamental questions about miRNA, but to use these advances to realize the full potential of this new and exciting chapter in biological science.

Capture the sequence and expression level of every small RNA in your sample

The discovery of new small RNAs is ongoing; 2,000 new sequences have been added to the miRBase Registry in the last two years [13]. The Applied Biosystems SOLiD™ RNA Analysis Solution includes a suite of products that enable rapid discovery of previously unreported small RNAs, sensitive quantitation of expression levels, and for the first time, the ability to assess allele-specific expression patterns.

Unlike other detection methods that only assay known or predicted small RNA sequences, the SOLiD™ System provides a digital readout of the small RNA molecules in a particular sample without prior sequence information. This capability enables both the discovery of novel small RNA molecules, and the detection of different isoforms generated by alternative processing [11].

Genome-wide small RNA discovery and profiling workflow

Features of the workflow:

- → Ability to discover novel small RNA molecules and isoforms
- → Sensitivity to detect small RNAs present at less than one copy per cell
- → Orientation information is preserved for strand-specific expression analysis
- → Enable cost-effective analysis of multiple samples in a single run using barcodes

How it works

Small RNAs are isolated from the sample and reverse transcribed to make a cDNA library of the small RNA population. The cDNA in the library is then amplified and sequenced, generating sequence fragments, commonly called "tags", corresponding to the cDNA of each small RNA. The tags are then mapped back to reference sequence databases to identify both new and known small RNA species. Relative expression levels can be calculated based on the number of tags obtained for each small RNA (Figure 13.4).

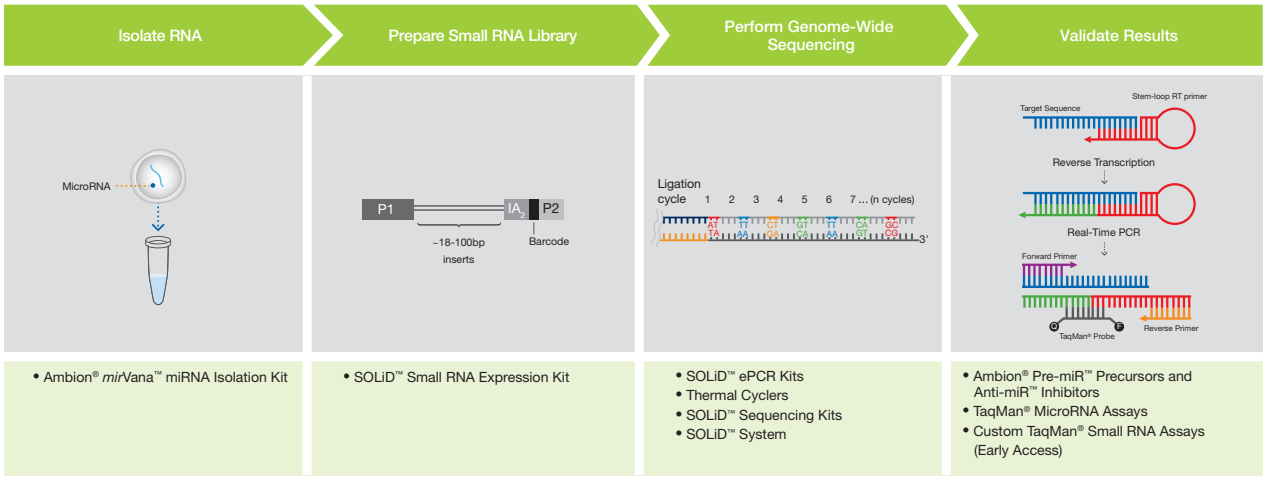

Figure 13.4. Genome-wide small RNA discovery and profiling workflow.

Robust small RNA discovery and profiling using the leading next-generation sequencing platform

The SOLiD™ RNA Analysis Solution is a robust method for global discovery and expression profiling of RNA. The SOLiD™ Small RNA Expression Kit provides a streamlined protocol for library construction enabling strand-specific expression analysis from as little as 10 ng of total RNA. It provides a simple means to convert RNA into a library of double-stranded DNA molecules using a robust one-day procedure (Figure 13.5). This unique process preserves the orientation information of the original molecule and enables strand-specific expression analysis. The small RNA libraries are then fed into the emulsion PCR (ePCR) step of the SOLiD™ system sample preparation workflow.

The SOLiD™ System generates over 400M mappable reads per run. This level of throughput allows researchers to analyze multiple samples per run while maintaining the sensitivity necessary for quantitative analysis of molecules present at low levels. The ability to multiplex multiple samples, due to the integration of barcode sequences during library preparation, dramatically reduces the cost, time, and labor associated with downstream ePCR and sequencing reactions.

The SOLiD™ System is a hypothesis-generating method for global small RNA discovery and quantitation. Figure 13.6 shows the proportions of data from a SOLiD™ System sequencing experiment that mapped to four known sequence databases. Approximately 51% of the tags mapped to known miRNAs, and more than 40% of the tags mapped to genomic regions but not to any known RNA species. Some of this latter set could represent previously uncharacterized miRNAs.

Data from the sequence tags that mapped to known miRNAs were evaluated to determine the relative expression of the corresponding miRNAs in human lung compared to placenta. MicroRNAs that showed significantly different expression in the two tissues were selected for differential expression analysis using TaqMan® MicroRNA Assays. Figure 13.7 shows a comparison of miRNA expression data from the two methods. With an R value of 0.9, this analysis suggests that the SOLiD™ platform is a valid profiling tool for gene expression analysis.

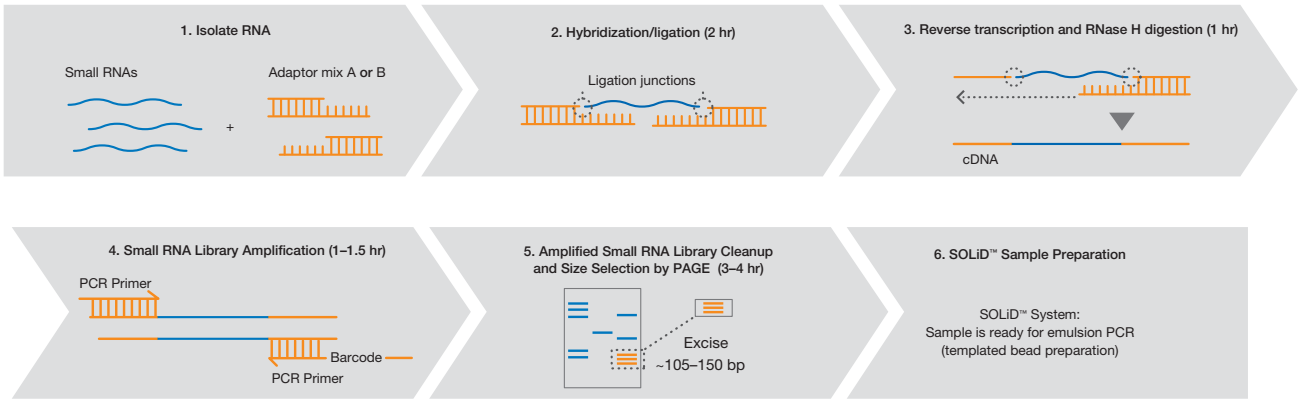

Figure 13.5. SOLiD™ Small RNA Expression Kit protocol.

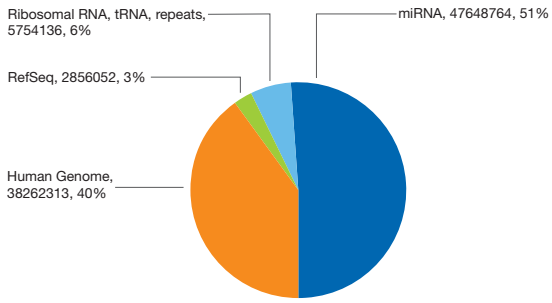

Figure 13.6. Distribution of 94 million mappable sequence reads to known sequence databases. Four human RNA samples (three from placenta and one from lung) were prepared and sequenced using a single SOLiD™ System run in the quad slide configuration. A total of 173M sequence tags were generated and 94M were mapped to the indicated databases with 0–1 mismatches. The small percentage (9%) of tags that mapped to known rRNAs, tRNAs, repeat regions, and the RefSeq database indicate that library preparation using the SOLiD™ Small RNA Expression Kit successfully enriched for the small RNA fraction. A subset of the sequence tags that mapped to the human genome, but not to known miRNAs, could represent previously uncharacterized small RNAs. These species are currently being validated as novel small RNAs using Custom TaqMan® Small RNA Assays.

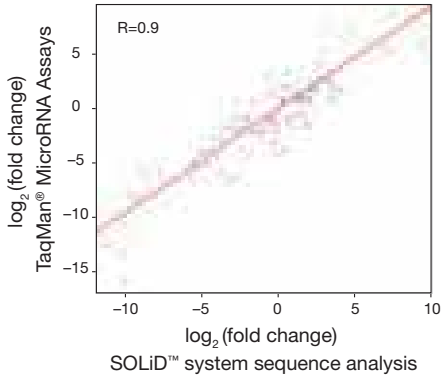

Figure 13.7. Good correlation between microRNA expression data from SOLiD™ System sequencing and analysis with TaqMan® MicroRNA Assays. The $\log_2$ of the fold-change between differentially expressed miRNAs in human lung and placenta samples obtained by SOLiD™ system sequencing was compared to the same data obtained by analysis using TaqMan® MicroRNA Assays. The resulting R value of 0.9 indicates that there is good correlation between relative miRNA expression data from the two platforms.

Profile microRNA expression in a single day using gold standard TaqMan® assay technology

Profiling the differences in global miRNA expression among 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 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 Applied Biosystems' 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 13.8)
- → 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 using real-time PCR. For the final quantitation step, 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 micro fluidic card containing 384 TaqMan® MicroRNA Assays. The real-time PCR is run on the Applied Biosystems® 7900HT Fast Real-Time PCR System.

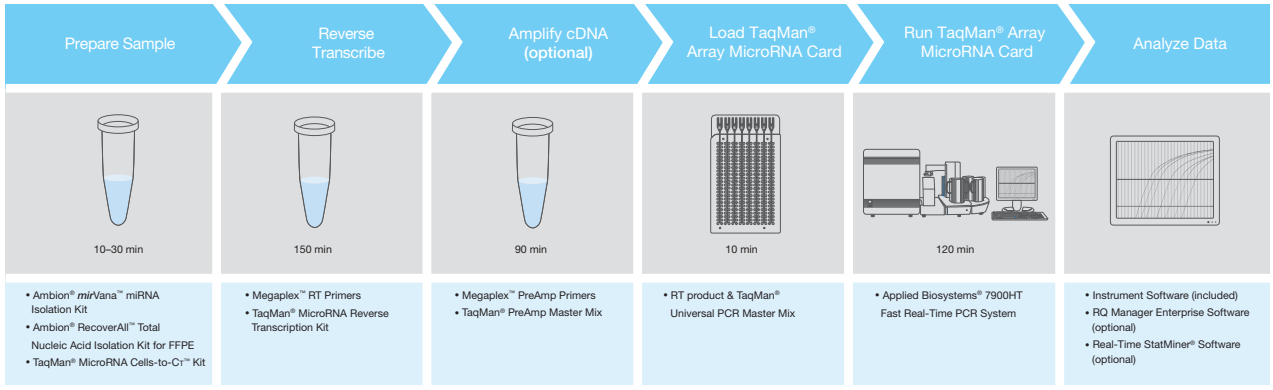

Figure 13.8. MicroRNA profiling workflow using TaqMan® technology.

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 13.9). 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 13.10). 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.

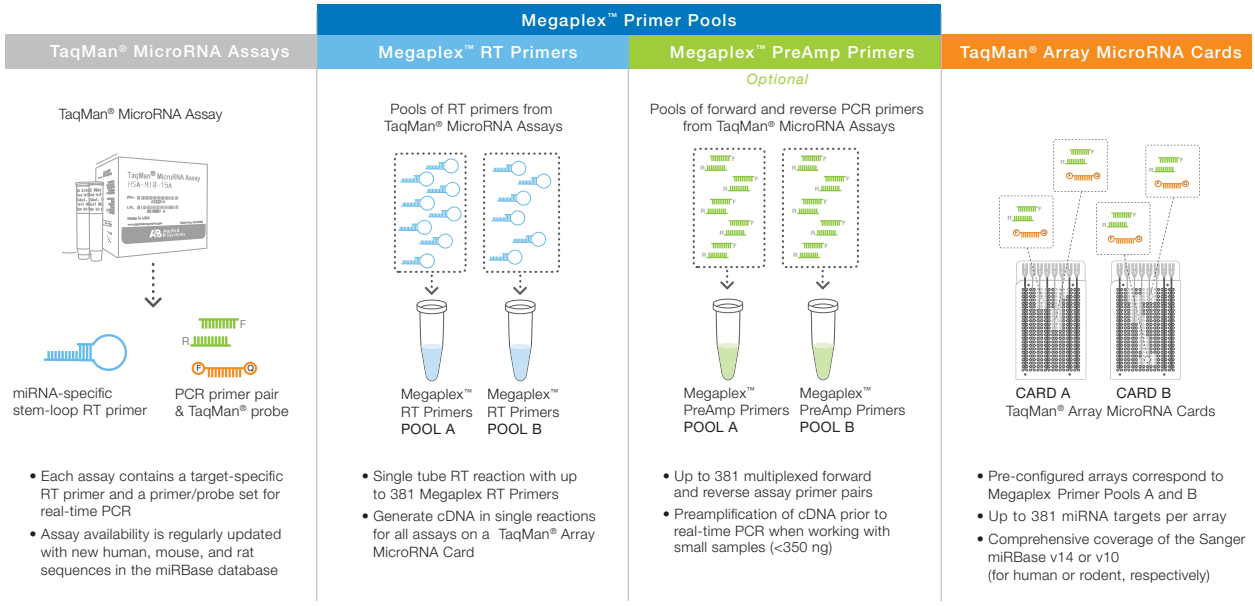

Figure 13.9. Megaplex™ Primer Pools bring the power of real-time PCR to microRNA profiling experiments.

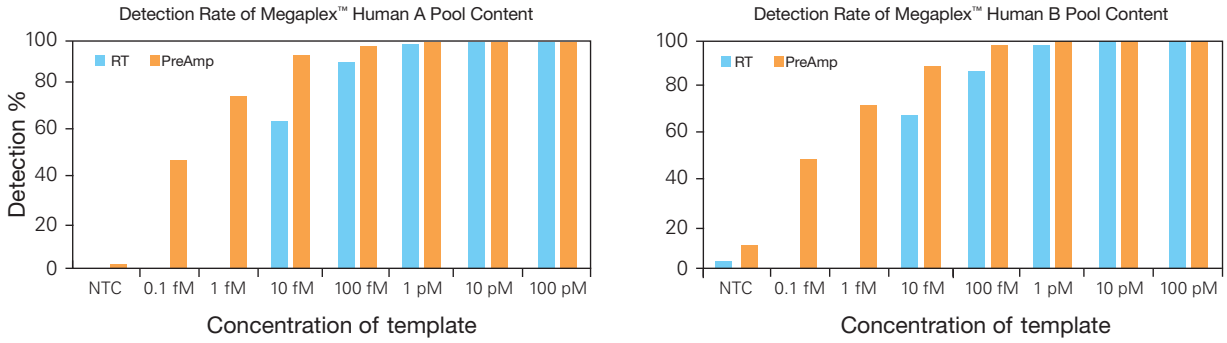

Figure 13.10. MicroRNA detection using Megaplex™ Primer Pools and TaqMan® Array MicroRNA Cards. Synthetic artificial targets (10 pM) for each assay represented on the A and B TaqMan® Array MicroRNA Cards were spiked into a complex total RNA background (10 ng/µL). The mixture was then serially diluted across a range of 6 or 4 logs, and detection of the artificial targets was tested 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. Subsequently, real-time PCR quantitation is performed using corresponding TaqMan® Array Human or Rodent MicroRNA Cards, on an Applied Biosystems® 7900HT Fast Real-Time PCR System.

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 micro fluidic 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 TaqMan® Array cards eight sample loading ports. 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.

Integromics StatMiner® Software

Real Time StatMiner® Software from Integromics is a powerful, easy-to-use bioinformatics tool for quality control and differential expression analysis of real-time PCR data. With just a single click, the software imports raw data or C_T values from any Applied Biosystems® Real-Time PCR System, and then guides you though every step of analysis to enable accurate results in minutes. The software is ideal for medium- and high-throughput PCR experiments and is compatible with TaqMan® Array Cards, and TaqMan® Assays in plate format.

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.

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, Applied Biosystems brings the benefits of TaqMan® Assays and quantitative real-time PCR—unparalleled sensitivity, specificity, and dynamic range—to miRNA detection and quantitation. 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. Applied Biosystems offers 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 13.11):

- → 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 continuously added for human, mouse, and rat species to stay aligned with the Sanger miRBase Registry

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 13.12). 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.

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. Applied Biosystems is continuously 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.

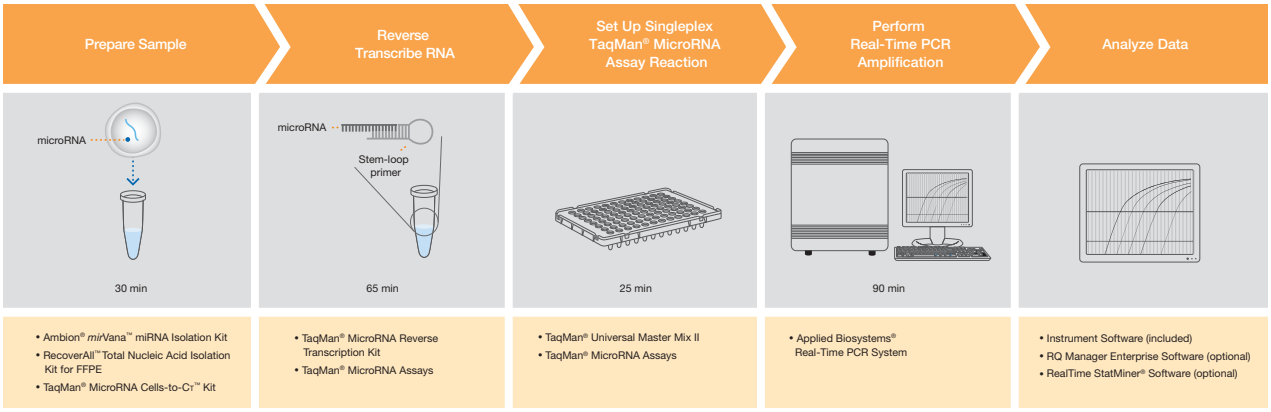

Figure 13.11. Targeted microRNA quantitation workflow.

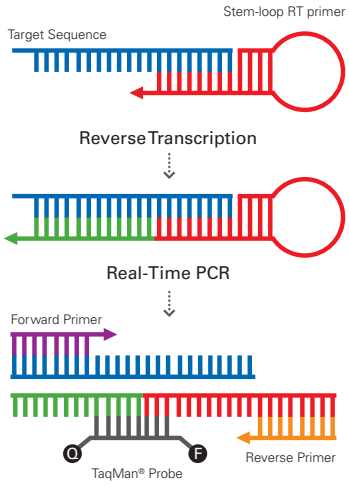

Figure 13.12. TaqMan® MicroRNA Assay approach. A simple two-step process brings the advantages of real-time PCR to miRNA research.

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 non-coding 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.

Custom TaqMan® Small RNA Assays (Early Access)

The novel adaptations in TaqMan® Assay design developed for the study of miRNAs using TaqMan® MicroRNA Assays are ideal for analysis of any small nucleic acid less than 200 bases long. With Custom TaqMan® Small RNA Assays, the benefits of these assays are available for any small RNA, from any species. This includes newly discovered miRNAs that are not yet in the registry and other classes of small RNAs, such as piwi-interacting RNA (piRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA).

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 13.13)
- → Sensitive—requires only 1–10 ng of total RNA or equivalent to conserve limited samples
- → Wide dynamic range—up to 9 logs—detect high and low expressors in a single experiment (Figure 13.14)
- → 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 Applied Biosystems 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 13.13. 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 x 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.

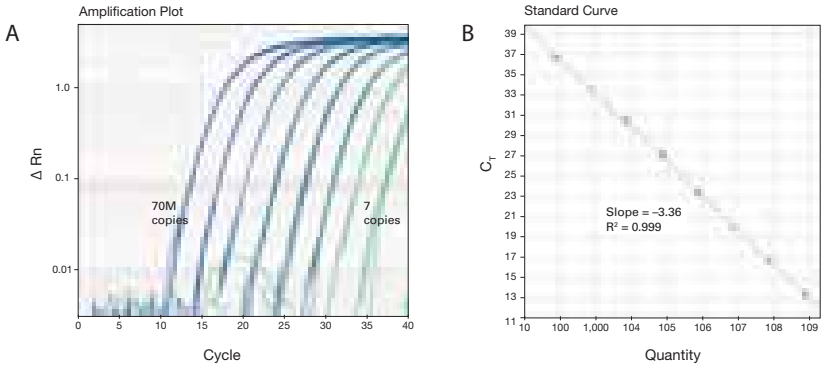

Figure 13.14. TaqMan® MicroRNA Assays provide wide dynamic range. This wide dynamic range enables miRNA targets that vary in abundance from a few copies to millions of copies to be accurately quantitated in the same experiment—an important factor given the wide range of miRNA concentrations within and 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 x 10^{-3} fM (equivalent to 7 copies per reaction) to 1.3 x 10^4 fM (equivalent to 7 x 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-encoding genes. Transfecting cultured cells with miRNA mimics can help identify gain-of-function phenotypes; down-regulation or inhibition experiments using miRNA inhibitors can be conducted to identify loss-of-function phenotypes. The combination of up-regulation and down-regulation 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.

