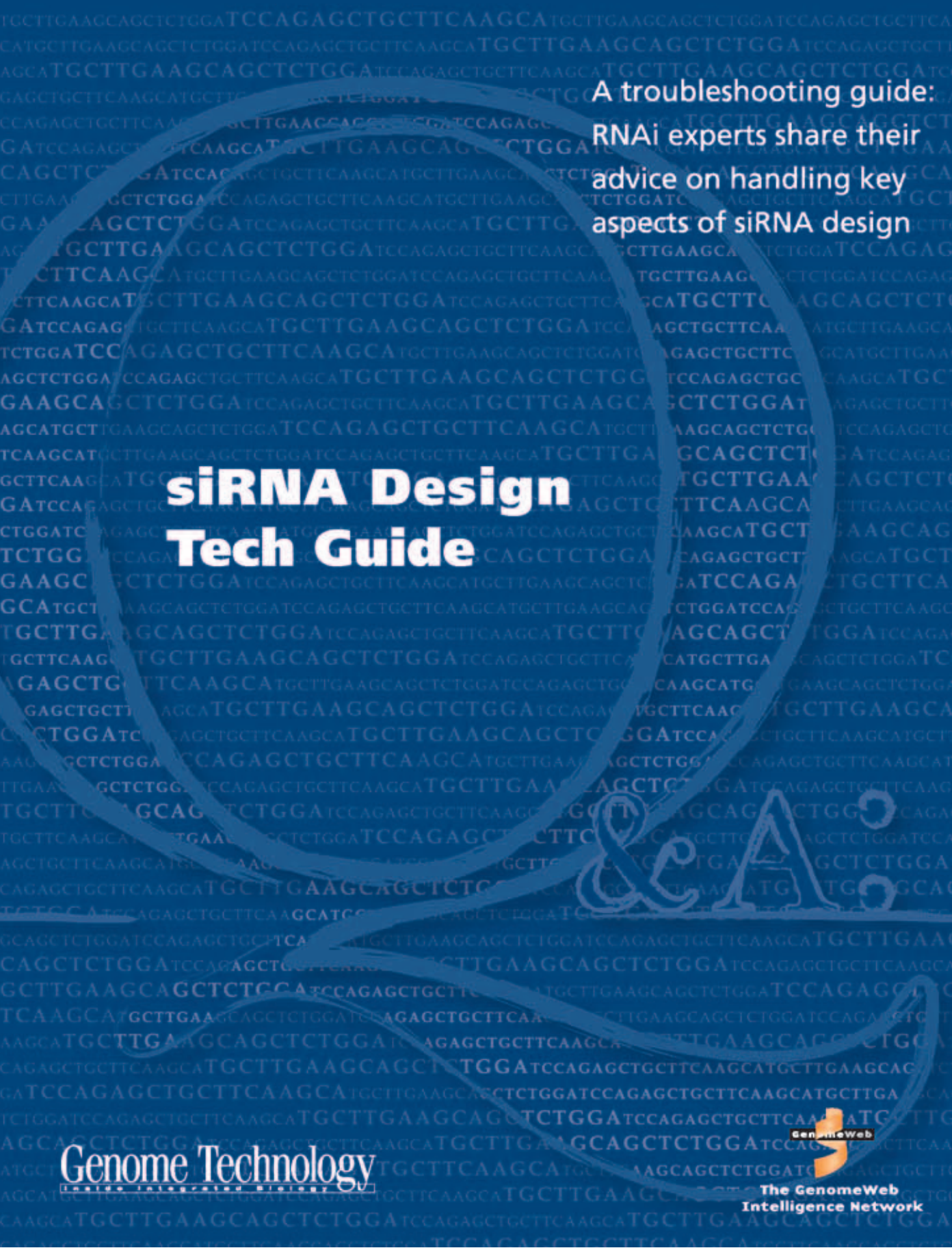
siRNA Design Tech Guide

A troubleshooting guide: RNAi experts share their advice on handling key aspects of siRNA design