Ambion® Pre-miR™ miRNA Precursors and Anti-miR™ mimics and inhibitors and the means to deliver them to cells

Ambion® 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 13.15). Both can be introduced into cells using transfection or electroporation parameters 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 mature miRNAs. The chemical modifications ensure that the correct strand, representing the desired mature miRNA, is taken up into the RNA-induced silencing complex (RISC)-like responsible for miRNA activity. 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.

Ambion® Anti-miR™ miRNA Inhibitors are chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNAs. 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.

There is a suite of products based on this technology that includes positive and negative controls, as well as Precursor and Inhibitor Libraries available as premade or custom sets. Figure 13.16 shows an experiment in which a library of Anti-miR™ miRNA Inhibitors was screened to identify miRNAs that are involved in cell proliferation. Note that Ambion® Pre-miR™ miRNA Precursors and Anti-miR™ miRNA Inhibitors keep pace with new additions to the miRBase database, and for sequences that are not published, customer-defined mimics and inhibitors are available.

Order Pre-miR™ and Anti-miR™ products at www.invitrogen.com/ordermirna.

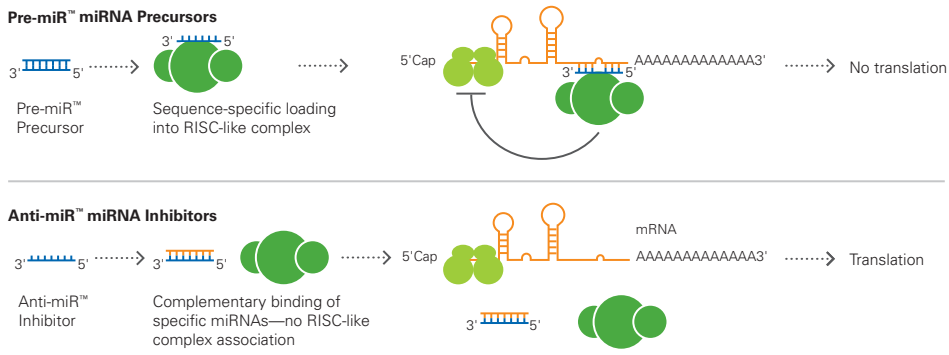

Figure 13.15. Activity of Ambion® Pre-miR™ miRNA Precursors and Anti-miR™ miRNA Inhibitors.

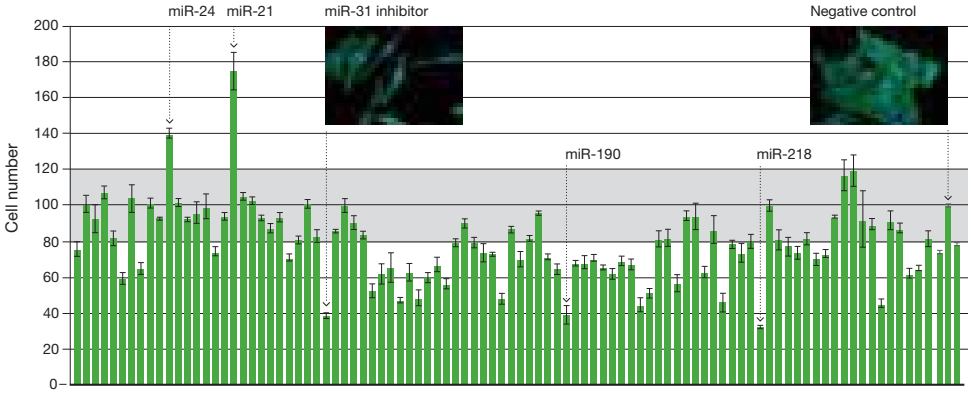

Figure 13.16. Identification of miRNAs involved in cell proliferation by screening with Anti-miR™ miRNA Inhibitors. HeLa cells were transfected with individual Anti-miR™ miRNA Inhibitors. After 72 hours, cells were fixed, stained with propidium iodide, and treated with an antibody to detect β-actin. 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 up-regulation of miR-31 resulted in elongated cells and reduced cell numbers.

Pre-miR™ miRNA Precursor Libraries

The Pre-miR™ miRNA Precursor Library-Human V3 consists of 470 miRNA mimics corresponding to 470 human mature miRNAs cataloged in version 9.2 of the miRBase Sequence Database. This collection of miRNA mimics enables rapid study of miRNA function (Figure 3) and can be used to simplify identification of miRNAs that interact with a particular target transcript. Each Pre-miR™ miRNA Precursor is in a quantity of 0.25 nmol, dried in individual wells of a 96-well plate. This amount is sufficient for 250 transfections when used at 10 nM in 96-well plates.

The Pre-miR™ miRNA Precursor Library–Mouse V3 consists of 379 miRNA mimics corresponding to 379 mouse mature miRNAs cataloged in version 9.2 of the miRBase Sequence Database. This collection of miRNA mimics enables rapid study of miRNA function and can be used to simplify identification of miRNAs that interact with a particular target transcript. Each Pre-miR™ miRNA Precursor is supplied dried at 0.25 nmol in individual wells of a 96-well plate. This amount is sufficient for 250 transfections when used at 10 nM in 96-well plates.

Custom Pre-miR™ miRNA Precursor Libraries, which include 50 or more Pre-miR™ miRNA Precursors of your choice in the amount you specify, are also available. Contact us at episcientist@invitrogen.com for additional details.

Anti-miR™ miRNA Inhibitor Libraries

The Anti-miR™ miRNA Inhibitor Library-Human V2 includes 327 miRNA inhibitors (0.25 nmol each) that correspond to 327 miRNAs in miRBase Sequence Database version 8.0.

The Anti-miR™ miRNA Inhibitor Library–Mouse V3 consists of 379 miRNA inhibitors corresponding to 379 mouse mature miRNAs cataloged in version 9.2 of the miRBase Sequence Database. This collection of miRNA inhibitors enables rapid study of miRNA function and can be used to simplify identification of miRNAs that interact with a particular target transcript. Each Anti-miR™ miRNA Inhibitor is supplied dried at 0.25 nmol in individual wells of a 96-well plate. This amount is sufficient for 50 transfections when used at 50 nM in 96-well plates.

Custom Anti-miR™ miRNA Inhibitor Libraries, which include 50 or more Anti-miR™ miRNA Inhibitors of your choice in the amount you specify, are also available. Contact us at episcientist@invitrogen.com for additional details.

Order at www.invitrogen.com/epi.

Reporter vector and combined chemiluminescent assay systems

To evaluate the interaction between miRNAs and their target sites, the Ambion® 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 down-regulation or up-regulation of reporter gene expression after transfection with Pre-miR™ miRNA Precursors or Anti-miR™ miRNA Inhibitors, respectively. With a Pre-miR™ miRNA Precursor Library, pMIR-REPORT™ Luciferase is an ideal screening tool to study miRNA-mediated regulation of a target gene.

The resulting luciferase and β-galactosidase activity can be measured using the Tropix® Dual-Light® Luciferase and β-Galactosidase Reporter Gene Assay System. With this innovative system, both assays are performed in less than one hour using a single extract for greater convenience and precision. Moreover, the wide dynamic range of this dual assay enables accurate measurement of luciferase and β-galactosidase concentrations over seven orders of magnitude, with 100- to 1,000-fold greater sensitvity than colorimetric and fluorescent assays for β-galactosidase.

Protein expression analysis

miRNA functional studies may require simultaneous analyses of RNA and protein expression. The Western-SuperStar™ Immunodetection System is a highly sensitive chemiluminescent immunodetection system, providing an ideal solution for measuring the effect of miRNA function on the expression of specific proteins.

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.

Applied Biosystems offers more than 1,000,000 TaqMan® Gene Expression Assays for 19 species, the most comprehensive set of pre-designed, 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 functional analysis workflow

Features of the workflow (Figure 13.17):

→ 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

Ambion® Pre-miR™ miRNA Precursors and Anti-miR™ miRNA Inhibitors are designed to mimic or inhibit specific miRNAs for artificial up-regulation and down-regulation of target mRNA translation, respectively. miRNA targets are validated by quantitating target protein and/or messenger RNA levels in reponse to miRNA up-regulation or down-regulation. Western blot analysis is used to investigate the impact on protein expression.

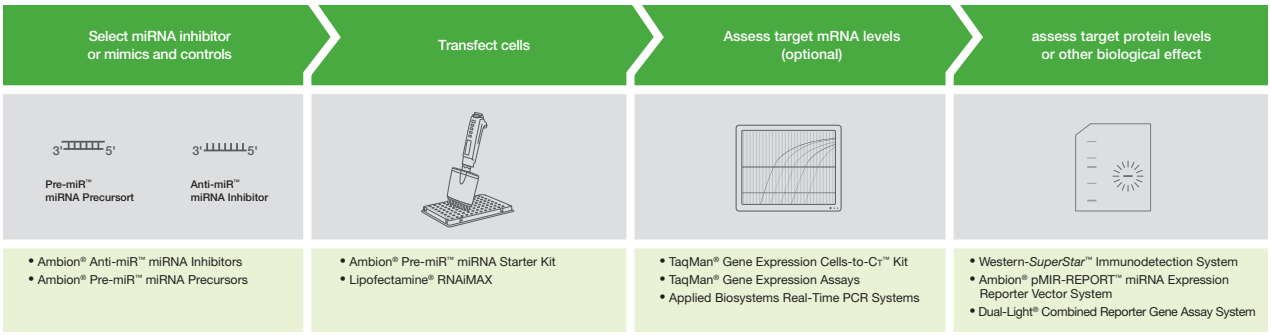

Figure 13.17. MicroRNA functional analysis workflow.

Sample preparation tailored for microRNA experiments

Most RNA isolation kits were developed to recover messenger RNA, and eliminate smaller molecules such as miRNAs. Invitrogen offers a range of products that were designed for optimal recovery of miRNA and other small RNAs from a wide variety of sample types (Figure 13.18). 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

MicroRNA Sample Preparation Kits

Invitrogen offers a variety of kits for the isolation and analysis of miRNA and other small RNAs. Each kit is ideal for use in miRNA analysis because they are optimized to enable:

→ Quantitative recovery of small RNA (<200 nt)

→ Maintenance of representative amounts of small RNA (eliminating experimental bias)

Ambion® *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 small RNA-containing total RNA, or for enrichment of the small RNA fraction (<200 nt), to increase sensitivity in downstream analyses.

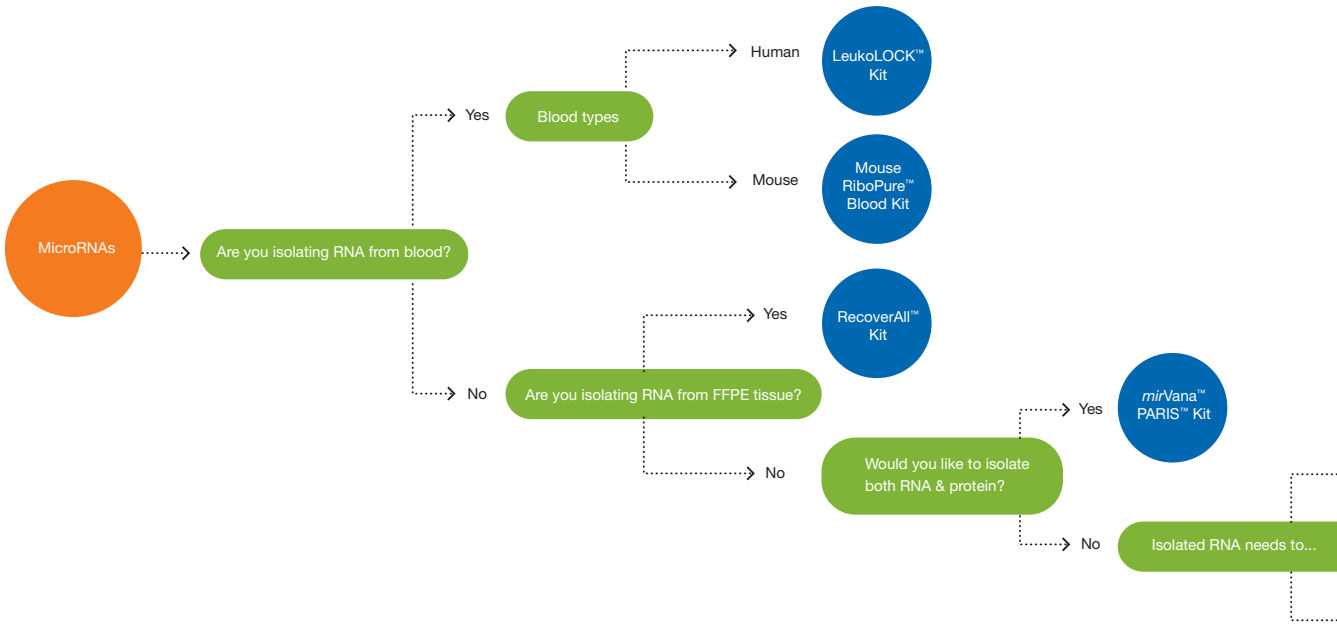

Figure 13.18. MicroRNA sample preparation selection guide.

Ambion® RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Tissues

The 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. The degree of 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-CT™ 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 TaqMan® MicroRNA Cells-to-CT™ 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-CT™ Kit

Similar to the Cells-to-CT™ Kit for microRNA, the TaqMan® Gene Expression Cells-to-CT™ 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.

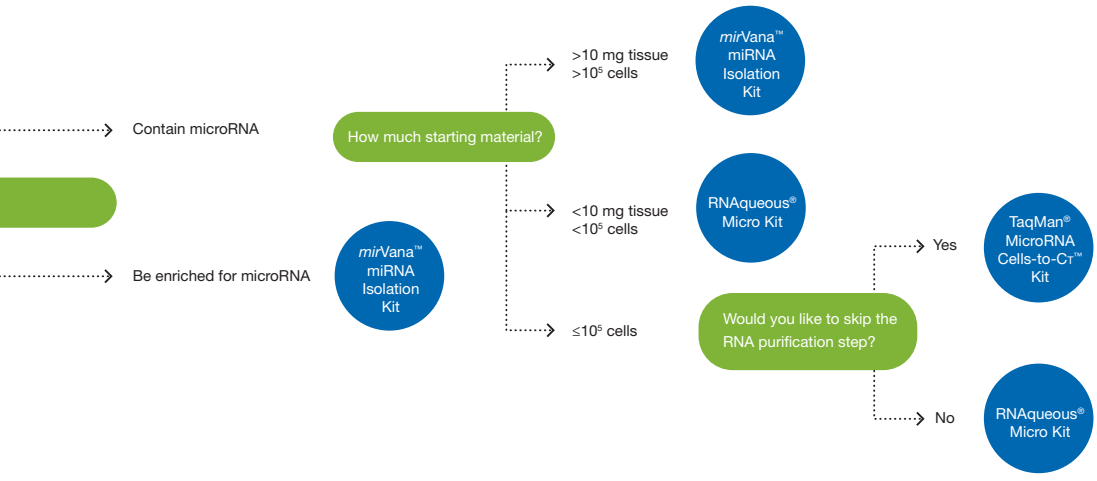

Table 13.1. MicroRNA sample preparation kits: quantitative recovery of small RNAs from a variety of sample types.

	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	10^3–10^7 cultured cells or 0.5–250 mg tissue	100–10^7 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 minute 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 of >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 and part numbers	100 lysis rxns/500 PCRs P/N 4391848 400 lysis rxns/2,000 PCRs P/N 4391996	Up to 40 purifications P/N AM1560	Up to 40 purifications P/N AM1556	40 purifications P/N AM1975	96 purifications P/N AM1839

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CHAPTER 14

Optimized miRNA profiling

NCode™ platform—for optimized miRNA profiling

miRNA expression profiling came into prominence in part because of the expectation that a highly expressed miRNA for a given tissue or cell type (or developmental stage) is likely to play a regulatory role. Since the first published article to report on miRNA profiling using an oligonucleotide microarray [1], microarray analysis has become the preferred tool for profiling miRNA expression patterns to gain insight into their relevance in development and disease. The NCode™ platform is optimized to investigate global miRNA expression patterns. From sample preparation to array analysis to qRT-PCR validation, the NCode™ platform provides optimized reagents for every step of the miRNA profiling workflow (Figure 14.1).

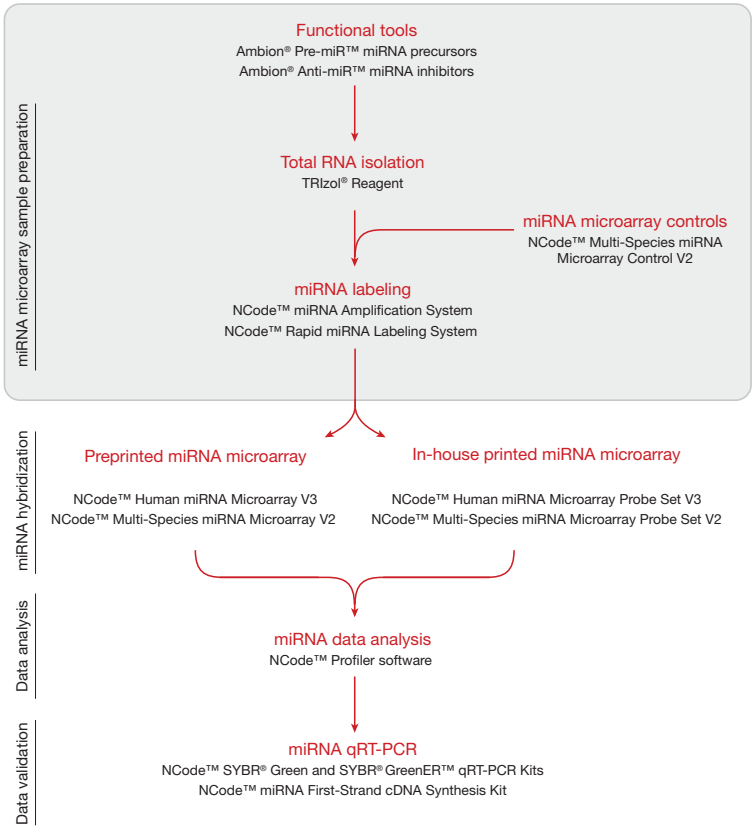


Figure 14.1. NCode™ platform workflow. During sample preparation, total RNA is isolated and quantified. Amplification of the small RNA molecules may be necessary when there is insufficient starting material. Next, a direct labeling method polyadenylates the enriched small RNA molecules and ligates a fluorescently conjugated, branched DNA structure to the tailed RNA. Tagged and tailed miRNAs are subsequently hybridized to the array. Bound miRNAs are detected by the hybridization of branched DNA structures containing dye molecules. The miRNA expression profile is calculated from relative signal intensity detected by a microarray scanner for each spot on the array. Data validation is accomplished using qRT-PCR.

Efficient isolation of total RNA (including miRNA) using TRIzol® Reagent

Isolating high-quality total RNA from your sample is a step crucial to the success of your research. TRIzol® Reagent purifies all RNAs, including those in the 10–200 nucleotide range, and is thus recommended for isolating total RNA for studying miRNAs. Extraction procedures involve ready-to-use monophasic solutions of phenol and guanidine isothiocyanate, and are based upon improvements to the single-step RNA isolation method developed by Chomczynski and Sacchi [2]. Using TRIzol® Reagent enables:

→ More effective purification of total RNA than column-based purification, without depletion of small RNAs
→ A simple, flexible protocol, completed in less than 1 hr
→ High-purity RNA from a wide range of animal tissues and cells

Product	Quantity	Cat. No.
TRIzol® Reagent	100 mL	15596026

Reliable recovery of total RNA (including miRNA) from FFPE samples with the RecoverAll™ Total Nucleic Acid Isolation Kit

→ Optimized for isolation of total nucleic acids, including microRNAs, from FFPE tissue
→ No overnight proteinase K digestion required—deparaffinize in the morning and perform qRT-PCR in the afternoon
→ Obtain typical yields of >50% that of unfixed tissue from the same sample source
→ Recovered nucleic acids are suitable for qRT-PCR, qPCR, mutation screening, and microarray analyses

The RecoverAll™ Total Nucleic Acid Isolation Kit is designed to extract total nucleic acid from formalin or paraformalin-fixed, paraffin-embedded (FFPE) tissues (Figure 14.2). Up to four 20 µm sections, or up to 35 mg of unsectioned core samples, can be processed per reaction.

The ability to isolate nucleic acid from archived tissue samples that is suitable for molecular analysis enables retrospective studies of diseased tissue at both the genomic and gene expression level. While standard preservation techniques that employ formalin are ideal for maintaining tissue structure and preventing decomposition, formalin preservation creates protein–protein and protein–nucleic acid crosslinks that make it difficult to perform molecular analyses. In addition, RNA (and to some extent DNA) is often fragmented and chemically modified to such a degree that it is incompatible with many molecular analysis techniques.

The degree of 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 (Figure 14.3) of RNA fragments of all sizes, including microRNA, in a relatively short amount of time.

The RecoverAll™ Total Nucleic Acid Isolation Kit procedure requires about 45 minutes of hands-on time and can easily be completed in less than 1 day when isolating RNA. FFPE samples are deparaffinized using a series of xylene and ethanol washes. Next, they are subjected to a rigorous protease digestion with an incubation time compatible for recovery of either RNA or DNA. The nucleic acids are purified using a rapid glass-filter methodology that includes an on-filter nuclease treatment and are eluted into either water or the low-salt buffer provided.

The recovered nucleic acids are suitable for downstream applications such as microarray analyses, qRT-PCR (see Figure 14.4), and mutation screening. However, as is the case with all FFPE tissue, sample fixation and storage typically cause nucleic acid fragmentation and modification. Therefore, downstream applications, such as microarray analysis, which require more pristine RNA than does qRT-PCR, may require modification for best results. Although DNA tends not to fragment as easily as RNA, DNA is more reactive to the formalin and requires a longer (2-day) protease digestion time to release substantial amounts of DNA. The recovered DNA can typically be used for PCR and other downstream applications.

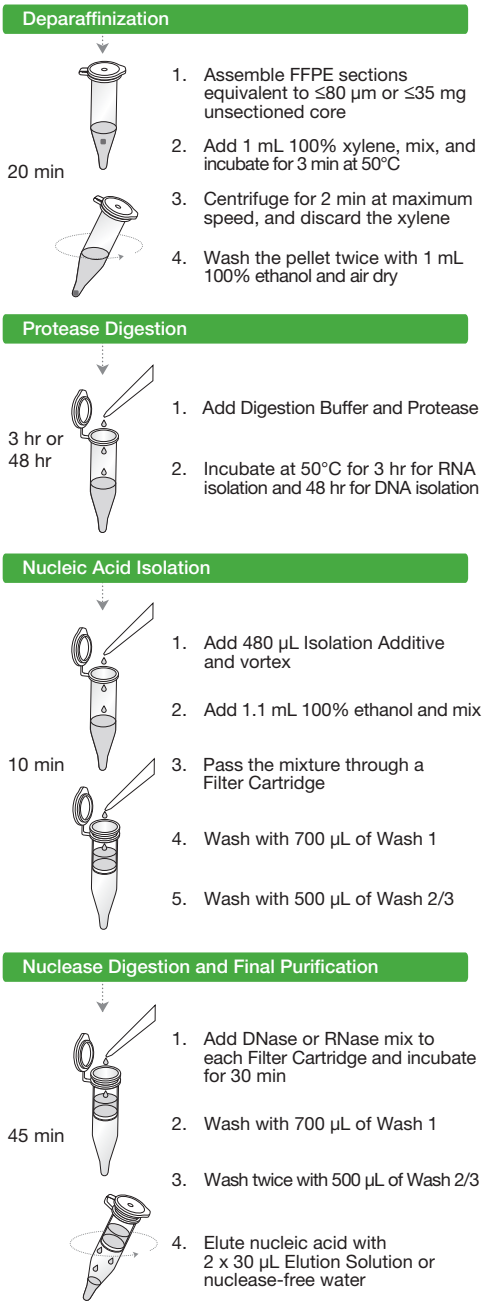


Figure 14.2. Overview of the RecoverAll™ Total Nucleic Acid Isolation Kit procedure.

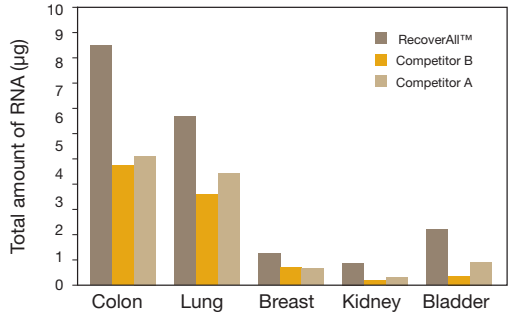


Figure 14.3. Yield of RNA from archived human FFPE tissue samples: RecoverAll™ kit vs. two competitor systems. A 10–20 µm section from each of the above archived human tissue blocks was isolated using each of three kits: Competitor A kit, Competitor B kit, and the Ambion® RecoverAll™ kit. Colon, lung, and breast samples were 1–2 years old; kidney was 3–5 years old; and bladder was 10–15 years old. Once the RNA was isolated, the concentration was determined via OD_{260}, and the amount of RNA recovered in micrograms was calculated. The RecoverAll™ Kit yielded the highest recovery of the three systems for all tissue types.

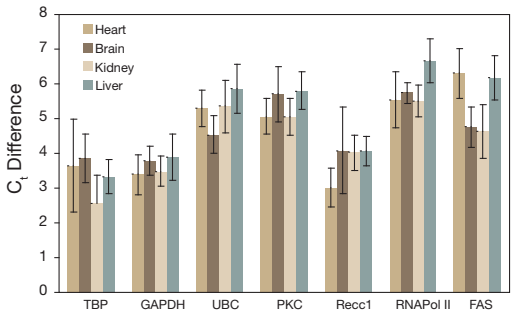


Figure 14.4. C_t values from real-time RT-PCR of frozen and FFPE mouse tissues. Four different tissues (brain, kidney, heart, and liver) were dissected from four different mice. Each tissue was split in half—one half was flash-frozen in liquid nitrogen and then stored at –80°C, while the other half was fixed and embedded using standard hospital protocol. RNA was isolated from one 20 µm section from each FFPE sample using the RecoverAll™ Kit. RNA from the frozen controls was isolated using the mirVana™ miRNA Isolation Kit. The RNA (400 ng) was then subjected to two-step real-time RT-PCR. The cDNA was generated using the RetroScript® Kit. Ambion® SuperTaq™ polymerase and other necessary reagents, plus an aliquot of the RT reaction, were used for the PCR. All gene targets had an amplicon ranging from 80 to 100 nt. The C_t values for all reactions of the same tissue were averaged together, then the C_t values for the frozen controls were subtracted from the C_t values for the FFPE samples to generate the graph above.

Product	Quantity	Cat. No.
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	40 purifications	AM1975

Simplified miRNA fluorescent labeling

The NCode™ Rapid miRNA Labeling System is a fast, reliable method to label endogenous miRNAs with fluorescent tags. Using this system, miRNAs from total RNA samples are polyadenylated and labeled with fluorescent Alexa Fluor® dyes, and hybridized to microarrays printed with species-specific antisense miRNA probes. This system has been optimized to enable sensitive and accurate profiling of miRNA expression patterns from minimal RNA input. Starting with 250 ng to 5 µg of total RNA, miRNAs are tagged directly in a quick and easy protocol with the NCode™ Rapid miRNA Labeling System (Figure 14.5). Lower starting amounts (100 ng) may be used with similar sensitivities, depending on the type of scanner and hybridization method used. Dynamic hybridization is required to achieve maximum sensitivity at lower input RNA levels. Maximum sensitivities are typically seen in the 500 ng to 1 µg range, depending on sample type. The protocol takes approximately 1 hour to complete and consists of just two steps prior to hybridization: poly(A) tailing, and ligation of a fluorescent molecule. After 8–16 hours of hybridization, the microarrays are ready to scan and analyze. The fluorescent dendrimer—a branched structure of single- and double-stranded DNA conjugated with Alexa Fluor® dyes—appends approximately 15 fluorophores to the RNA strand. The high sensitivity achieved with this method is due to the signal amplification effect of the labeled branched DNA structure [1], enabling maximum signal-to-background ratios and strong signal correlation for increased sensitivity (Figure 14.6, Figure 14.7).

Using the NCode™ Rapid miRNA Labeling System enables:

- → Less time at the bench—hybridize in one step, with no miRNA enrichment required
- → Increased sensitivity—reliably detect attomolar levels of miRNA
- → Reliable miRNA profiling results
- → Rapid turnaround—complete your experiments in a single day using the 8 hr hybridization protocol with dynamic hybridization

1. This method uses 3DNA® Reagent, manufactured under exclusive license from Genisphere, Inc.

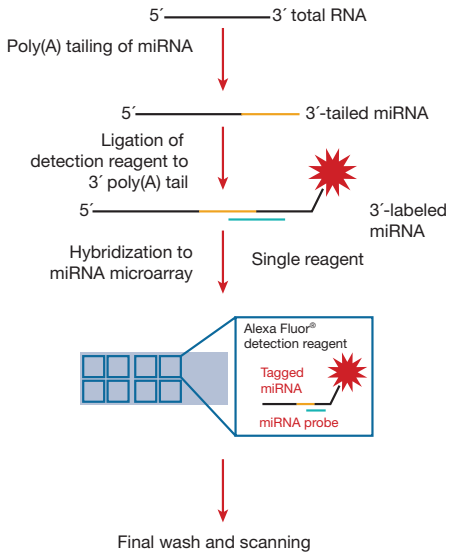


Figure 14.5. NCode™ Rapid miRNA Labeling System titration. The NCode™ Rapid miRNA Labeling System is recommended when starting with 250 ng to 5 µg total RNA. When starting with less than 50 ng of total RNA, we recommend using the NCode™ miRNA Amplification System to increase the quantity of miRNA that can be detected and provide sufficient material for replicate experiments.

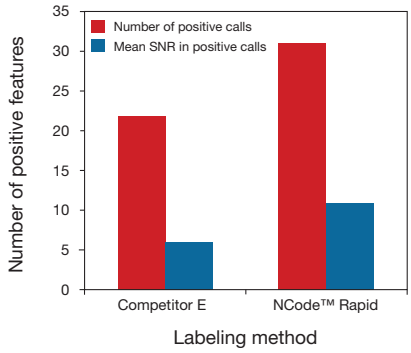


Figure 14.6. Sensitivity of the NCode™ Rapid miRNA Labeling System compared to competitor "E". Starting with 100 ng pooled total human RNA, samples were labeled with the NCode™ Rapid or labeling system from "E". Replicate samples were arrayed on either NCode™ microarrays or competitor microarrays in both red and green channels. Images scanned via an Axon scanner and data acquired in a GenePix Pro® scanner. Data were background-corrected and compared across common human features for signal-to-noise ratios (SNR) and positive-call designation. Positive call is determined as SNR >3 in all replicate features across all replicate arrays. Mean SNR was calculated from positive calls. The NCode™ Rapid miRNA Labeling System is brighter and detects more features at low input (100 ng of total RNA).

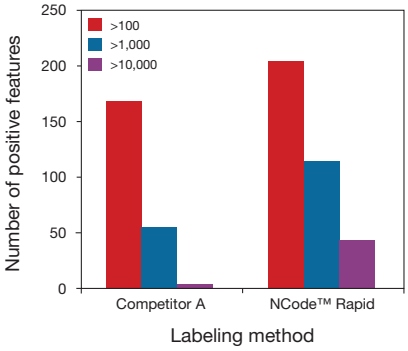


Figure 14.7. Sensitivity of the NCode™ Rapid miRNA Labeling System compared to competitor "A". Starting with 100 ng pooled total human RNA, samples were labeled with the NCode™ Rapid or labeling system from "A". Replicate samples were arrayed on either NCode™ microarrays or competitor microarrays in the green channel. Images were scanned via an Axon scanner and data acquired in a GenePix® scanner. Data were background-corrected and compared across common human features for signal-to-noise ratios (SNR) and positive-call designation. Positive call is determined as SNR >3 in all replicate features across all replicate arrays. Mean SNR was calculated from positive calls. The number of features having signals greater than 10,000 FU (purple bars), 1,000 FU (dark blue bars), and 100 FU (red bars) are shown above. The NCode™ Rapid miRNA Labeling System is brighter and detects more features at low input.

Product	Quantity	Cat. No.
NCode™ Rapid miRNA Labeling System	20 rxns	MIRLSRPD-20
NCode™ Rapid Alexa Fluor® 3 miRNA Labeling System	20 rxns	MIRLSA3-20

Ultrasensitive miRNA amplification

In many instances, RNA samples derived from laser capture microdissection (LCM), flow-sorted (FACS) samples, or needle biopsies do not yield sufficient starting amounts of RNA for standard profiling methods or for the experimental replicates needed for statistical reliability. In such cases, it is useful to apply linear miRNA amplification methods to the enriched miRNA population, thereby increasing the relative target abundance levels. The NCode™ miRNA Amplification System, based on mRNA amplification methods, enables robust and efficient linear amplification and microarray profiling of small amounts of RNA species, such as miRNA, from as little as 50 ng of total RNA or the enriched equivalent. The NCode™ miRNA Amplification System enables:

- → Increased sensitivity, with consistent 2,000- to 5,000-fold amplification of miRNA
- → Amplified product that faithfully represents the initial miRNA sample (Figure 14.8)
- → Precise amplification from reaction to reaction

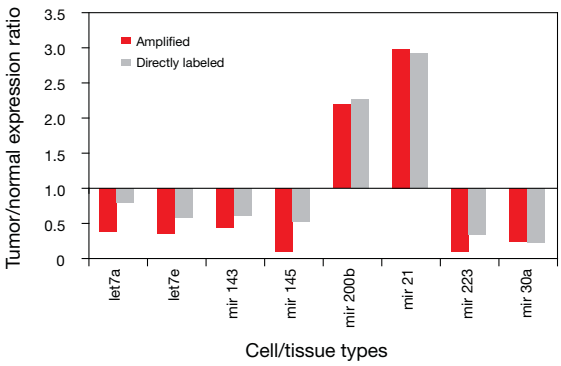


Figure 14.8. Ultrasensitive and accurate miRNA microarray profiling from small samples. Total RNA from large cell carcinomas and adjacent normal tissue was isolated from multiple patients and enriched for miRNA with the PureLink™ miRNA Isolation Kit. Enriched miRNA from 300 ng of total RNA was amplified using the NCode™ miRNA Amplification System and labeled for array analysis with the NCode™ miRNA Labeling System. Additionally, the miRNA from 10 µg of total RNA from the same source was labeled with the NCode™ miRNA Labeling System without amplification and hybridized to NCode™ Multi-Species miRNA Microarrays for analysis. The ratios of miRNA expression in tumor samples vs. adjacent normal tissues were calculated for all human miRNA.

Product	Quantity	Cat. No.
NCode™ miRNA Amplification System	20 rxns	MIRAS20

Comprehensive miRNA expression profiling

Whether you prefer to profile miRNAs using in-house printed arrays or preprinted arrays, the NCode™ platform allows you to comprehensively screen most known miRNAs in a variety of species (Figure 14.9). Enjoy the advantages of a leading preprinted miRNA profiling microarray, or gain the flexibility of a probe set and controls for array fabrication in your lab. The NCode™ microarrays offer:

- → Arrays spotted in triplicate with positive and negative controls throughout for monitoring hybridization specificity
- → Increased sensitivity from maximum hybridization intensities, and normalized melting temperatures for uniform hybridization
- → Easy normalization of signal intensities during scanning, using Alexa Fluor® dye control probes
- → Detection and identification of miRNAs conserved in other species but not yet validated for your model

NCode™ Human miRNA Microarray

The NCode™ Human miRNA Microarray V3 consists of Corning® epoxide–coated glass slides printed with optimized probe sequences that target nearly all known human miRNAs in the Sanger miRBase Sequence Database, Release 10.0, plus 373 novel putative human miRNAs (Table 14.1) discovered through deep sequencing and validated by both microarray hybridizations and qPCR analysis. Each microarray slide comes fully blocked and ready to use. Access to this putative novel content enables researchers to access and profile biologically relevant small non-coding RNAs (putative miRNAs) that are not available on other platforms (Figure 14.10). This content is not derived from *in silico* predictions.

NCode™ Multi-Species miRNA Microarray

The NCode™ Multi-Species miRNA Microarray V2 consists of optimized probe sequences targeting nearly all of the known and predicted mature miRNAs in the Sanger miRBase Sequence Database, Release 9.0, for human, mouse, rat, *D. melanogaster, C. elegans*, and zebrafish sequences (Figure 14.11, Table 14.1). These species-specific, unmodified oligonucleotides are 34 to 44 bases in length, and each microarray slide comes fully blocked and ready to use. Interrogation of samples with the NCode™ Multi-Species miRNA Microarray allows researchers to compare cross-species miRNA expression, and validate the existence of predicted miRNA species (or those that are conserved but not yet validated in a particular organism).

Controls

The NCode™ Multi-Species miRNA Microarray Control V2 is a synthetic 22-nucleotide miRNA positive control that is used to assess the efficiency of array labeling and hybridization. This control does not exhibit any detectable cross-hybridization or interference with endogenous miRNAs from any sequenced organism. The NCode™ Multi-Species miRNA Microarray and NCode™ miRNA Microarray Probe Sets include oligonucleotide probes that are complementary to the control.

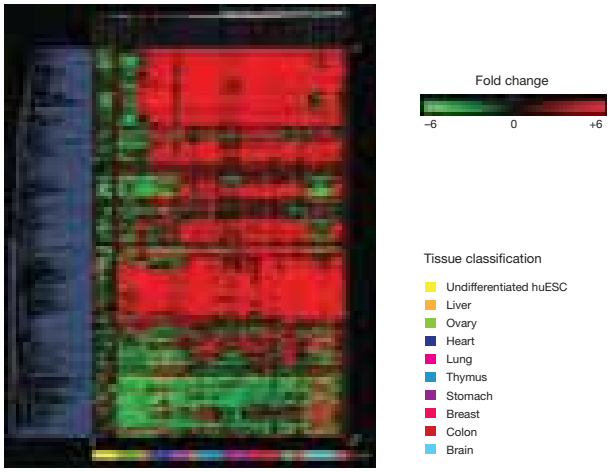


Figure 14.9. Profiling and cluster analysis of total RNA from various tissues on the NCode™ Human miRNA Microarray V3. Both known and novel content show tissue-specific differential expression.

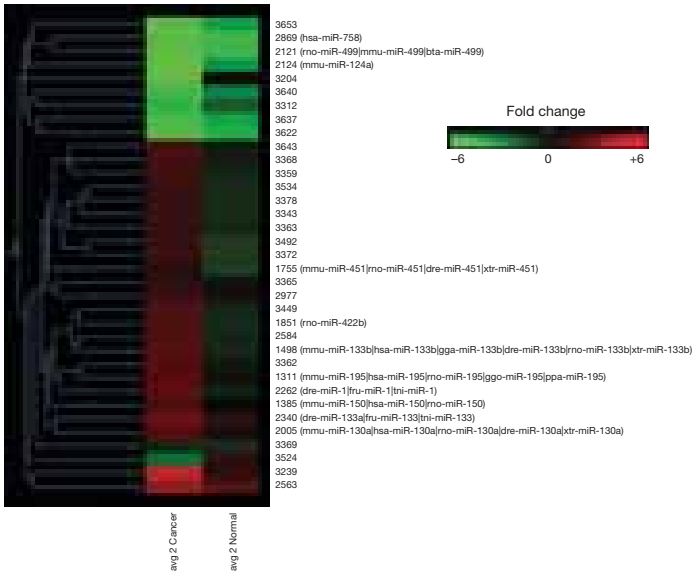


Figure 14.10. Novel putative miRNA content in adenocarcinoma. The NCode™ Human miRNA Microarray V3 was used to profile adenocarcinoma samples, and profileswere compared to matched normal tissue. Significant expression changes were seen within the novel putative miRNA content on the array, in addition to the expected known miRNAs from the Sanger miRBase.

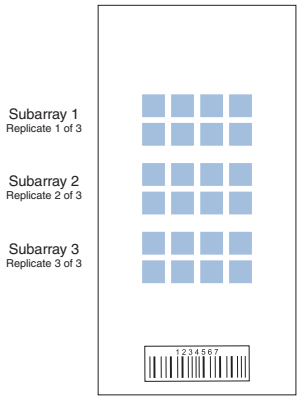


Figure 14.11. NCode™ miRNA microarray content. Each epoxide slide contains probes for profiling designated miRNA sequences, spotted in triplicate. NCode™ probes for the NCode™ miRNA Microarray Control are printed throughout the array to facilitate analysis, as are mismatch and shuffled controls for monitoring hybridization specificity.

Table 14.1. Number of probe sequences in the NCode™ miRNA microarrays.

Probe content	Human miRNA array V3 probes	Multi-species miRNA array V2 probes
Based on Sanger miRBase Sequence Database release	Version 10.0	Version 9.0
Human	710 plus 373 putative	553
Mouse		427
Rat		261
C. elegans		115
D. melanogaster		85
Zebrafish		371
Small nucleolar RNAs	29	10
Mismatch controls	76	76
NCode™ positive control	1 (72)*	1 (72)*
NCode™ dye normalization controls	5	5

* The NCode™ positive control is spotted throughout three subarrays (72 wells).

Product	Quantity	Cat. No.
NCode™ Human miRNA Microarray V3	5 arrays	MIRAH305
NCode™ Multi-Species miRNA Microarray V2	5 arrays	MIRA2-05
NCode™ Multi-Species miRNA Microarray Probe Set V2	500 pmol	MIRMPS2-01
NCode™ Multi-Species miRNA Microarray Control V2	10 µL	MIRAC2-01
NCode™ Human miRNA Microarray Probe Set V3	500 pmol	MIRHPS301

Probes

For researchers planning to spot their own miRNA microarrays, Invitrogen has probes from the NCode™ Human miRNA Microarray V3 and NCode™ Multi-Species miRNA Microarray V2 available in 384-well plates. Each well contains 500 pmol of lyophilized oligonucleotide probe, ready for resuspension and printing.

Simplified data analysis using the NCode™ Profiler

Use of Latin squares loop design/dye swap methodology. There are a number of methods used to analyze expression profiling microarray data, but some are not ideal for miRNA microarrays due to the reduced number of features present and the dynamic levels of miRNA expression across samples. We recommend using the Latin squares normalization and loop design/dye swap model described by Kerr et al. [3], because this method provides reliable statistical data with the smallest number of microarray chips. This experimental design generates a loop without identical chip replicates, though each tissue will be replicated twice, once with each Alexa Fluor® dye. Using this array layout enables comparison of each test sample with both the reference sample and comparison of each test sample with any other test sample. This normalization method attempts to reduce or eliminate any general miRNA effect, array effect, dye effect, overall tissue effect, array–miRNA interaction, and dye–miRNA interactions from the data (Figure 14.12).

Experimental design. NCode™ Profiler software enables simple design and analysis of miRNA microarray data using loop design and dye swap normalization methods that support sample-to-sample comparison within the experiment. They are designed with statistical considerations to reduce or eliminate any general miRNA effect, array effect, dye effect, overall tissue effect, array–miRNA interaction, and dye–miRNA interaction from the data.