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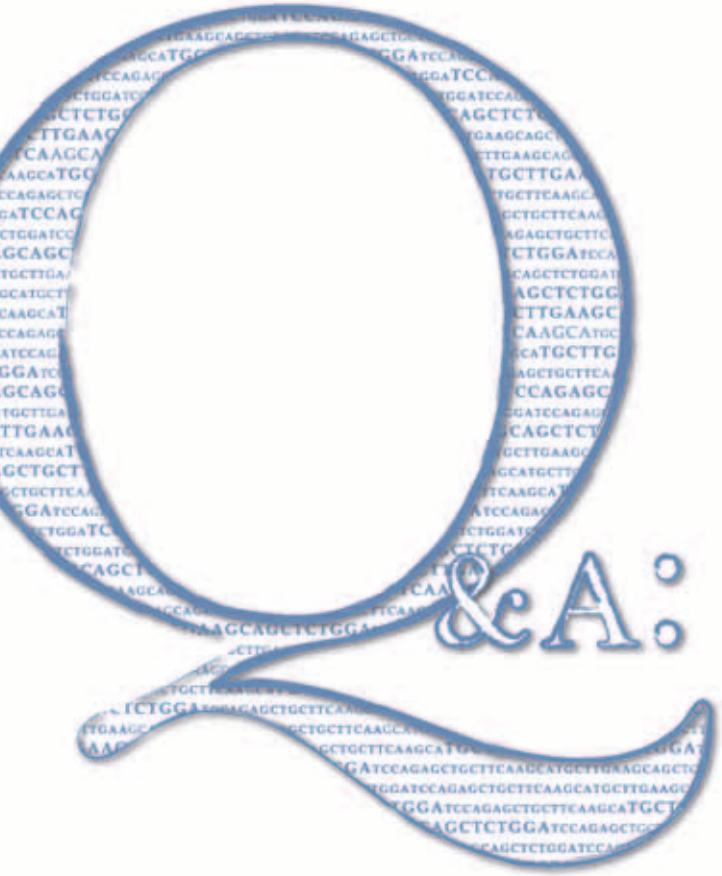

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Letter from the editor

For this latest installment of *Genome Technology*'s technical reference guide series, we've assembled a team of experts well-versed in the field of RNA interference. In the pages to come, these contributors share valuable and time-tested suggestions for designing effective short interfering RNAs.

From the field's fascinating genesis in petunia studies to the first use of the term "RNA interference" by researchers studying *C. elegans*, gene silencing has become one of the hottest topics in molecular biology. In the last few years, the rate of discovery in the field of RNAi has skyrocketed, with new papers touting novel applications hitting the literature databases every day.

In the meantime, siRNAs have gained popularity as the tool of choice for gene knock-down experiments. Their application is broad and cost-effective, with the result that their use as *in vitro* research tools is well-established. Although *in vivo* delivery issues are still being ironed out, the potential use of siRNAs as therapeutic agents holds great promise.

The field is still relatively young, and discoveries regarding the functionality of siRNAs are continually being made. Most researchers agree that, as more becomes known about siRNA mechanisms of action, design parameters for creating high-quality siRNA will surely undergo modification. With this in mind, we attempted to formulate questions that get to the principles underlying improved design of siRNAs, from target selection to delivery.

Keep this guide on hand for detailed advice on selecting highly effective siRNA sequences, reducing off-target silencing effects, optimizing transfection efficiency, and more. Also, be sure not to miss the resource guide, which is comprised of recommended reading and websites sure to make your silencing experiments a success. *— Jennifer Crebs*

Index of experts

Genome Technology would like to thank the following contributors for taking the time to respond to the questions in this tech guide.

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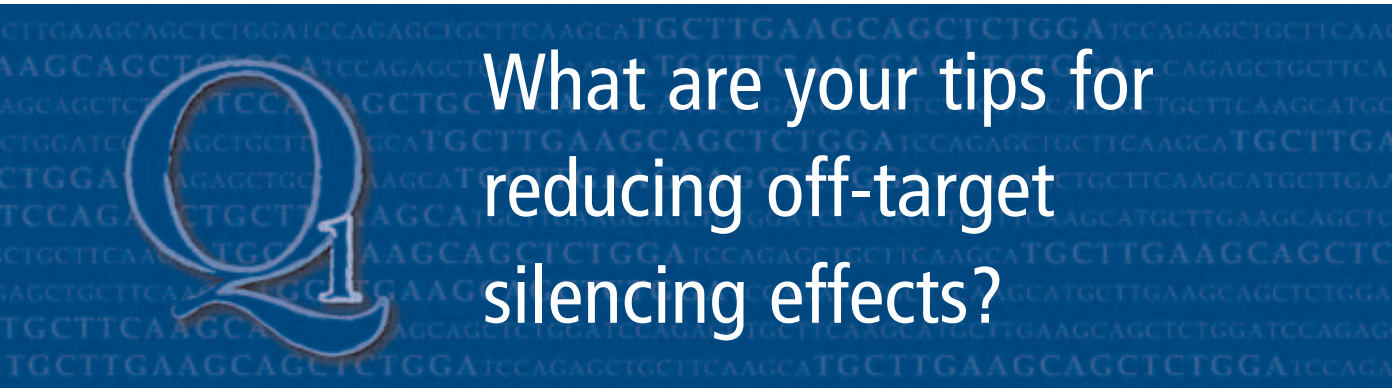


What are your tips for reducing off-target silencing effects?

The best way to avoid off-target silencing effects is to check the siRNAs for specificity using sequence database search methods like NCBI Blast. Special settings can be used (E-value of 1000, word-size of 7, and no filtering) to find short, nearly exact matches. DEQOR and a number of other siRNA design tools already offer a specificity check of siRNAs by Blast searches against a number of selected species.

In case your favorite Web tool for siRNA design does not include specificity checks, you can use your candidate siRNAs as queries to perform a Blast search against your model species, for instance at the NCBI.

— Bianca Habermann

To reduce the off-target silencing effects, Blast the chosen siRNA sequence against the host genome using BlastN (search for short, nearly exact matches) at NCBI online. Make sure the chosen siRNA [is] only specific for the target gene (no more than 16nt matches to any other host genes for 21nt siRNA, if possible).

— Ming Jiang

One of the things that we know causes off-targeting is the first 2-7 bases — a seed sequence. There are two different pathways you have to worry about: if you're using lipids, depending on what cell types you're working with, you could be activating the toll-like receptors or even get some PKR activation periodically. That could probably be attenuated by putting some backbone modifications on the siRNA, but then you kind of dicker with the potency.

So what we like to actually first do is Blast search to make sure that bases 2-7 are not going to be picking up any endogenous targets, because that's really where all of the action takes place. If it's [bases] 4-15, we don't care as this really isn't going to make a difference. But [bases] 2-7 is how the siRNA is aligned with the target, so we eliminate those siRNAs that are a potential problem. Then we try to use the lowest possible concentration. So we like to be in the subnanomolar range whenever possible. That's why we try to use this combination of 27mer and highly-selected siRNAs.