Data analysis. miRNA researchers now have a tailored tool to identify differentially expressed miRNA markers on microarrays. NCode™ Profiler software is an advanced experimental design and analysis solution designed for two-dye expression profiling microarray experiments. Once an experiment has been conducted, the raw data can be imported and analyzed using the statistical methods designed into the array. For each tissue on the array, pairwise differential expression is determined and the test statistics, fold expression changes, and p-values are generated. Additionally, the ranking of each miRNA with respect to the other miRNAs in terms of overall expression level within the tissue is given. Using NCode™ Profiler software enables:

- → Simplified experimental design steps
- → Confidence interpreting results with proven statistical analysis using dye swap or loop design normalization models
- → Output includes test statistic, fold expression changes for each pair-wise comparison, and p-values and rankings of miRNA markers for all samples
- → Easy export of normalized data to visualization software for clustering (tree) and heat map analysis

Visit www.invitrogen.com/ncode to download a free copy. NCode™ Profiler software runs on Microsoft Windows® XP and Windows Vista® operating systems.

Figure 14.12. The NCode™ Profiler software for miRNA microarray data analysis has a simple user interface and provides both data analysis using Latin squares normalization and experimental design guidance.

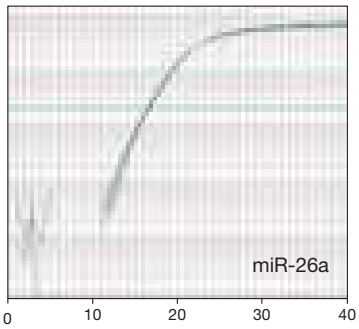
Robust data validation using the NCode™ miRNA qRT-PCR Kits

Quantitative RT-PCR (qRT-PCR) is the standard for validating microarray data and is an invaluable tool for highly sensitive and accurate profiling of miRNA population subsets. Most commercially available miRNA qRT-PCR systems use proprietary, predesigned, miRNA-specific primers for real-time PCR and/or reverse transcription. Unfortunately, such primers require that the miRNA sequence is publicly available, and that a commercial qRT-PCR assay has been developed for that specific sequence. This limits the availability of qRT-PCR assays for many model organisms, recently discovered miRNAs or non-coding RNAs, or proprietary miRNA sequences.

The NCode™ VILO™ miRNA cDNA Synthesis and NCode™ EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kits overcome these limitations by combining a carefully optimized combined polyadenylation and reverse transcription step using the SuperScript® VILO™ enzyme and a universal primer. The miRNA-specific amplification occurs during the PCR reaction, in which the sequence of the miRNA of interest is used as the target-specific PCR primer.

Use the NCode™ EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kits to:

- → Amplify using minimal total RNA input (no additional enrichment needed, conserve precious samples) (Figure 14.13)
- → Detect miRNA, ncRNA, or mRNA sequences from the same universal cDNA
- → Archive cDNA for later validation—the use of the cDNA for multiple experiments will enable more reliable comparison and allow future interrogation of new sequences
- → Obtain excellent sensitivity over a broad dynamic range of miRNA abundance
- → Profile closely related miRNAs with single-nucleotide discrimination
- → Use multiple real-time PCR instrument platforms, including standard and fast- cycling modes



MicroRNA	Total RNA	Enriched RNA
26a	16.53	16.53
195	17.19	17.28
1	24.97	25.36

Figure 14.13. miRNA detection from total RNA or enriched miRNA starting materials. cDNA was generated from brain reference RNA or an equivalent volume of enriched miRNA. Average C_t values for 10 ng/µL total RNA or equivalent per qPCR reaction are shown.

Match the NCode™ qPCR tool to your research needs

NCode™ EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kits—incorporate proven Invitrogen enzymes and the newest SYBR® GreenER™ dye specifically tailored for superior results on high-throughput, fast-cycling instruments. These kits provide:

- → Fast-activating, antibody-mediated Platinum® *Taq* DNA Polymerase in association with the brighter and less PCR-inhibitory SYBR® GreenER™ dye—complete, rapid activation that enables the best sensitivity and reproducibility
- → Flexible format with premixed or separate ROX—designed to work with default instrument protocols on a wide range of high-throughput fast instruments (e.g., AB 7500 FAST, AB 7900 HT Fast, AB StepOne™ System, Roche LightCyler® 480, Corbett Rotor-Gene™
- → Significantly reduced carryover contamination with UDG carryover protection—uracil-DNA-glycosylase (UDG)/dUTP incorporated in all kits

NCode™ VILO™ miRNA cDNA Synthesis Kit—provides reagents only for the first-strand cDNA reaction allowing you to use your own optimized qPCR supermix. Tried and tested SuperScript® III Reverse Transcriptase is formulated in an enhanced buffer system to provide you with the most reliable first-strand synthesis and higher cDNA yields.

Product	Quantity	Cat. No.
NCode™ VILO™ miRNA cDNA Synthesis Kit	50 rxns	A11193050
NCode™ EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kit With Premixed ROX	200 rxns	A11193052
NCode™ EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kit Universal	200 rxns	A11193051

NCode™ Non-coding RNA Arrays

Accelerate discovery with the first commercially available microarrays for profiling long non-coding RNA (ncRNA)

NCode™ Human and Mouse Non-coding RNA Microarrays are first-generation high-density arrays designed to profile longer ncRNA (>200 bases) and to analyze mRNA simultaneously on the same array. In addition to the ncRNA content, probes targeting mRNA allow discovery of coordinated expression with associated protein-coding genes.

NCode™ Long Non-coding RNA Database

The NCode™ Long ncRNA Database contains information relating to the long ncRNA and mRNA sequences profiled by the NCode™ Human and Mouse Non-coding RNA Arrays (Figure 14.14). The 17,000+ long (>200 bases) ncRNAs from human and 10,000+ ncRNAs from mouse are predominantly derived from published sources. Search probe-specific information for the ncRNA Microarray by selecting species and the Search, Browse, or BLAST functions.

Figure 14.14. The NCode™ Long Non-coding RNA Database.

Profile ncRNA with your existing expression array workflow

→ Using the NCode™ microarrays, you can:

→ Profile more than 17,000 long (>200 bases) ncRNAs from human and more than 10,000 ncRNAs from mouse, derived from published sources* (Table 14.2)

→ Correlate ncRNA expression with mRNA expression for known pathways, with simultaneous profiling of coding and non-coding RNAs (Figure 14.15)

→ Perform ncRNA research using standard expression array labeling technologies and instrumentation–including the Agilent® Technologies system

The NCode™ Human and Mouse Non-coding RNA Microarrays are available in a duplex array format in two sizes (1 slide and 5 slides).

Table 14.2. Each slide contains non-coding RNA and mRNA features and controls for monitoring experimental success.

Format	Human ncRNA Array	Mouse ncRNA Array
Total features	22,074 coding featues 17,112 non-coding features*	25,179 coding featues 10,802 non-coding features*
Control features	1,325 on each array	1,325 on each array

*The ncRNA probes predominantly target full-length cDNAs cataloged from published studies. Specifically, the ncRNA content has been developed from the following sources: manual curation from literature, Functional Annotation of Mouse (FANTOM3) project, Human Full-length cDNA Annotation Invitational (H-Invitational) project, antisense ncRNAs from cDNA and EST database for mouse and human using a computation pipeline [4], human snoRNAs and scaRNAs derived from snoRNA-LBME-db, RNAz [5], Non-coding RNA Search [6], and EvoFold [6].

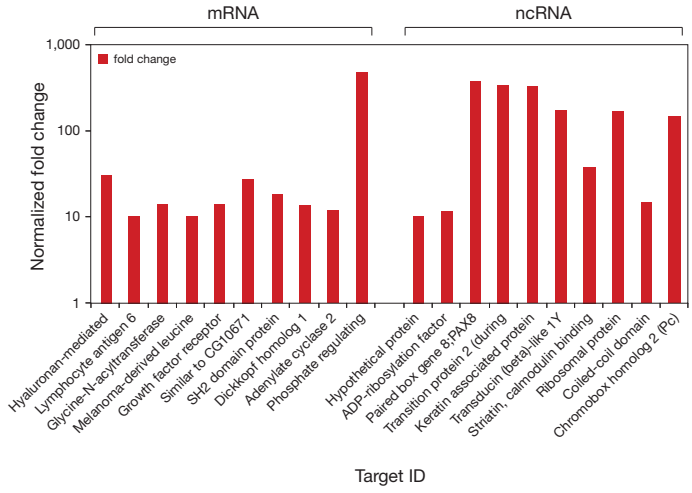


Figure 14.15. NCode™ Human Non-coding RNA Array permits simultaneous detection of mRNA and ncRNA expression in biological samples. The histogram describes the observed fold change of both mRNA and ncRNA transcripts in breast cancer tumor samples vs. adjacent normal tissues in a single patient. Fold change from expression levels from normal tissue were calculated for known breast cancer associated transcripts (both coding and non-coding) derived from the tumorgene.org database. Non-coding sequences were identified by an algorithm that scores various characteristics of protein-coding genes including open reading frame length, synonymous/nonsynonymous base substitution rates, and similarity to known proteins [1]. Along with putative long ncRNAs, the array also targets characterized ncRNAs, such as Xist, Air, PINC, Neat1, and Neat2. Although the array targets some miRNA precursors, it is not designed to detect miRNAs. Refer to the NCode™ miRNA products for tools optimized for miRNA labeling, arrays, and qRT-PCR.

Product	Quantity	Cat. No.
NCode™ Human Non-coding RNA Microarray	1 slide	NCRAH-02
NCode™ Human Non-coding RNA Microarray	5 slides	NCRAH-10
NCode™ Mouse Non-coding RNA Microarray	1 slide	NCRAM-02
NCode™ Mouse Non-coding RNA Microarray	5 slides	NCRAM-10

Genome-wide analysis of long non-coding RNA (ncRNA) expression in solid tumors using the NCode™ Non-coding RNA Microarray platform

Numerous studies have demonstrated that in higher organisms, most of the nonrepetitive genome is transcribed in a developmentally regulated fashion. Interestingly, only a small percentage (<20%) of these transcripts are associated with genes that encode proteins [1]. Non-coding transcripts come in a wide range of sizes (18 nt to 100 kb), and, in stark contrast to what is known of protein-coding transcripts, very few non-coding transcripts have experimentally derived functions. Recent evidence suggests that thousands of long non-coding RNAs are expressed whose functions have yet to be studied but which appear to play important roles in gene regulation. Microarray studies have demonstrated that a majority of the mammalian genome is transcribed [8], often on both strands, to produce overlapping and interlacing transcripts, many of which are cell- and tissue-specific. While most are as yet unstudied, increasing evidence suggests these non-coding transcripts affect many of the developmental and regulatory processes [9] that are critical in cancer research investigations. More recent data on large intervening non-coding RNAs, or lincRNAs [10], further define a group of functional ncRNAs that are conserved and implicated in diverse biological processes. Here we describe the utility of the NCode™ Non-coding RNA Microarray platform for simultaneously profiling coding and non-coding transcripts from three types of solid tumors compared to adjacent normal tissue.

New microarrays to examine ncRNAs

With the maturation of next-generation sequencing technologies, more ncRNA transcripts, especially the longer classes, are becoming the subject of gene expression research. In 2008, two novel microarrays were launched to assist researchers in the study of ncRNA biology: the NCode™ Human and Mouse Non-coding RNA Microarrays. These two high-density arrays contain content developed by John Mattick (University of Queensland, Australia), using algorithms that identified non-coding transcripts present in public cloning and sequencing databases like FANTOM3. The human array contains probes targeting 17,000 unique non-coding transcripts from various databases and 22,000 unique protein-coding transcripts from the RefSeq collection, printed in duplicate along with thousands of standard positive and negative controls. The mouse array contains features for 10,800 unique non-coding transcripts and 23,000 unique protein-coding transcripts from the RefSeq collection, also printed in duplicate with the standard positive and negative controls (Figure 14.16).

NCode™ Human and Mouse ncRNA Microarrays

Data presented here were obtained using the Invitrogen NCode™ Human Non-coding RNA Microarray platform (Cat. No. NCRAH10). This high-density microarray is the first commercially available tool capable of simultaneously profiling both long non-coding RNA (ncRNA) expression and messenger RNA (mRNA) expression from a single sample (Table 14.3). The NCode™ Human Non-coding RNA Microarray is manufactured with Agilent SurePrint® technology, and is printed in a 2 x 105K format, which allows for two individual sample hybridizations per slide. Non-coding sequences were identified by a proprietary algorithm that scores various characteristics of protein-coding genes, including open reading frame length, synonymous/nonsynonymous base substitution rates, and similarity to known proteins [1]. Characterized ncRNAs manually curated from literature as well as from public database repositories are also included. In addition to the ncRNA content, probes targeting mRNA content from the RefSeq database and other collections are also included, allowing discovery of coordinated expression with associated protein-coding genes.

Data analysis

The microarrays were scanned with an Agilent DNA Microarray Scanner, and Feature Extraction Software was used to generate raw data files. Raw data were uploaded into GeneSpring GX 7.3.1 Software (Agilent) for normalization and analysis. Normalization was performed using the default parameters for Agilent one-color array data. Expression levels of differentially expressed transcripts, both coding and non-coding, were validated using the EXPRESS SYBR® GreenER™ qPCR SuperMix (Invitrogen Cat. No. 11784-200). Threshold cycle (C_t) values were normalized to the GAPDH housekeeping gene.

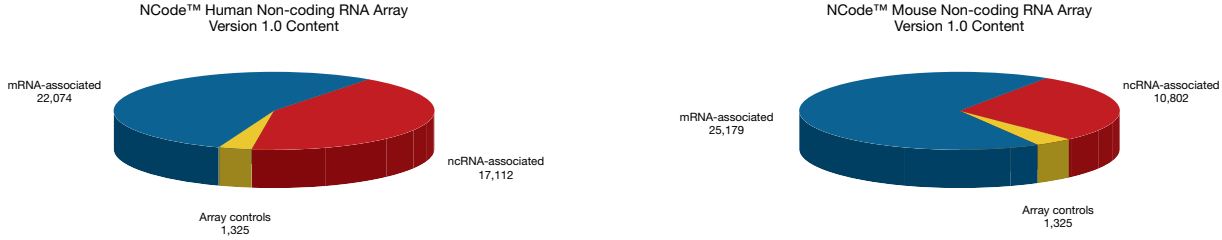


Figure 14.16. Charts representing content of the Human and Mouse Non-coding RNA Microarrays, highlighting the ncRNA and mRNA percentages.

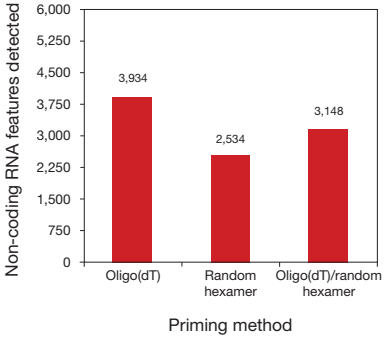
Table 14.3. Non-coding and coding-associated transcripts targeted in the NCode™ microarrays.

	Number of unique sequences (60-mer, printed in duplicate)	Number of putative long non-coding RNAs (>200 nt) targeted	Number of coding-associated transcripts targeted
NCode™ Human Non-coding RNA Microarray*	39,186	17,112	22,074
NCode™ Mouse Non-coding RNA Microarray*	35,981	10,802	25,179

* 1,325 Agilent positive control features are also included in each array, and are used for generating automated QC reports.

Evaluation of labeling strategies for NCode™ ncRNA array analysis

As a validation of the recommended RNA labeling approaches, we compared RNA expression profiles from a lung tumor sample labeled using cDNA synthesis primed with either an oligo(dT) primer, similar to the primer used in the SuperScript® Indirect RNA labeling method, or random primers, or a combination of both. This allowed us to examine the efficacy of using a poly(A) tail–targeted priming method to analyze ncRNA transcripts, many of which are thought not to be poly(A)-tailed. As can be seen in Figure 14.17, more ncRNA features were detected with oligo(dT) priming than with random priming or with random priming in combination with oligo(dT) priming. Additionally, our comparison revealed very high Pearson correlations (r^2 = 0.87 and 0.90, respectively) between poly(A)-primed samples and randomly primed, or randomly primed in addition to oligo(dT)-primed samples. These data indicate that although many of these transcripts may not have poly(A) tails, the majority are still detectable by poly(A)-specific priming due to internal poly(A) sequence stretches. This is not surprising given that the non-coding content was mined from public expression library databases that were generated using oligo(dT)-primed cDNA synthesis methods. We also examined the normalized signal intensities of a subset of transcripts (Figure 14.18) and observed that both the oligo(dT)-primed cDNA probes and oligo(dT)-primed plus randomly primed cDNA probes provided similar signal intensities.



	Oligo(dT)	Random hexamer	Oligo(dT)/ random hexamer
Oligo(dT)	1.00		
Random hexamer	0.87	1.00	
Oligo(dT)/ random hexamer	0.90	0.94	1.00

Figure 14.17. Comparison of RNA labeling strategies. The histogram shows the overall number of transcripts detected using cDNA-labeled probes generated with oligo(dT)-primed vs. randomly primed cDNA templates. Additionally, the table shows the Pearson correlation between data sets from each priming method.

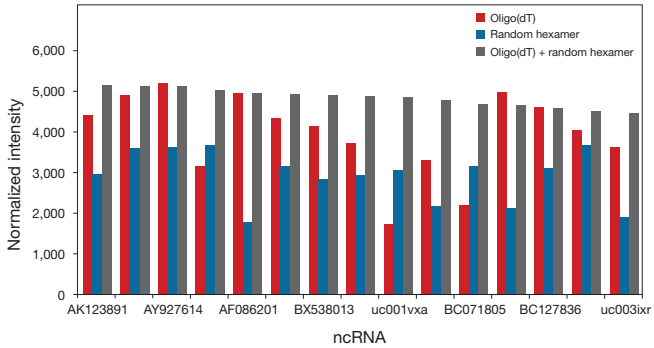


Figure 14.18. Relative signal intensities of different cRNA labeling strategies. The histograms above show the relative signal intensities of a number of ncRNA transcripts, detected as significantly above background, with each priming method.

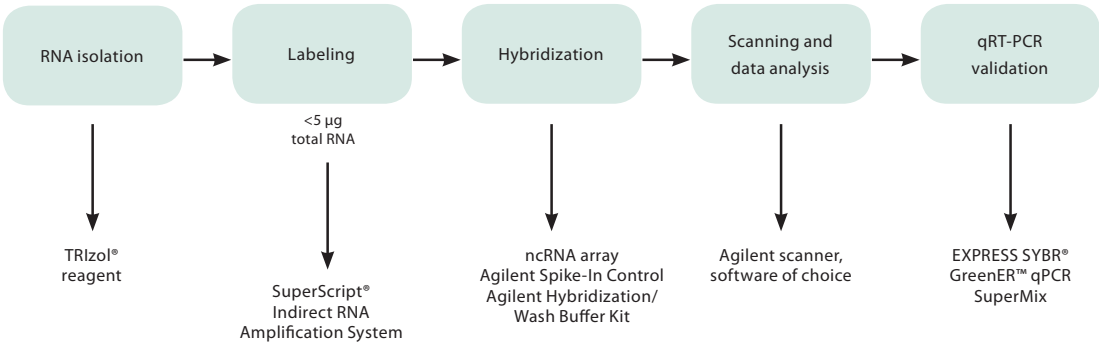


Figure 14.19. Recommended workflow from RNA isolation to qRT-PCR validation of microarray data.

Figure 14.19 shows the generalized workflow from RNA isolation to results validation. cDNA probes were generated using the SuperScript® Indirect RNA Amplification System (Invitrogen Cat. No. L1016-02) according to the manufacturer's protocol, using 1 µg of total RNA in each labeling reaction. Three matched pairs of human primary tumors and adjacent normal tissue were profiled. Total RNA isolated from breast, colon, and lung tissue (obtained from BioChain, Hayward, CA) was extracted with TRIzol® reagent (Invitrogen Cat. No. 15596026). A 1,000-fold dilution of Agilent One-Color RNA Spike-In Mix (containing exogenous mRNA transcripts that are specific to the Agilent control features found on the Agilent array) was added to 1 µg of total RNA sample in each tube. The RNA samples were amplified and labeled using the SuperScript® Indirect RNA Amplification System following the suggested protocol. The resulting aminoallyl cRNA was purified and indirectly labeled with Alexa Fluor® 555 reactive dye (Invitrogen Cat. No. A32756) using the supplied protocol. The labeled cRNA was quantitated using a NanoDrop instrument. Additional RNA from each sample was labeled using the SuperScript® Indirect cDNA labeling method, primed with either oligo(dT) or random primers. The Gene Expression Hybridization Kit (Agilent) was used to prepare 1.5 µg of labeled cRNA following the suggested protocol. Hybridization buffer (2X) was added to the fragmented samples, and carefully loaded onto the array using Hybridization Gaskets (Agilent Part Number G2534-60002) and SureHyb chambers (both from Agilent). After an overnight hybridization at 65°C, the arrays were processed using Agilent wash buffers.

Profiling mRNA and ncRNA expression in solid tumors with the SuperScript® Indirect RNA Amplification System

All of the primary solid tumor comparisons yielded specific relative expression changes (compared to adjacent normal tissue) that can be associated with tumorigenesis, as well as distinctly tissue-specific expression profiles not associated with disease state but rather tissue type. Solid tumor–specific transcripts, both coding and non-coding, were detected from many genomic loci in both the sense and anti-sense orientations (Figure 14.20). The annotation files supplied with the microarray allowed complex genomic location analysis of specific chromosomal locations that could be associated with relative changes in expression.

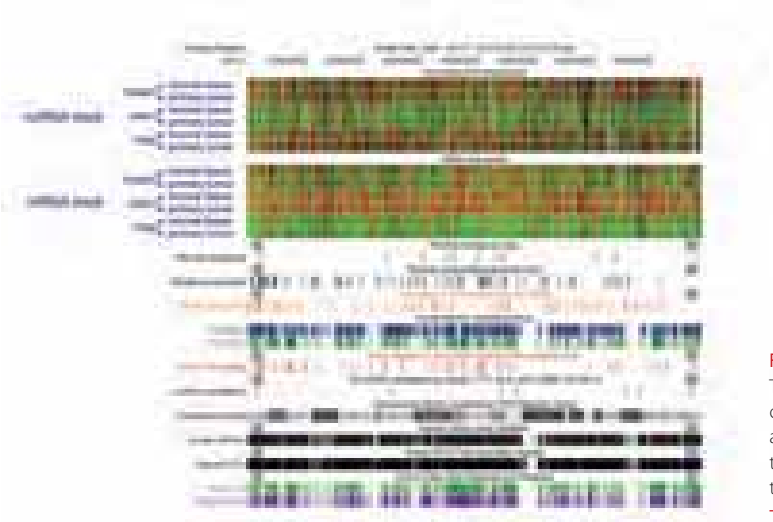


Figure 14.20. Profiling mRNA and ncRNA expression in three tumor types. The heat maps and genomic maps depict changes and location of both coding and non-coding transcription on chromosome 17 associated with each tissue and tumor type. The specific genome tracts displayed compare publicly available transcriptome annotations to our observed expression profiles.

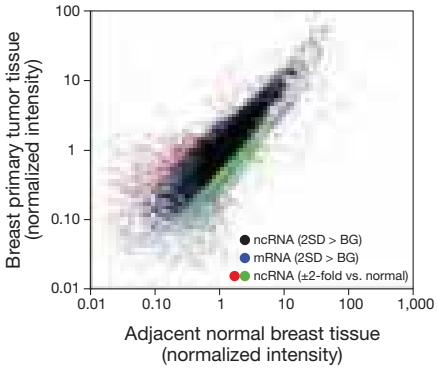


Figure 14.21. Differentially expressed transcripts in breast tumors. The scatter plot above depicts (1) ncRNA expression within at least two standard deviations above background in primary breast tumor and/or adjacent normal breast tissue (black), (2) mRNA expression within at least two standard deviations above background in primary breast tumor and/or adjacent normal breast tissue (blue), and (3) changes in ncRNA expression greater than 2-fold up-regulated (red) and down-regulated (green). This genome browser view represents the relative change and genomic location for the Her2/neu gene and surrounding areas.

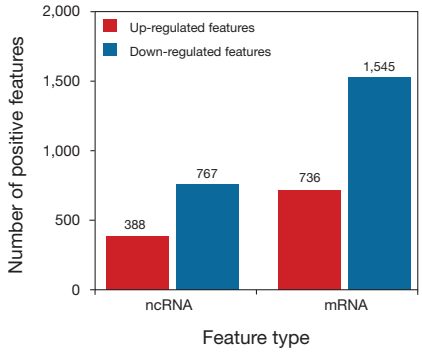


Figure 14.22. Differentially expressed transcripts in breast tumors. The histogram depicts the coding and non-coding transcripts exhibiting >2-fold up- or down-regulation in breast tumor vs. normal breast tissue.

To gain a more detailed picture of global changes in gene expression associated with primary tumors, efforts were focused on gene expression profiles in primary breast tumor tissue compared to adjacent normal tissue. The scatter plot in Figure 14.21 shows global expression of ncRNAs and mRNAs in breast tumor compared with adjacent normal breast epithelial cells. As can be seen from this graph, the majority of both coding and non-coding transcripts show equivalent levels of expression between the two samples; however, there is clear evidence of differential expression for both types of transcripts, both up- and down-regulated, in the tumor sample. Overall, 1,155 non-coding transcripts and 2,281 mRNA transcripts showed statistically significant differences in expression levels in the breast tumor sample (Figure 14.22).

Furthermore, differential expression of a known breast cancer–specific mRNA marker, HER2, was seen. Specifically, a 3-fold up-regulation of HER2 transcript in the tumor samples was observed, which is consistent with the previously reported observation that HER2 is up-regulated in ~15–20% of breast cancer types [8]. Interestingly, we also detected significant changes in a previously unidentified ncRNA transcript in the vicinity of the HER2 gene (array probe ID: IVGNh21558) (Figure 14.23).

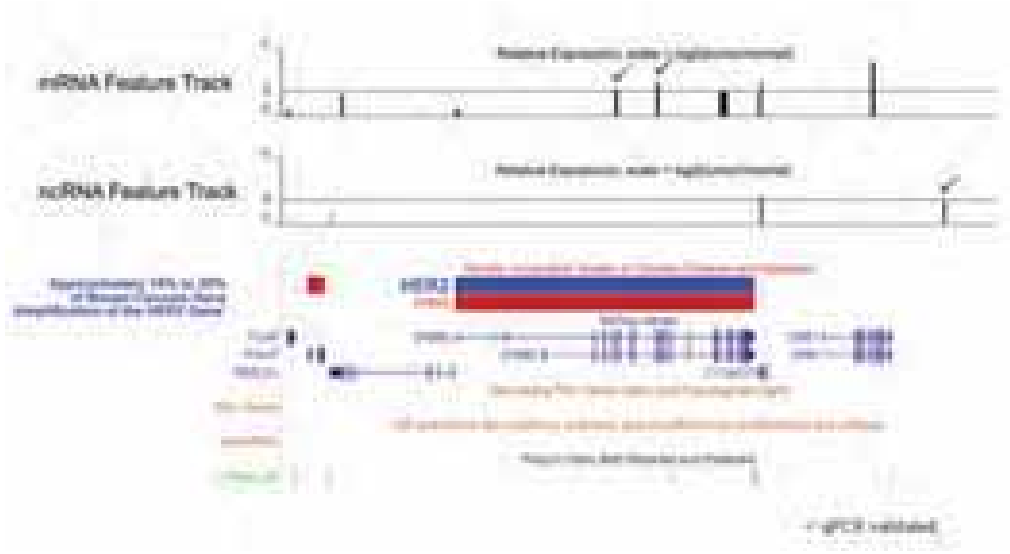


Figure 14.23. Differentially expressed genes in the vicinity of HER2. This figure depicts the relative expression and genomic location for the HER2 gene and surrounding areas. The levels of up-regulation of HER2 expression and the adjacent ncRNA transcript are shown.

As shown in Figures 14.24 and 14.25, differentially expressed transcripts, both coding and non-coding, were validated using the EXPRESS SYBR® GreenER™ qPCR SuperMix (Invitrogen Cat. No. 11784-200). Most importantly, differential expression of the HER2 gene and the adjacent ncRNA transcript was confirmed to be up-regulated in breast tumors.

Conclusions

The importance of non-coding RNA (ncRNA) in cellular functions, development, and disease has only been recently appreciated, but is now widely accepted. One of the critical issues hindering the elucidation of ncRNA function is the lack of research tools to study them. Here we describe the use of a high-density microarray to profile the expression patterns of 17,000 ncRNAs in humans, which should help expedite this functional investigation. In addition, this array contains features for 22,000 mRNAs, allowing researchers to simultaneously profile both coding and non-coding RNA expression in their model systems.

We describe the profiling of three solid tumors from three separate patients and show the power of this array for identifying differentially expressed transcripts, both mRNAs and ncRNAs associated with these cancers. In one primary breast tumor, we confirm the overexpression of the HER2 gene, which has been described previously. Interestingly, we also identify a novel ncRNA transcript downstream of the HER2 gene that is also up-regulated in this breast tumor. Although the function of this previously unknown ncRNA transcript and its relatedness to HER2 expression and function are not known, researchers using standard mRNA expression arrays would not have been able to even discover this transcript. This demonstrates the power of this novel array product. Subsequent steps in this workflow might include the examination of function by up- and down-regulation of specific sequences to study endogenous or reporter gene regulation and phenotypic response.

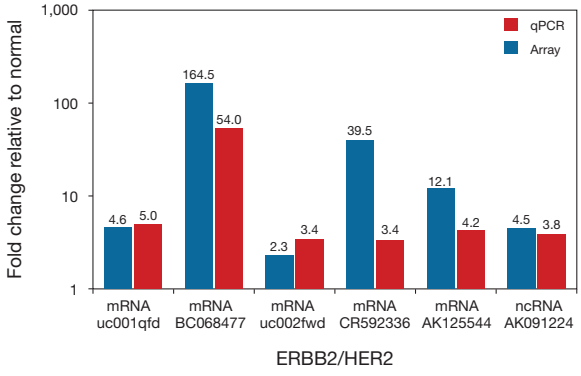


Figure 14.24. NCode™ qRT-PCR validation of NCode™ ncRNA array results. The figure above shows the relative change (compared to the donor's normal breast tissue) of mRNA and ncRNA targets, measured by qRT-PCR and microarray.

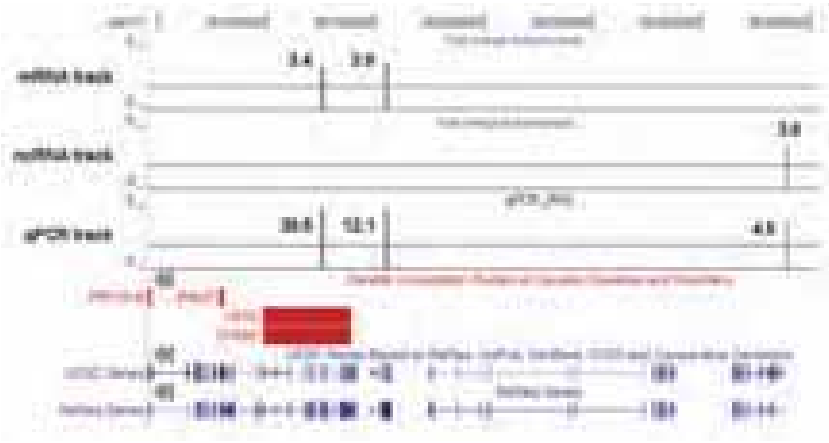


Figure 14.25. NCode™ qRT-PCR validation of HER2/neu and adjacent ncRNA. The figure above shows the relative expression of HER2/neu mRNA and adjacent ncRNA as measured by qRT-PCR and microarray.

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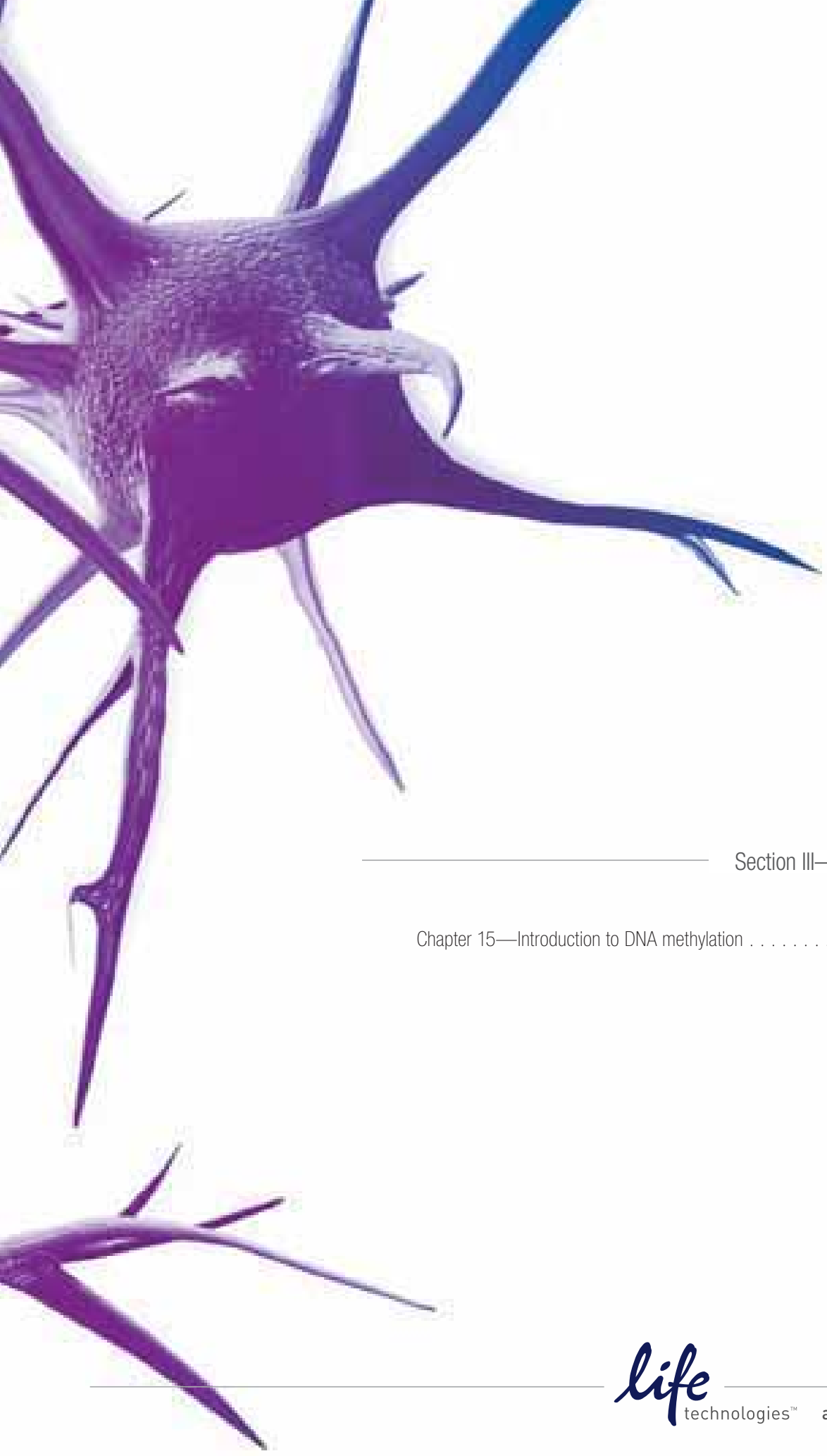

Section III—miRNA Profiling

Chapter 15—Introduction to DNA methylation 134

CHAPTER 15

Introduction to DNA methylation

Overview of DNA methylation

DNA methylation is a DNA modification that occurs normally in both eukaryotic and prokaryotic organisms. In many plants and animals, it is characterized by the biochemical addition of a methyl group (–CH3) to the cytosine C5 in cytosine-phosphate-guanine (CpG) dinucleotides via a methyltransferase enzyme [1]. In plants, the cytosine can be methylated in the CpG, CpNpG, and CpNpN contexts, where N represents any base except guanine (Figure 15.1). Although CpG dinucleotides occur rather infrequently in mammalian genomes (approximately one-fourth the expected frequency), DNA segments abundant with CpG dinucleotides do exist. Called CpG islands, these segments are typically 100–500 base pairs long and often correspond to transcription start sites and adjacent exons. CpG dinucleotides occurring within promoters or first exons are much less likely to be methylated compared to those appearing elsewhere. DNA methylation studies have gained prominence, in part, because aberrant hypermethylation of CpG islands can occur and has been linked to changes in transcription and gene expression. These events have been shown to play a central role in cancer, gene imprinting, embryonic development, X-chromosome gene silencing, cell cycle regulation, and many other biological functions [2,3,4]. For more information, visit the epigenetics learning center on the Invitrogen website at www.invitrogen.com/epigenetics.

DNA methylation analysis

Methods for DNA methylation analysis can be generally categorized as either global or locus-specific. Global methylation analysis measures the overall level of methylated cytosines in a sample. Locus-specific methylation analysis encompasses a large number of techniques that determine specific methylation patterns at a single location (within a promoter region, for example). Genome-wide methylation analysis techniques include some microarray methods, bisulfite sequencing, antibody-based detection such as MeDIP, and DNA methyltransferase assays. The most common locus-specific methylation technique is bisulfite conversion followed by a variety of PCR methods or bisulfite sequencing of PCR fragments. Other techniques include enzymatic digestion followed by Southern blot analysis or PCR. Mass spectrometry has also been used for DNA methylation analysis to determine specific methylation patterns. As the next-generation sequencing technology matures, new methods for locus-specific methylation detection are eagerly anticipated.

Methylated DNA enrichment

Highly sensitive enrichment of methylated DNA

MethylMiner™ Methylated DNA Enrichment Kit enables superior enrichment and differential fractionation of double-stranded DNA based on CpG methylation density, with increased sensitivity over antibody-based methods (Figure 15.2). Fractionation permits important comparisons between samples and enables researchers to focus analysis on only the methylation densities of interest.

Advantages of MethylMiner™ Methylated DNA Enrichment Kit include:

- → Partitioning with high-affinity binding—at least 4-fold greater sensitivity than antibody-based methods (Figure 15.3)
- → Fractionation based on CpG methylation density—dsDNA capture is achieved with MBD2 protein and facilitates ligation of double-stranded adaptors for next-generation sequencing
- → Rapid and easy elution with salt eliminates the need for proteinase K treatment and phenol/chloroform extraction
- → Precise answers—fractionated DNA permits distinctions to be made regarding methylation status and density
- → Fast protocol—completed in less than 4 hours
- → Easy—simple handling with Dynabeads® magnetic beads—the gold standard in magnetic beads

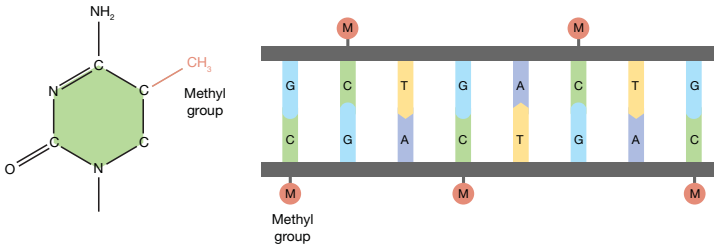

Figure 15.1. DNA methylation. A methyl group addition to the cytosine carbon 5 in cytosine-phosphate-guanine (CpG) and other nucleotide sequences inhibits the binding of transcription factors to promoters.

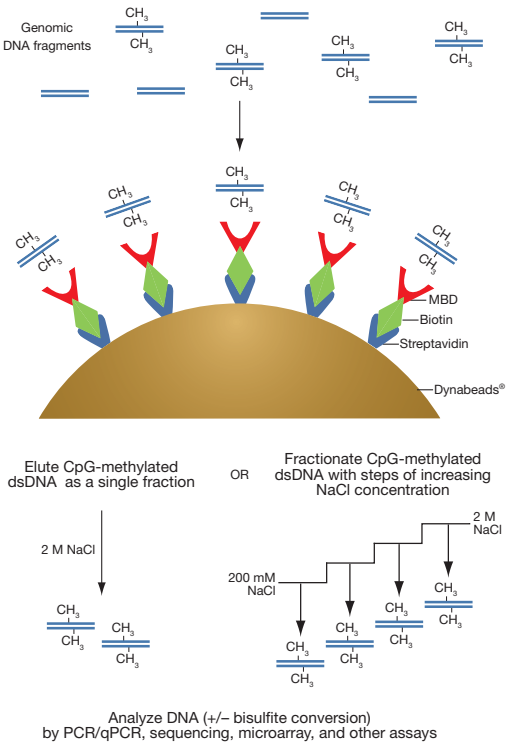

Figure 15.2. MBD-biotin–based CPG methylated DNA affinity capture. The MethylMiner™ Methylated DNA Enrichment Kit allows for a diversity of elution strategies, depending on you preferred workflow and downstream application. It supports: 1) a single elution using undiluted high-salt elution buffer; 2) a single elution using proteinase K ; 3) a series of stepwise elutions with buffers containing successively greater NaCl concentrations; or 4) stepwise fractional elutions followed by a final proteinase K treatment. This method overcomes challenges associated with denatured DNA using antibody-based methods. The dsDNA is more compatible with downstream epigenetic analysis applications—including next-generation sequencing.

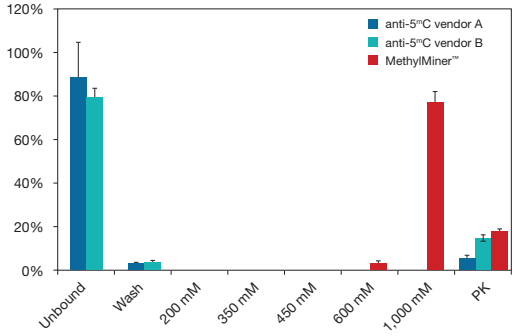

Figure 15.3. MethylMiner™ Methylated DNA Enrichment Kit captures more heavily methylated DNA compared to an antibody-based method. Relative capture and recovery of qPCR amplicon 1 DNA by MethylMiner™ Methylated DNA Enrichment Kit and anti-5^{m}C from two different vendors. In parallel, equal moles of anti-5^{m}C antibody molecules were coupled to M-280 Protein G Dynabeads® and MethylMiner™ MBD-biotin molecules were coupled to M-280 Streptavidin Dynabeads® magnetic beads. After coupling, triplicate aliquots of 10 µL of antibody-beads were incubated with 0.5 µg of fragmented, heat-denatured MCF-7 ssDNA and triplicate aliquots of 10 µL of MethylMiner™ beads were incubated with 0.5 µg of fragmented, nondenatured dsDNA. After mixing at 22°C for 1 hour, the supernatants were recovered, the beads were washed and then serially eluted with buffers of increasing NaCl concentration. As a final step, all beads were treated with 20 µg of proteinase K at 56°C for two hours. The proteinase K digest supernatants were collected and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). All fractions were ethanol-precipitated, redissolved in water, and assayed with amplicon 1 specific primers by qPCR using a SYBR® GreenER™ master mix. Recovery of amplifiable DNA was measured relative to a standard curve of input fragmented genomic DNA. As shown, ~100% of the heavily methylated DNA sequence was captured by the MethylMiner™ beads and ~80% could be eluted with 1 M NaCl. In contrast, the anti-5^{m}C antibody from two different vendors could only capture ~10–20% of this heavily methylated DNA and elution could only be achieved with proteinase K treatment.