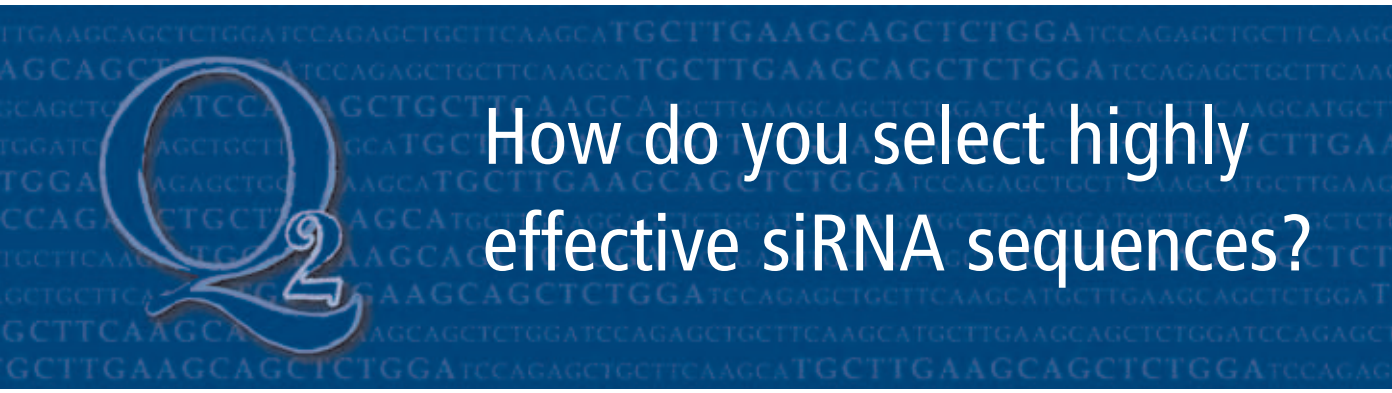
The other thing that we do is just monitor for any cell toxicity. But that's something that's harder to avoid. The other thing that this asymmetric design parameter that the 27mer does is it actually greatly eliminates the upper strand from being incorporated into RISC. The way the dicing reaction goes, it ends up forcing incorporation of the bottom strand, so you end up losing most of the upper strand-based off-target effects.

— John Rossi

Finding siRNAs that can function at very low concentrations is currently the only way to reduce non-specific effects (of course sequence homology search also helps to some extent; however, siRNAs can also work as miRNAs).

If an siRNA does not show activity at 50nM concentration, then it should be discarded. Since the 5′ end of an siRNA strand is critical for incorporation into RISC, 5′ end of the sense strand can be modified to make it incapable of RISC incorporation. This will reduce any sense strand-mediated non-specific effects.

We do not use ***(continued on page 12)***



How do you select highly effective siRNA sequences?

The tool DEQOR that was developed in my lab was designed for esiRNA-based RNA interference (Henschel, et al., 2004). Instead of looking for single 21nt stretches of high quality, DEQOR looks for entire stretches on the mRNA that contain high-quality siRNAs. The quality criteria of selecting a high-quality siRNA are effectively identical to those that are used for siRNA-based design. DEQOR could therefore also be used for siRNA design with the output containing the top quality windows within the input sequence. We have currently implemented three rules for quality control of siRNAs in DEQOR:

1. Based on experimental findings by Schwarz et al. (2003) and Khvorova et al., (2003), siRNAs are more efficient in gene silencing when the 5′ end of the antisense strand is thermodynamically less stable than its 3′ end. DEQOR therefore checks for the correct asymmetry of the siRNA. We have implemented this rule in a simple but efficient manner: DEQOR penalizes those siRNAs that have a reverse asymmetry, which have an A/T pair at the 3′ end of the antisense and a G/C pair at its 5′ end.
2. We avoid secondary structure formation and premature transcription termination of RNA pol III transcribed genes by penalizing poly (-A), (-T), (-C) and (-G) stretches.
3. Unpublished data from the lab of Frank Buchholz have shown that the GC-content of an siRNA should be within the range of 20 percent and 50 percent. In DEQOR, we penalize siRNAs with a GC-content below 20 percent and above 50 percent in an incremental manner.

> "The quality parameters of a high-quality siRNA are almost certainly going to change over the next years."
>
> *— Bianca Habermann*

Another observation made in the Buchholz lab was that esiRNA-based RNA interference works more effectively when part of the 3′ UTR is used for esiRNA production (up to 500nt after the stop codon). When selecting stretches within the mRNA for esiRNA production, we therefore consider the position of high-quality regions within the target mRNA.

RNA interference is a relatively new field of research and continuous discoveries are made also in terms of what makes a functional siRNA. The quality parameters of a high-quality siRNA are almost certainly going to change over the next years. Researchers working with RNA interference as a tool for gene silencing are therefore well advised to follow research reports on RNAi to improve design of siRNAs. With DEQOR, we try to implement the newest research data published on siRNA efficiency for the quality control step of the program.

DEQOR has been extensively tested for esiRNA-based RNA interference and we get an up to 10-fold improvement for gene silencing with regions of mRNAs that have been optimized using DEQOR as

opposed to randomly selected regions.