Product	Quantity	Cat. No.
MethylMiner™ Methylated DNA Enrichment Kit	1 kit	ME10025

Enrichment of differentially methylated regions with MethylMiner™ kit fractionation and deep sequencing with the SOLiD™ System

Introduction

DNA methylation is an important epigenetic modification involved in the remodeling of chromatin structure and, ultimately, the control of gene expression. Proper control of DNA methylation patterns is essential for normal embryonic development and tissue differentiation [5], X-chromosome inactivation [6], and gene imprinting [7]. Aberrant methylation has been linked to many diseases, including cancers [8]. The modified base 5-methylcytosine constitutes about 1% of all DNA bases in mammalian genomes. Although the presence of 5-hydroxymethylcytosine in brain [8] and cytosine methylation at non-CpGs, namely CpNpG in early embryogenesis, has been reported [10], mammalian DNA methylation is found almost exclusively in the symmetrical CpG dinucleotide. While DNA methylation in CpG-rich promoter regions correlates with transcriptional silencing, it is becoming clear that CpG methylation in regions of lower CpG density and distal to promoters also correlate with gene expression [11,12]. Thus, it would be quite useful to effectively partition densely methylated regions from moderately methylated regions to better understand the physiological role of this DNA chemical modification

To fully understand the role of DNA methylation in normal and disease states, it is important to first determine genomic methylation patterns. Methods of characterizing methylation are generally based upon one of three techniques: bisulfite conversion, digestion with methylation-sensitive restriction enzymes, and antibody- or 5-methylcytosine binding protein–based purification of methylated DNA [13]. Next-generation sequencing platforms enable ultrahigh-throughput sequencing, mapping, and counting of short DNA reads (tags) and, in combination with any of the above methylation profiling strategies, can be used for comprehensive, genome-wide mapping of methylation sites.

Methylated cytosines constitute a small percentage of all bases in mammalian genomes. In the human genome, for example, only about 1% of the bases are 5-methylcytosine. It is in many cases neither cost-effective nor necessary to sequence the entire genome with deep coverage in order to interrogate the genomic methylation patterns. Thus, sensitive, accurate, and versatile tools for the enrichment of methylated sequences from the genome are highly useful for genome-wide studies of methylation patterns.

Different methods for selective enrichment of methylated genomic DNA fragments are available. Those based upon antibodies are termed methylated DNA immunoprecipitation (MeDIP) [14]. Other methods use proteins that naturally recognize methylated cytosines in double-stranded DNA. In the case of the MethylMiner™ system, the capture medium is the methyl-CpG binding domain (MBD) of the human MBD2 protein coupled to superparamagnetic Dynabeads® M-280 Streptavidin via a biotin linker. Here we describe a genome-scale study of the patterns of DNA methylation in the MCF-7 breast cancer cell line, using enrichment with the MethylMiner™ system on the SOLiD™ sequencing system.

Methods

Comparison of methylated DNA enrichment using the MBD-based MethylMiner™ kit to MeDIP-based enrichment identified numerous workflow advantages to using the MethylMiner™ system (Figure 15.4). First, with antibody-based enrichment, the fragmented DNA must be denatured and kept single-stranded during the binding step for efficient capture. Denaturation of the DNA is necessary because all of the validated antibodies have been selected to bind a fully accessible methylated cytosine ($5^{m}C$) and cannot bind $5^{m}C$ occurring naturally in double-stranded DNA. This constraint can reduce binding efficiency, as fragments can reanneal during the binding reaction. To compensate for this, antibody–DNA binding is often performed for several hours to overnight at 4°C. After washing the beads, the DNA can only be recovered after digestion with proteinase K, usually with heat, which necessitates further clean-up, such as phenol/chloroform extraction followed by precipitation with ethanol. In contrast, the MethylMiner™ system is fast, convenient, and more versatile. The DNA remains double-stranded during the binding step, which only takes 1 hour at room temperature (longer times at 4°C can also be used). After incubation, the MethylMiner™ beads are washed and the captured methylated DNA eluted. Following adapter ligation, the eluted double-stranded DNA fragments are ready for next-generation sequencing, which provide a significant advantage over single-stranded fragments produced in MeDIP-based enrichment. The system uses Dynal® superparamagnetic beads, which provide high-quality and efficient capture kinetics as well as rapid and convenient magnet-based bead capture for washing and changing solutions. Batch elution is easily achieved by simply mixing the beads with a high-salt (2 M NaCl) buffer [15]. Furthermore, since the affinity of MBD2 for methylated DNA can be modulated by ionic strength, fractionation of the captured DNA based on its degree of methylation can be performed with graded changes in ionic strength. Varying degrees of CpG methylation density can influence gene regulation so the ability to fractionate the genome according to the degree of methylation is a bonus for functional studies. Finally, only ethanol precipitation is needed to obtain concentrated and purified double-stranded methylated DNA that is ready for further analysis, such as by PCR methods or microarray or high-throughput sequencing (Figure 15.4).

Results

Human DNA from the breast cancer–derived cell line MCF-7 was used to map the methylation status of CpG sites on a genome scale. Briefly, 50 µg of genomic DNA was sheared using a Covaris™ S2 non-contact sonicator (SOLiD™ 3 fragment library protocol) to generate short random DNA fragments with a median size of ~150 bp (Figure 15.5A, 15.5B). Fragmented DNA was then subjected to methylated DNA enrichment according to the protocol supplied with the MethylMiner™ Methylated DNA Enrichment Kit (Cat. No. ME10025), and two methylated fractions (500 mM and 1 M NaCl eluates) were isolated [15]. Figure 15.5C shows

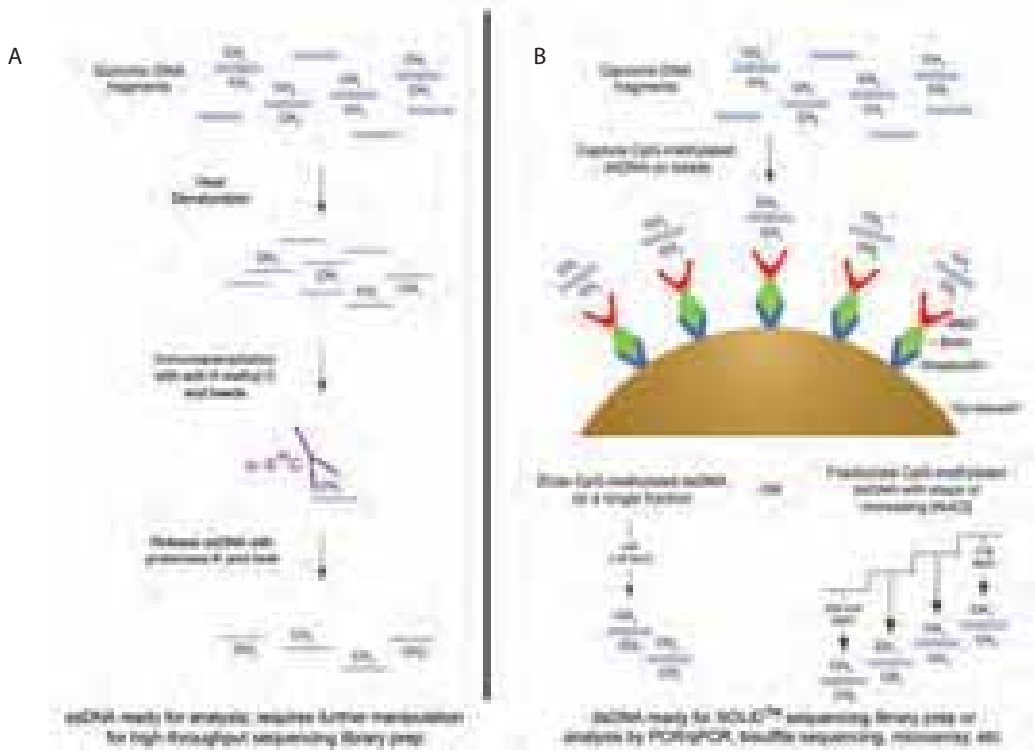

Figure 15.4. Comparison of MethylMiner™ and antibody-based MeDIP workflows. (**A**) Workflow for antibody-based MeDIP. (**B**) Workflows for MethylMiner™ MBD bead–based enrichment of CpG-methylated DNA

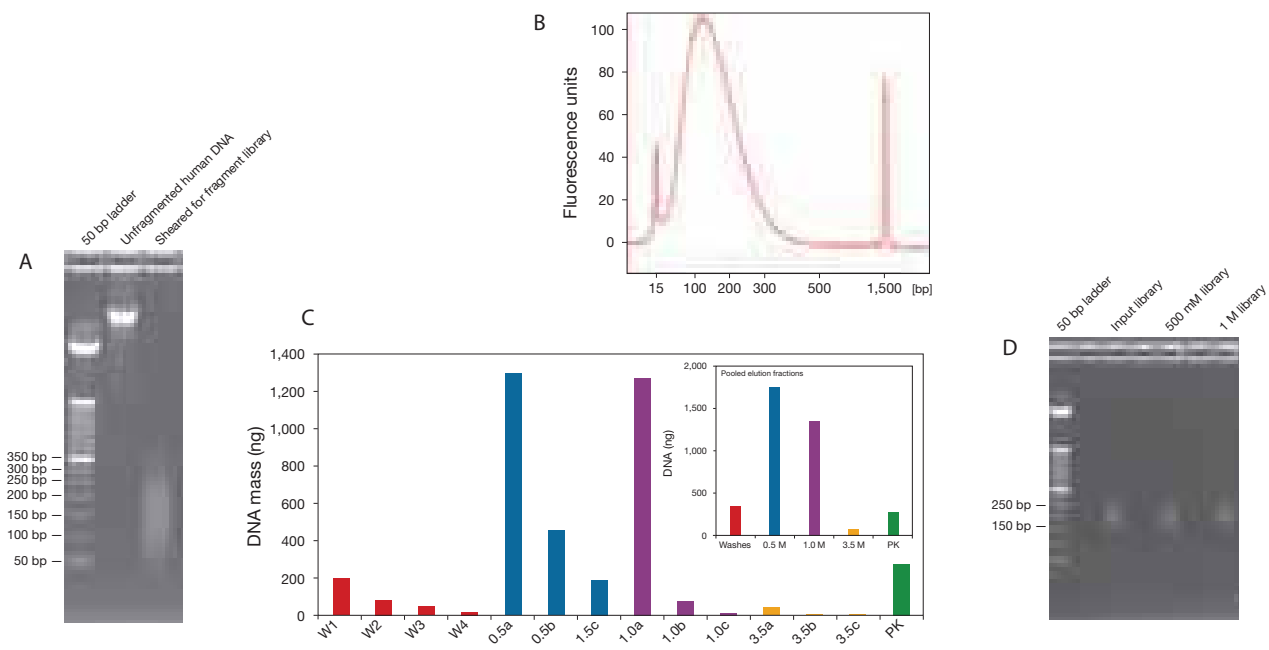

Figure 15.5. SOLiD™ 3 fragment library preparation after enrichment and elution using the MethylMiner™ kit. (**A**) Agarose gel and (**B**) 2100 Bioanalyzer trace of sheared human genomic DNA subjected to MethylMiner™ enrichment. (**C**) Recovery of DNA at different stages of MethylMiner™ enrichment. W1–W4 are successive wash fractions. 0.5a–c are successive elutions with buffer containing 500 mM NaCl; likewise, 1.0a–c and 3.5a–c are successive elutions with buffer containing 1 M and 3.5 M NaCl, and PK refers to residual DNA recovered with a final proteinase K treatment. The inset shows the mass of DNA pooled from these iterations. (**D**) Gel of library DNA.

the typical elution profile of DNA from the MethylMiner™ beads following serial salt elution and multiple washes. Greater than 90% of the captured DNA is sequentially eluted with 500 mM and 1 M NaCl. The eluted mass of DNA was 6.9% (3.47 µg) of the total mass loaded (50 µg). Subsequent elution at a very high NaCl concentration (3.5 M) followed by digestion with proteinase K showed that less than 10% of the captured DNA remained on the beads after elution with 1 M NaCl.

DNA fractions were then used to construct standard SOLiD™ fragment libraries according to the manufacturer's instructions (SOLiD™ Fragment Library Construction Kit). Library DNA was size-selected either with E-Gel® SizeSelect™ 2% agarose gels or by gel purification from 2% agarose E-Gel® EX gels (Figure 15.5D). The MethylMiner™ SOLiD™ libraries were sequenced at 50-base read length in a 4-well deposition chamber on a SOLiD™ 3 System, and the sequenced tags were mapped to the human reference genome (hg18) using the SOLiD™ primary and secondary analysis software package. Each quarter-slide chamber yielded ~20 million uniquely mappable sequencing reads.

To confirm that MethylMiner™ fractionation was indeed partitioning DNA fragments based on methylation density, the overall number of CpG dinucleotides within the fragments in each fractionation library was calculated after SOLiD™ system sequencing (Figure 15.6). There was a significantly higher density of CpG dinucleotides in the MethylMiner™ kit–enriched fractions compared to the unbound fraction. In addition, the 1 M NaCl eluate had a higher density than the 500 mM NaCl eluate. Notably, 25% of the unenriched sequenced human genomic DNA fragments had no CpGs. In contrast, only 0.65% of the 500 mM NaCl-enriched sequences and 0.11% of the 1 M NaCl-enriched sequences contained no CpGs. Despite the fact that the exact methylation status of the CpG sites was not interrogated directly, our results suggest that fragments containing two or more methylated CpGs per 150 bp were captured by MethylMiner™ kit fractionation and were progressively enriched from 2- to 10-fold with increasing degrees of methyl-CpG content. Overall, our data demonstrate that MethylMiner™ treatment can fractionate DNA based on CpG density [16].

To validate the performance of MethylMiner™ kit fractionation, we labeled the MCF-7 DNA from enriched and unenriched fractions with Alexa Fluor® dyes (BioPrime® Total for FFPE Genomic Labeling System, Invitrogen) and hybridized them to both a human chr21/22 tiling array (Nimblegen) and a human CpG island/promoter-focused array (Agilent) in parallel with DNA enriched by traditional antibody-based MeDIP [14]. Summary results for high-intensity microarray probe hits are shown in Figure 15.7. While there is overlap between the combined MethylMiner™ kit fractions (500 mM and 1 M NaCl) and MeDIP data sets, there are more than 2,400 unique strong hits from the combined MethylMiner™ kit fractions and only 147 unique array hits in the MeDIP sample. In total, there are more than five times as many hits overall and more than 16 times as many unique hits with MethylMiner™ kit fractionation as compared to MeDIP.

Our results indicate that MethylMiner™ fractionation pulls down more sequences containing CpG-rich sites and sequences within promoters than does the antibody-based approach. In addition to "global" data demonstrating successful enrichment with MethylMiner™ kit capture, detailed information on locations and CpG densities can also be directly visualized and inspected with SOLiD™ system reads. Figure 15.8 depicts the uniquely mappable SOLiD™ system sequencing read locations and densities on a single chromosome (chr 21). Sequencing read distributions are depicted for unenriched DNA, successive MethylMiner™ elution fractions (500 mM and 1 M NaCl), and full

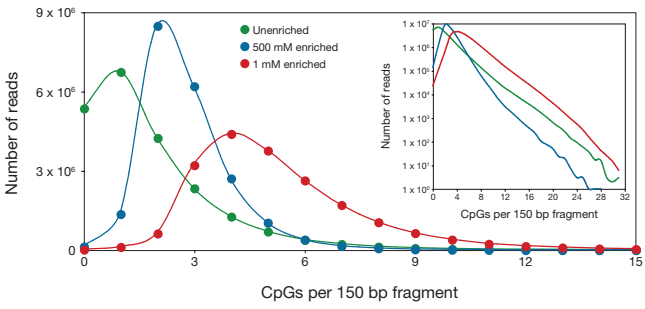

Figure 15.6. CpG dinucleotide density in MethylMiner™ kit–enriched SOLiD™ 3 fragment libraries. Numbers of CpG dinucleotides found within each 150 bp nuclear fragment sequenced from unenriched DNA and MethylMiner™-enriched fractions (500 mM and 1 M NaCl) are shown. The number of CpGs per fragment DNA was determined by counting within 150 bp downstream of uniquely observed read starts. The inset graph shows the 10-fold depletion of CpG-rich fragments from the 500 mM NaCl fraction and concomitant 10-fold enrichment of these fragments in the 1 M NaCl fraction, both relative to unenriched DNA.

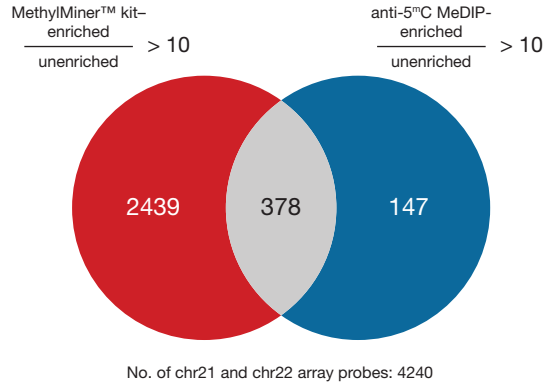

Figure 15.7. Strongly positive features on microarrays: MethylMiner™ kit enrichment vs. antibody MeDIP. The Venn diagram shows the strong positive features from chromosomes 21 and 22 (signal intensities >10-fold over background) detected on human CpG island/promoter focused arrays. The number of strong positive features for combined MethylMiner™ fractions (red, pooled 500 mM and 1 M NaCl eluates) and antibody MeDIP (blue) are shown. Overlap of strong features (gray) is also shown.

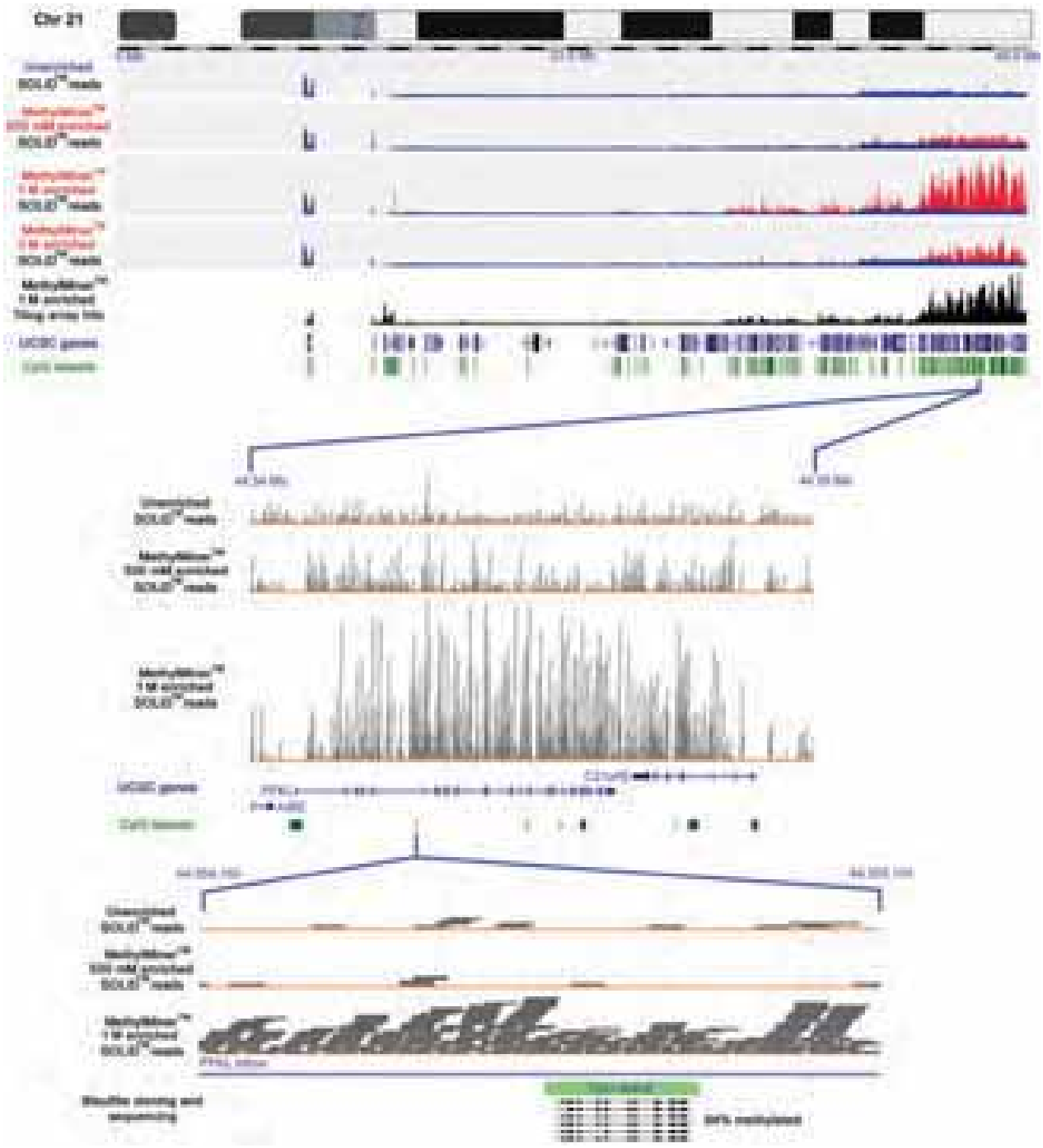

Figure 15.8. MethylMiner™ kit enrichment and SOLiD™ system sequencing across chromosome 21. Tracks illustrating sequencing reads from unenriched MCF-7 DNA, MethylMiner™ kit–enriched 500 mM and 1 M NaCl elution fractions, and full MethylMiner™ kit enrichment with 2 M NaCl elution are shown. Sequencing read depth of coverage for the enriched fractions is shown in red relative to the more uniform low coverage obtained from unenriched DNA shown in blue. Positive features for the MethylMiner™ 1 M NaCl-enriched fraction on a tiling array are also shown. Locations of annotated genes and CpG islands are indicated. Zoomed-in views of 50 kb and 1 kb regions are discussed in the text.

MethylMiner™ elution with 2 M NaCl; further, tiling microarray data for the 1 M NaCl fraction are shown. For the MethylMiner™ kit–enriched uniquely mapped reads, the read coverage without enrichment is superimposed on the enriched read coverage to illustrate the relative enrichment obtained for equivalent amounts of sequencing time and expense; each sequencing reaction comprised ~20 million uniquely mappable reads from one chamber of a 4-well slide. The annotated genes and CpG islands are also shown for reference. The unenriched track suggests that there has been amplification of chromosome 21 DNA in this validated cancer cell line, and the enriched tracks indicate that the distal end of the long arm of the chromosome is densely methylated. This is not surprising since widespread methylation of large chromosomal segments has been seen in other cancer genome studies and since the vast majority of annotated genes lie within this region of chromosome 21 [17,18].

At this high level, good concordance can be seen between the data sets, and the profiles of the serially eluted 1 M NaCl fraction reads and the batch-eluted 2 M NaCl reads are remarkably similar, indicating good reproducibility of sequence capture and significant sequence overlap between these two different elution schemes. The greater depth of coverage seen in the 1 M NaCl fraction attests to the decomplexing power of fractionation. Methylated sequences that resist elution with 500 mM NaCl are better enriched in a subsequent 1 M NaCl elution fraction than in the more complex sample that is eluted as a whole with 2 M NaCl.

For a more detailed view, the analysis focused on a 50 kb region in chromosome 21 (middle of Figure 15.7), and at this level of resolution, a broad peak of enrichment that spans the neighboring genes PFKL and C21orf2 can be seen in the SOLiD™ system data from the 1 M NaCl elution fraction. Closer inspection of the 1 kb region that encompasses one of the PFKL gene's intronic CpG islands clearly demonstrates the enrichment in this fraction (lower portion of Figure 15.8). The average depth of coverage is less than 1 in both the unenriched and 500 mM NaCl elution fractions; in contrast, coverage depth ranges from 5 to 15 in the 1 M NaCl elution fraction. Bisulfite cloning and sequencing of this CpG island verified that its CpGs are 94% methylated (Figure 15.8, bottom row). Notably, this CpG island failed to be identified as methylated in our MeDIP-microarray experiments. To further corroborate the MethylMiner™ SOLiD™ system data with the methylation status of the genomic DNA, we focused on a 200 kb region harboring the ADAMTS1 and ADAMTS5 genes on chromosome 21 (Figure 15.9). The differential enrichment between the MethylMiner™ fractions is evident at this localized view. Sequences spanning the body and the upstream promoter region of the ADAMTS1 gene are exclusively enriched in the 500 mM NaCl fraction. With respect to sequences covering the Methylated CpG Unmethylated CpG CpG island in the promoter of the ADAMTS5 gene, however, there is preferential enrichment in the 1 M NaCl fraction (Figure 15.8). We further carried out bisulfite cloning and sequencing across a portion of the CpG island in the ADAMTS5 promoter to determine the exact methylation pattern, since this locus, like the PFKL CpG island shown in Figure 15.8, had also been identified in our microarray experiments as significantly enriched (>10-fold) with MethylMiner ™ treatment but not captured by MeDIP. As shown in Figure 15.8, the bisulfite-sequenced region shows an intermediate degree (57%) of CpG methylation. In all other loci tested (5 regions that were similarly detected on microarrays with MethylMiner™ kit but missed with MeDIP), bisulfite sequencing has confirmed the presence of >80% CpG methylation (data not shown). These results further indicate that MethylMiner™ kit enrichment permits the isolation of fractions of genomic DNA that harbor differing degrees of methylation. This capacity to capture moderately methylated regions, and direct compatibility with SOLiD™ system sequencing, are notable advantages over antibody-based methods.

Conclusions

When it comes to whole-genome methylation analysis, MethylMiner™ enrichment provides several significant advantages over traditional antibody-based enrichment. The DNA binding step is rapid, efficient, and more sensitive to low levels of CpG methylation with the biotinylated MBD protein in MethylMiner™ kit fractionation. In addition, the incorporation of Dynal® superparamagnetic beads facilitates effective, high-quality capture kinetics. Furthermore, with the MethylMiner™ kit method, genomic DNA can be fractionated according to its methylation density. Finally, the capacity to capture and release double-stranded DNA greatly expedites library construction for current generation high-throughput sequencing. The multiplexing capabilities of SOLiD™ system sequencing permits bar coding of numerous samples that can be sequenced simultaneously. Moreover, samples can be further divided into distinct fractions according to their methylation density with MethylMiner™ kit fractionation. In summary, MethylMiner™ kit fractionation combined with SOLiD™ system sequencing is a seamless, easy workflow that enables robust enrichment and deep coverage across focal areas for cost-effective sequencing of methylated sequences.

Product	Quantity	Cat. No.
MethylMiner™ Methylated DNA Enrichment Kit	1 kit	ME10025

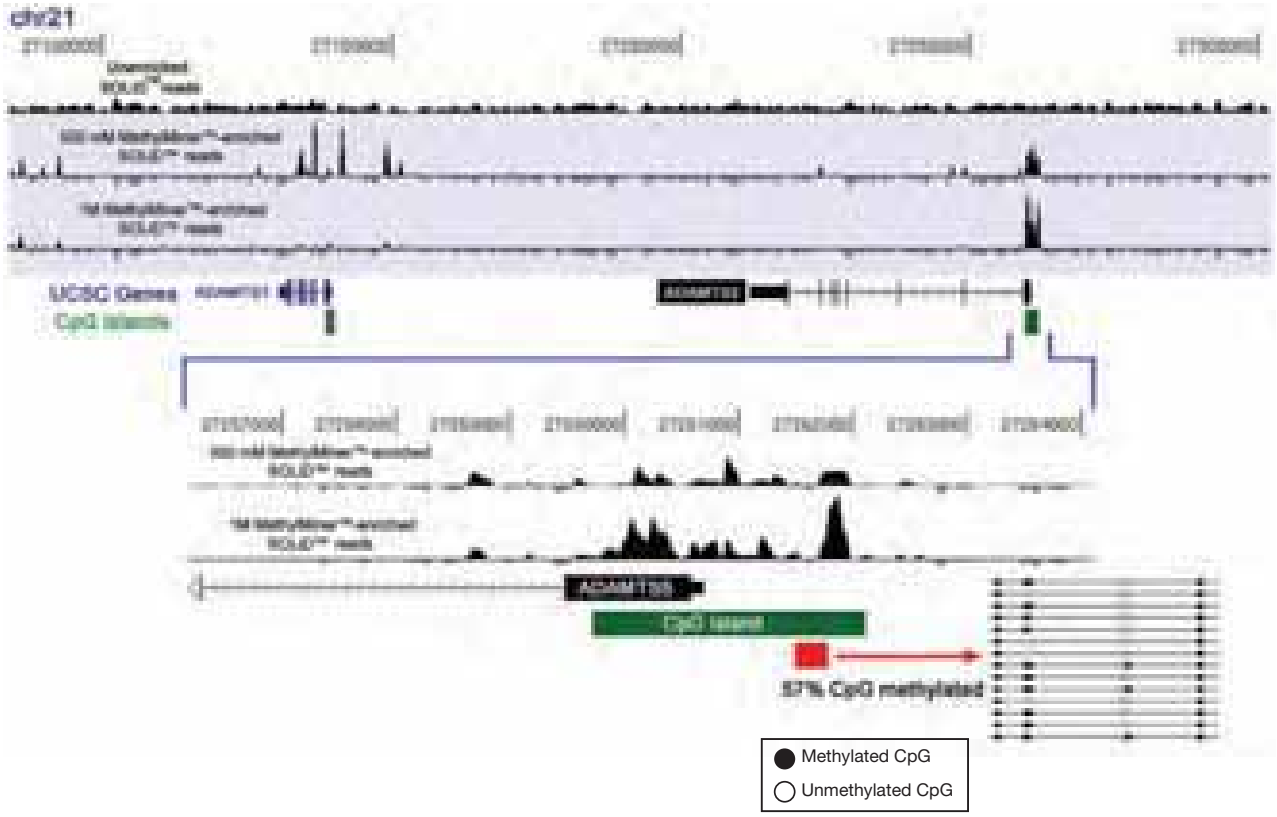

Figure 15.9. Annotated view of a 200 kb region of chromosome 21. Tracks of SOLiD™ system reads from unenriched and MethylMiner™ kit–enriched 500 mM and 1 M NaCl fractions. For MethylMiner™ kit–enriched sequences, the read distribution from an unenriched sequence data set of the same size has been subtracted to highlight regions of enrichment. Locations of annotated genes and CpG islands are also shown. Results of bisulfite sequencing of a small segment in the promoter CpG island of ADAMTS5 are also shown. Open circles: nonmethylated CpGs; filled circles: methylated CpGs.

Locus-specific DNA methylation analysis

Since the first published article on the sequencing of bisulfite-converted DNA in 1992 [19], the bisulfite conversion method has become a preferred tool for methylation analysis. In this method, DNA is denatured and treated with sodium bisulfite, causing unmethylated cytosines to be converted to uracils while methylated cytosines remain unchanged [19]. Converted DNA can be PCR-amplified and analyzed by DNA sequencing or restriction endonuclease digestion and the methylation patterns determined by comparison to untreated DNA (Figure 15.10, Figure 15.11).

Genome-wide methylation

Methylation of cytosines in mammalian genomic DNA is now a recognized epigenetic phenomenon with wide-ranging implications in basic gene regulation and medical research. In particular, changes in DNA methylation, including genome-wide losses and hypermethylation of promoter regions, are strongly correlated with the onset and progression of cancer. One common strategy for monitoring the methylation status of the genome, and particular loci within it, involves fragmenting the DNA, and then capturing the methylated fragments with an anti–5-methyl cytosine antibody. The MeDIP (methylated DNA immunoprecipitation) assay has been used in conjunction with bisulfite sequencing to determine the precise distribution of methylated cytosines in three human chromosomes and in the Arabidopsis genome. Variations on this assay (MCIP and MIRA) have been described that utilize methyl-binding domain (MBD-family) proteins in lieu of an antibody.

Genomic DNA isolation and purification for methylation analysis

Successful results begin with effective genomic DNA isolation and purification. Invitrogen offers several options for genomic DNA isolation:

→ PureLink™ reagents—silica spin-column technology

→ ChargeSwitch®- magnetic bead based purification, amenable to high-throughput isolation

Visit www.invitrogen.com/oligos for information on primer design software to help with low-complexity DNA.

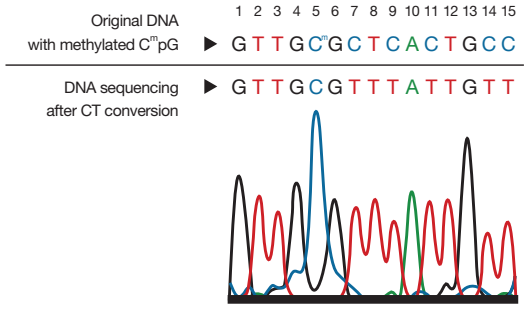

Figure 15.10. Complete conversion of unmethylated cytosines into uracils after bisulfite treatment. DNA with methylated CpG (position 5) was processed using the MethylCode™ Bisulfite Conversion Kit. The recovered DNA was amplified by PCR, cloned, and sequenced. Following bisulfite treatment, the methylated cytosine at position 5 remained intact while the unmethylated cytosines (positions 7, 9, 11, 14, and 15) were converted into uracils and detected as thymines following PCR.

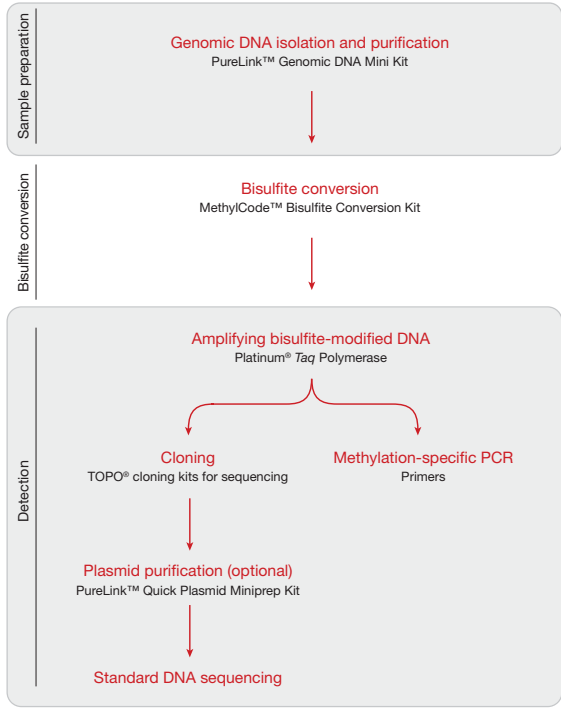

Figure 15.11. Streamlined MethylCode™ method produces better results. The most commonly used technique for detecting methylation in specific regions of DNA is the sodium bisulfite conversion method.

PureLink™ reagents—reliable isolation of genomic DNA

PureLink™ genomic DNA isolation reagents enable high-yield, high-purity DNA extractions from a wide variety of sample types, including blood, tissue, cells, bacteria, swabs, and blood spots, in a familiar silica spin-column or semi-skirted 96-well plate format. With Genomic DNA Kits allow you to:

- → Obtain higher gDNA yields and purity, with optimized spin-column membrane and buffer formulations
- → Save time and money—use with a large range of sample sizes (e.g., 100 µL to 1 mL blood); 96-well versions do not require special equipment
- → Increase flexibility—one kit works with a variety of sample types

Product	Quantity	Cat. No.
PureLink™ Genomic DNA Mini Kit	10 preps	K1820-00
PureLink™ Genomic DNA Mini Kit	50 preps	K1820-01
PureLink™ Genomic DNA Mini Kit	250 preps	K1820-02
PureLink™ 96 Genomic DNA Kit	4 x 96 preps	K1821-04
PureLink™ Genomic Digestion Buffer	70 mL	K1823-01
PureLink™ Genomic Lysis/Binding Buffer	80 mL	K1823-02
PureLink™ Genomic Wash Buffer I	100 mL	K1823-03
PureLink™ Genomic Wash Buffer 2	75 mL	K1823-04
PureLink™ Genomic Elution Buffer	160 mL	K1823-05
PureLink™ *Pro* 96 Genomic DNA Purification Kit	4 x 96 preps	K1821-04A
EveryPrep™ Universal Vacuum Manifold	1 each	K2111-01
PureLink™ Genomic Plant DNA Purification Kit	50 preps	K1830-01

MethylCode™ bisulfite treatment—fast and complete DNA conversion

The most commonly used technique for DNA methylation analysis consists of treating DNA with sodium bisulfite, which causes unmethylated cytosines to be converted to uracils while methylated cytosines remain unchanged (Figure 15.12). The MethylCode™ Bisulfite Conversion Kit improves upon this approach, increasing conversion efficiency, while reducing the number of steps and overall time required for results. The streamlined protocol (Figure 15.13) integrates the DNA denaturation and bisulfite conversion processes into one convenient step, replacing chemical denaturation using sodium hydroxide with a simple temperature denaturation. In addition, cumbersome postconversion DNA precipitation steps are eliminated by an innovative in-column desulfonation technology. This minimizes template degradation and DNA loss so that as little as 500 pg of starting material is required. Recovered DNA is ready for PCR amplification and downstream analysis applications including restriction endonuclease digestion, sequencing, and microarrays. Complete conversion of unmethylated cytosines, as well as DNA purification, can be achieved in less than 3 hours, compared to traditional methods that require overnight incubation. The MethylCode™ Bisulfite Conversion Kit offers many benefits:

- → Complete conversion of unmethylated cytosines—allows you to eliminate false positives
- → Minimal template degradation and DNA loss during treatment and clean-up
- → High yield of bisulfite-converted DNA
- → Less time at the bench—integrated one-step DNA denaturation and bisulfite conversion completed in 3 hr
- → Simplified procedure—enables elimination of cumbersome DNA precipitation steps using novel in-column desulfonation

Original DNA with methylated CpG: G T T G C^m G C T C A C T G C C
Same DNA after bisulfite treatement: G T T G C^m G U T U A U T G U U

Figure 15.12. Effects of bisulfite treatment. Unmethylated cytosines are converted to uracils, while methylated cytosines remain unchanged.

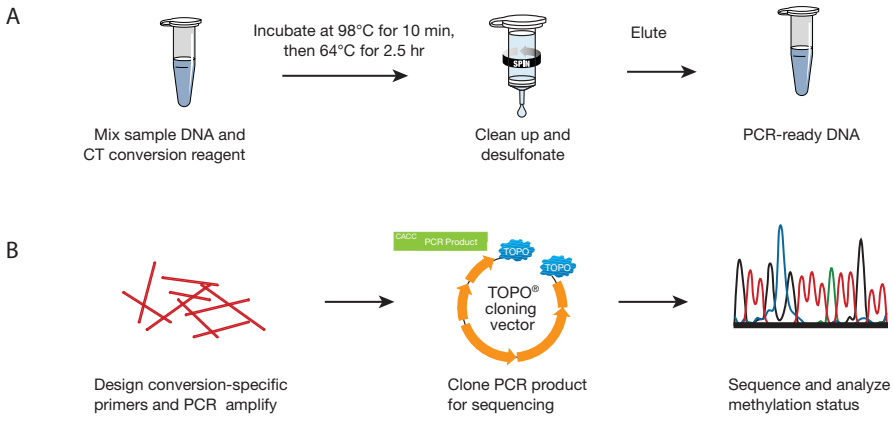

Figure 15.13. Streamlined MethylCode™ bisulfite conversion protocol is complete in just 3 hr. A. Sample DNA is temperature-denatured and converted, and the converted DNA is precipitated using an in-column desulfonation technology and then eluted. B. The DNA can then be PCR amplified, cloned, sequenced, and analyzed for methylation patterns.

Product	Quantity	Cat. No.
MethylCode™ Bisulfite Conversion Kit	50 rxns	MECOV-50

Detection of bisulfite-modified DNA

Detection of bisulfite-modified DNA can be facilitated through conventional PCR, methylation-specific PCR, and bisulfite genomic sequencing after PCR amplification, with or without cloning.

High-fidelity reagents for amplifying bisulfite-treated DNA and methylation-specific PCR (MSP)*

Following bisulfite conversion, the modified DNA template is distinguishable from the original template at methylated cytosines. This results in a pool of DNA fragments with altered nucleotide sequences due to differential methylation status. The methylation-specific PCR reaction involves amplifying the altered nucleotide sequences using methylation-specific primers for the CpG islands of interest (Figure 15.14). Invitrogen's PCR products are tailored to address the persistent challenges associated with amplifying bisulfite-modified DNA—such as a small initial sample of highly fragmented DNA. Not all polymerases are effective at amplifying converted DNA, due to variation in specificity and fidelity of the particular enzymes used. We have validated the use of Platinum® *Taq* DNA polymerases for amplification of bisulfite-converted DNA. Using the Platinum® PCR kits, you can expect:

→ Faithful amplification based on reliable and consistent single-nucleotide discrimination
→ High sensitivity and high yield of bisulfite-converted DNA
→ Room-temperature reaction assembly

* Methylation-specific PCR may be covered by one or more U.S. patents—nos. 5,786,146; 6,017,704; 6,200,756, and 6,265,171, and patents based on foreign counterpart applications. No license or rights under these patents to perform methylation-specific PCR is conveyed expressly or by implication to the purchase of Invitrogen's product. Users of Invitrogen products subject to this label license should determine whether they have all the appropriate licenses in place. Further, no warranty is provided that the use of these products will not infringe on the patents referred to above.

Product	Quantity	Cat. No.
Taq DNA Polymerase, native	100 units	18038-018
Taq DNA Polymerase, native	500 units	18038-042
Taq DNA Polymerase, recombinant	100 units	10342-053
Taq DNA Polymerase, recombinant	500 units	10342-020
Platinum® *Taq* DNA Polymerase	100 rxns	10966-018
Platinum® *Taq* DNA Polymerase	250 rxns	10966-026
Platinum® *Taq* DNA Polymerase	500 rxns	10966-034

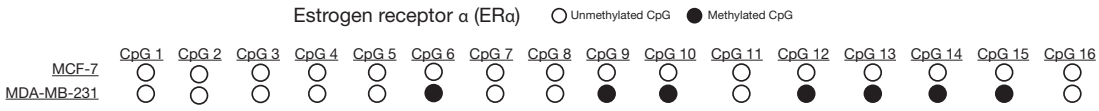

Figure 15.14. Methylation patterns of ERα as analyzed using the MethylCode™ kit. Genomic DNA from MCF-7 and MDA-MB-231 cell lines was isolated and treated with the MethylCode™ Bisulfite Conversion Kit. Bisulfite-converted DNA was then used as template in PCR to amplify a portion on the estrogen receptor alpha (ERα) gene. Next, the PCR product was cloned into a sequencing vector and sequenced. Finally, the sequences were analyzed to show a differential methylation pattern of the ERα gene between the MDA-MB-231 and MCF-7 cell lines (locus—X03635 6450 bp mRNA linear PRI 11-JUN-2003; definition—Homo sapiens mRNA for estrogen receptor; accession—X03635 M11457; version—X03635.1 GI:31233).

Simplify your preparation for bisulfite genomic sequencing

Combine effective plasmid DNA purification with quick and efficient cloning. The PureLink™ Quick Plasmid Miniprep Kit provides efficient and reliable purification of plasmid DNA for cloning with TOPO® technology. TOPO® technology presents the fastest, most efficient way to clone PCR products for sequencing. The key to TOPO® cloning is the enzyme DNA topoisomerase I, which functions as both a restriction enzyme and a ligase that cleaves and rejoins DNA during replication. To harness the religating activity of topoisomerase, we provide over 30 linearized vectors with topoisomerase I covalently bound to each 3' phosphate. Typically, after only 5 minutes at room temperature, ligation of DNA sequences with compatible ends ligations is complete and your DNA is ready for transformation into *E. coli*.

Top-rated sequencing vectors

The TOPO® TA Cloning Kits for Sequencing contain a vector with a minimized multiple cloning site that positions the T7 and T3 priming sites only 33 bp away from the PCR product insertion site. This allows you to sequence more of your insert and less of the vector, saving you time and money. It is designed for fast and efficient results from *Taq*-amplified PCR products.

Product	Quantity	Cat. No.
TOPO® TA Cloning® Kit for Sequencing with One Shot® MAX Efficiency™ DH5α-T1^R *E. coli*	1 kit	K4595-01
TOPO® TA Cloning® Kit for Sequencing with One Shot® MAX Efficiency™ DH5α-T1^R *E. coli*	40 rxns	K4595-40
TOPO® TA Cloning® Kit for Sequencing (with pCR 4-TOPO®) with One Shot® TOP10 Chemically Competent *E. coli* and PureLink™ Quick Plasmid Miniprep Kit	20 preps	K4575-02
TOPO® TA Cloning® Kit for Sequencing with One Shot® TOP10 Chemically Competent *E. coli*	10 rxns	K4575-J10
TOPO® TA Cloning® Kit for Sequencing with One Shot® TOP10 Electrocomp™ *E. coli*	20 rxns	K4580-01
TOPO® TA Cloning® Kit for Sequencing with One Shot® TOP10 Electrocomp™ *E. coli*	40 rxns	K4580-40
TOPO® TA Cloning® Kit for Sequencing	40 rxns	K4575-40
TOPO® TA Cloning® Kit for Sequencing	20 rxns	K4575-01
TOPO® TA Cloning® for Sequencing with One Shot® Mach1™ T1 Phage-Resistant Chemically Competent *E. coli*	20 rxns	K4530-20
TOPO® TA Cloning® Kit Dual Promoter (with pCR II-TOPO® vector) with One Shot® Mach1™ T1 Phage-Resistant Chemically Competent *E. coli*	20 rxns	K4610-20
TOPO® XL PCR Cloning Kit with One Shot® Mach1™ T1 Phage-Resistant Chemically Competent *E. coli*	20 rxns	K7030-20
TOPO® TA Cloning® Kit (with pCR 2.1-TOPO® vector) with One Shot® Mach1™ T1 Phage-Resistant Chemically Competent *E. coli*	20 rxns	K4510-20

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Section IV—Histone Modifications and Chromatin Remodeling

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CHAPTER 16

Histone modifications and chromatin remodeling

DNA is compacted and organized within chromatin in the nuclei of eukaryotic cells. As the fundamental subunits of chromatin, nucleosomes form a chain of ellipsoidal beads of histone proteins around which the DNA is wound. Four histone types (H2A, H2B, H3, and H4) form the octamer center of the nucleosome, with 147 nucleotide pairs wrapped 1¾ turns around the center [1]. Each histone has an amino-terminal tail region consisting of 25–40 amino acid residues that protrude beyond the nucleosome surface. H1 histone plays a role in linking the nucleosome structures together to condense the chromatin.

Chromatin exists in many configurations and undergoes dynamic structural changes. These range from local changes necessary for transcriptional regulation to global changes necessary for chromosome segregation. Several epigenetic mechanisms introduce variation to chromatin structure, including covalent histone modifications, histone variant composition, DNA methylation, and non-coding RNA. Changes to the chromatin structure, called chromatin remodeling, can facilitate gene transcription by loosening the histone-DNA complex, allowing other proteins such as transcription factors to access the DNA. Alternatively, chromatin remodeling where histones assume a more closed conformation, blocks transcription factor access to the DNA, resulting in loss of gene expression. Relatively little is known about how remodeling factors change nucleosome structure and how different factors work together to promote chromatin remodeling.

Chromatin remodeling is typically initiated by posttranslational modification of the amino acids that make up the histone proteins, as well as through methylation of neighboring DNA (Figure 16.1).

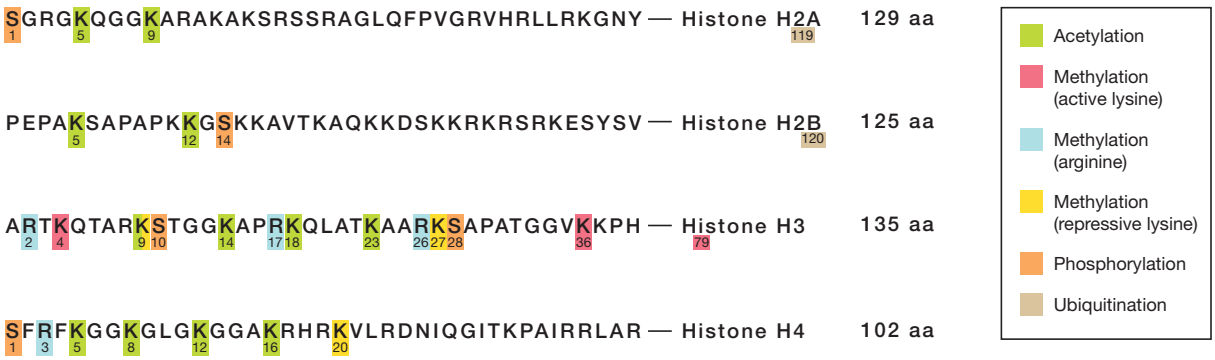

Figure 16.1. Histone modifications and variants. Posttranslational modifications to histone amino-terminal tails include acetylation (lysine and arginine), phosphorylation (serine and threonine), ubiquitination (lysine), and sumoylation, which are associated with a wide variety of biological processes including transcriptional activation and repression, mitosis, and apoptotic regulation.

Investigating histone modifications and chromatin remodeling

Histones are subject to a wide variety of posttranslational modifications, including but not limited to lysine acetylation, lysine and arginine methylation, serine phosphorylation, and lysine ubiquitination. These modifications occur primarily within the histones' amino-terminal tails and are thought to affect chromosome function. Posttranslational modifications of histones create an epigenetic mechanism for regulating a variety of normal and disease-related processes. Current research has focused on investigating the role of epigenetics in transcription, cell proliferation, differentiation, senescence, apoptosis, and the DNA damage response.

MAGnify™ ChIP

To date, the most widely used and powerful method to identify regions of the genome associated with specific proteins is the chromatin immunoprecipitation (ChIP) assay. The ChIP assay has been widely used to study both histone and nonhistone proteins, such as transcription factors, within the context of the cell. Antibodies that recognize a protein of interest are used to determine the relative association of that antigen in the context of chromatin at one or more loci in the genome.

In the ChIP assay, chromatin is sheared and immunoprecipitated with a highly specific antibody directed against a component of the chromatin complex. Because transcription factors and other DNA-binding proteins have a weaker affinity for chromatin than histones, a crosslinking step is incorporated before immunprecipitation to avoid dissociation of nonhistone proteins from the chromatin-binding site.

MAGnify™ Chromatin Immunoprecipitation System

This kit facilitates a faster and more reproducible solution for Chromatin Immunoprecipitation (ChIP) and includes all reagents needed to perform ChIP with an antibody of interest. The MAGnify™ Chromatin Immunoprecipitation System will allow you to:

- → Reduce overall ChIP protocol time by one day (Table 16.1)
- → Reduce input cell number per ChIP experiment (10,000–300,000 cells required) (Figures 16.2, 16.3)
- → Decrease background caused by nonspecific binding through the use of Dynabeads®
- → Improve reproducibility due to optimized magnetic DNA purification (avoid columns and phenol/chloroform steps) (Figure 16.4)
- → Increase confidence in results due to optimized and reproducible components and antibodies that have been qualified for chromatin immunoprecipitation
- → Easily increase throughput with small volumes, magnetic protocol, and magnet compatible with multichannel pipetting
- → Reduce experimental error with Dynabeads® Protein A/G Mix—worry less about antibody compatibility

The ChIP-ready DNA is ready for downstream analysis for most methods, including PCR/qPCR-based assays, bisuflite conversion followed by amplification, cloning, sequencing, direct sequencing, library preparation for high-throughput sequencing, and sample prep for DNA microarray analysis. This kit is unique in its utility for permitting researchers to take advantage of lower starting cell numbers for ChIP, thus preserving precious samples such as primary cells, stem cells, and biopsies. Another key aspect for successful ChIP analysis is antibodies that recognize the target protein in the context of chromatin. We have developed an extensive catalog of CHiP-tested antibodies to expedite success.

Table 16.1. Comparison to a conventional ChIP protocol.

Workflow step	MAGnify™ ChIP timeline	Conventional ChIP timeline
Preclearing	N/A	1–2 hr
Antibody/chromatin incubation	2 hr	Overnight
Bead pulldown	1 hr	2 hr
Washes	30 min (2 buffers)	1–3 hr (4 buffers)
Reverse crosslinking	1.5 hr	Overnight
Proteinase K digestion		2 hr
DNA elution from beads		15–30 min
DNA purification		2 hr–overnight
Average time	**5 hr**	**36–48 hr**

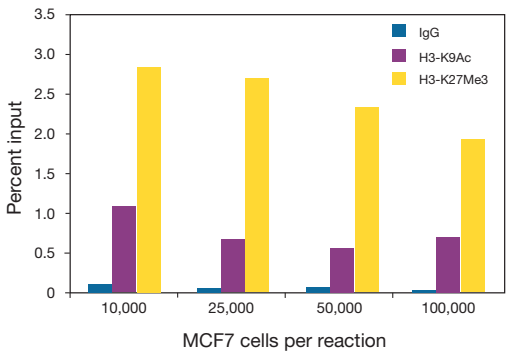

Figure 16.2. Titration of cell number with MAGnify™ ChIP. Sheared chromatin from MCF7 cells was prepared from 10^6 cells in 50 µL lysis buffer and diluted to indicated number of cells per ChIP according to the MAGnify™ ChIP protocol. 1 µg of antibody (IgG included in kit: H3-K27Me3, Cat. No. 49-1014; H3-K9Ac, Cat. No. 49-1009) were used for each ChIP experiment. 1% of the sonicated chromatin was set aside as input control. Optimized qPCR primers were used to amplify the SAT2 locus (Cat. No. 49-2026). Data are graphed as percent input.

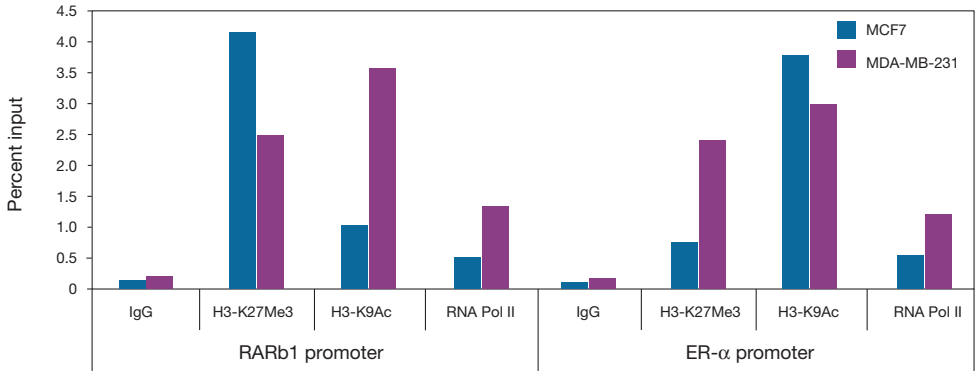

Figure 16.3. 10,000 cell input with MAGnify™ ChIP. Sheared chromatin from MCF7 or MDA-MB231 cells was prepared from 10^6 cells in 50 µL lysis buffer and diluted to 10,000 cells per ChIP, according to the MAGnify™ ChIP protocol. 1 µg of antibody (IgG included in kit: H3-K27Me3, Cat. No. 49-1014; H3-K9Ac, Cat. No. 49-1009) or 3 µL RNA Pol II (Cat. No. 49-1033) were used for each ChIP experiment. 1% of the sonicated chromatin was set aside as input control. Optimized qPCR primers were used to amplify to different loci: RARb1 promoter (Cat. No. 49-2027) or ER-α promoter (Cat. No. 49-2028). Data are graphed as percent input.

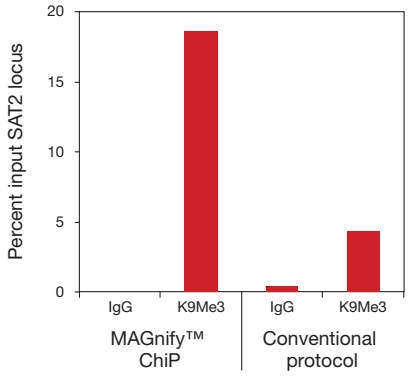

Figure 16.4. MAGnify™ ChIP vs. conventional protocols. Sheared chromatin from 293Gt cells was prepared and 50,000 cells used for ChIP (MAGnify™ ChIP) and 10^6 cells per ChIP used for the conventional ChIP protocol. 1 µg of antibody (IgG included in kit: H3-K9Me3, Cat. No. 49-1008) was used for each ChIP experiment. 1% of the sonicated chromatin was set aside as input control. Optimized qPCR primers were used to amplify the SAT2 locus (Cat. No. 49-2026), a known target of heterochromatin-associated H3-K9Me3. Key elements of a conventional protocol use an overnight immunoprecipitation step of antibody and chromatin, followed by enrichment with Protein A sepharose beads and extensive washes with buffers containing variable salt concentrations. Reverse crosslinking was done overnight at 65°C and DNA purification performed by phenol/chloroform extraction.

Product	Quantity	Cat. No.
MAGnify™ Chromatin Immunoprecipitation System	1 kit	49-2024

Antibodies qualified for chromatin immunoprecipitation

Antibodies are used in ChIP to capture the crosslinked DNA–protein complex. Performing a successful ChIP assay requires that the antibody has high affinity for the fixed protein that is bound to the chromatin complex. Because antibodies are crucial for a successful ChIP experiment, it is important to select an antibody that has high affinity for the protein or proteins of interest. The affinity for particular epitopes can differ between different antibodies and antibody preparations. Differences in affinity of the antibody will affect the strength of association with the chromatin complex, and the resulting signal levels. Further, some antibodies may be more sensitive to inhibitory factors present in the chromatin sample, resulting in a decrease in binding efficiency with increasing input.

Unfortunately, the successful use of a specific antibody in other applications (i.e., western blotting) may not guarantee success in ChIP analysis. To ensure success, we have qualified a number of antibodies directed against histones, as well as other chromatin associated proteins for use in ChIP analysis. Please note that we carry an extensive selection of antibodies that have been prequalified for use in ChiP analysis.

NOTE: Please visit our antibodies page at www.invitrogen.com/chipantibody for an up-to-date listing of ChIP-qualified antibodies.

DynaMag™-PCR

The MAGnify™ ChIP system uses a novel magnet that is compatible with 0.2 mL PCR strip tubes (Figure 16.5). The new DynaMag™-PCR magnet is optimized for efficient magnetic separation of small sample volumes using Dynabeads® magnetic beads. This magnet has been designed for applications with reduced starting cell numbers and volumes used in immunoprecipitation and washing steps. The novel magnetic separator allows you to achieve results quickly and easily while offering the freedom to simultaneously perform multiple ChIP assays in PCR tubes with a multichannel pipettor.

→ Optimal working volume: up to 200 µL
→ Holds up to sixteen 0.2 mL PCR tubes (individual or strip tubes)

Figure 16.5. DynaMag™-PCR magnet.

Product	Quantity	Cat. No.
DynaMag™-PCR	1 each	49-2025

Quantitative PCR (qPCR) of ChIP-ready DNA

Good primer design is critical for high-quality ChIP data. In general, primers should be 20 to 30 bases long with a T_m between 55° and 60°C. Most primers do not require purification or special treatment prior to PCR. Primers should be designed so that amplification targets are 75 to 350 bp in length, as amplification efficiency is reduced substantionally with increasing length. In general, a final primer concentration of 1 µM works well for most primer sets, but in some instances, improved product specificity may be obtained by lowering the final primer concentration 5- to 10-fold.

High-quality primer pairs should result in ~1.9-fold amplification/cycle (this can be determined from quantitative analysis of raw fluorescence data for each cycle, which is generally available on commercial instruments). Amplified material at the completion of the PCR should contain only one product (as assayed on high-percentage agarose or polyacrylamide gels). Specificity information can also be obtained by running dissociation curves on reactions following the conclusion of the qPCR run.

In general, individual samples should be run in triplicate. For each primer pair examined, the input DNA samples should be run alongside the immunoprecipitated samples. Amplification efficiencies among different primer pairs vary slightly on a per-cycle basis, and these slight variations in efficiency can translate into substantially different amounts of amplified material in the cycle range used for analysis. Precise quantitation of relative binding cannot be accurately performed without a primer pair–specific input signal.

We have validated ChIP primer pairs for various promoter regions for validation of ChIP-qPCR. Primers were validated by testing 5-point dilutions of input DNA from MCF7 and PC3 cells, with primer efficiency and slope assessed for each pair.

Product	Quantity	Cat. No.
MAGnify™ SAT2 Primers	100 reactions	49-2026
MAGnify™ RARβ1 Primers	100 reactions	49-2027
MAGnify™ ERα Primers	100 reactions	49-2028
MAGnify™ c-Fos Primers	100 reactions	49-2029

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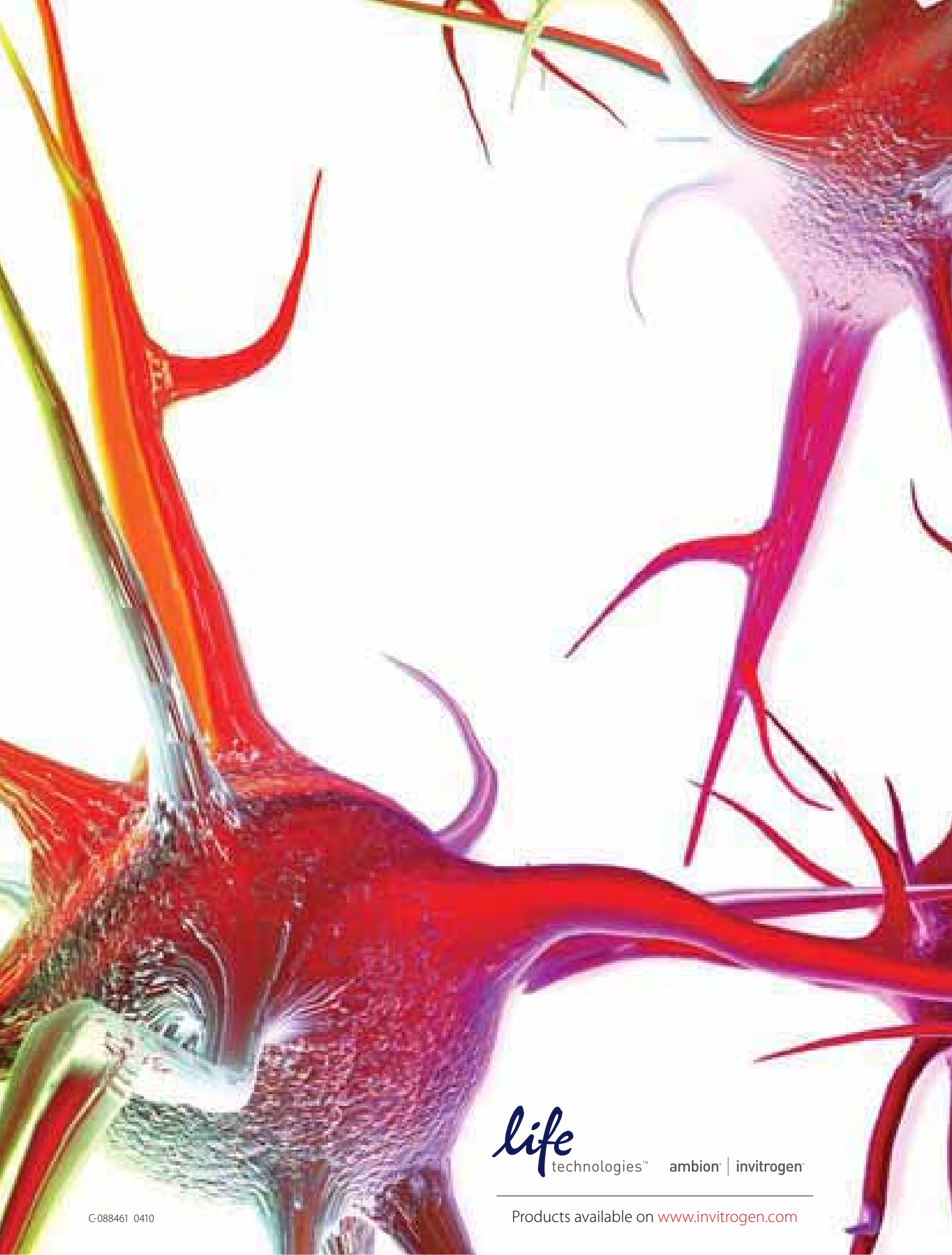

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