— Bianca Habermann

There are a few websites for designing siRNAs available for free online now: Dharmacon, Ambion, and Qiagen.

I use the Dharmacon site to select siRNA sequences. The website normally gives more than one sequence. Then I check their thermodynamic properties (dimmer duplexes and loops). I choose the one with the highest criterion matches and lowest possibility of dimmers and loops. I always use two Ts at 3′ of siRNA, but this is not essential. It's been reported that two Us has the same efficiency as Ts.

— Ming Jiang

We have a three-step process. The first step that we have is an algorithm that we developed here that allows us to look at the secondary structure of the target and the thermodynamic end stability of the siRNA. Then we get an ordered ranking of the best siRNAs based on these criteria. We take those siRNAs and run a Blast search against the genome to make sure that they don't have any extensive homology, basically from bases 2-10, with any expressed sequences. If they do, then we carefully look at that and see if it's going to be a potential problem.

Then the next step is we can either directly go and test those siRNAs in cell culture if we have a convenient way of monitoring the knockdown, or we can do a second test which is a cell extract assay. This basically allows us to look at the ability of a given siRNA to bind to RISC. We incubate four or five 32P labeled siRNAs along with reference siRNAs in human cell cytoplasmic extracts followed by a native gel electrophoresis. We've seen that there's a direct correlation between siRNAs that bind very highly to RISC and their efficacy in cell culture, so we can pre-screen them at that level. Then we just take the best one and go ahead and use it.

We also do one other thing: we convert it to a 27mer. The reason we do that is because we've actually found and published that making them substrates for the enzyme Dicer increases their potency quite considerably — up to 100-fold in some cases — so we can use much lower concentrations. The way we do that is the bottom strand is going to be from bases 1-21 that we identified in our algorithm, then we just add 6 extra bases upstream of that. The top strand is going to be a 25mer, which doesn't include the two-base 3′ overhang, and it has two deoxynucleatides at its 5′ end. This turns out to be very good Dicer substrate. So that's our siRNA.

— John Rossi

We tend to include the following parameters in [selecting] an siRNA sequence:

1. has a random set of nucleotides, in the region of 30 percent to 50 percent GC content.
2. lacks stretches of three or more nucleotides, especially Gs or Cs.
3. 5′ antisense end of the duplex has lower Tm than 3′ antisense end. However, I must add that some of the best siRNAs we have discovered in the past lacked

(continued on page 12)

What design parameters increase your confidence in target validation?

There are several mechanisms by which confidence in target validation can be increased.

Of course, there should be a positive and negative control to the experiment. The positive control is generally silencing of a protein, which leads to the expected phenotype. The negative control could either be a scrambled, unspecific oligo. Alternatively, one could use an siRNA that has been designed to a gene that is usually not present in the organism, like luciferase.

Titration of the siRNA to the lowest possible concentration is advisable, since siRNAs at high concentration can display unexpected side effects.

The level of mRNA and protein after silencing should be assessed within the experiment, for instance by quantitative RT-PCR or by quantitative western blotting, respectively.

Because off-target effects are frequently encountered when siRNAs or shRNAs are used, it is important to validate identified hits with a second, independent siRNA or shRNA. Only if the second silencing trigger results in the same phenotype can one be confident that the observed phenotype is real.

The most stringent control is of course a rescue experiment by transfection of a copy of the target gene that is not sensitive to the siRNA. This can for instance be a rodent orthologue of the gene or otherwise the target gene carrying silent mutations. Ideally, this is achieved by expression of the rescue construct under physiological conditions. Kittler et al. (2005) have for instance proposed a rescue strategy by expression of an orthologue of the targeted gene under physiological conditions using BACs. This leads to a physiological level of expression of the rescue transcript and therefore avoids side effects from overexpression of the gene, which should not be neglected. Though more costly and laborious, this is currently certainly the most reliable control that can be done in terms of target validation.

— Bianca Habermann

> "The most stringent control is a rescue experiment by transfection of a copy of the target gene that is not sensitive to the siRNA. "
>
> *— Bianca Habermann*

There are three parameters and they always work for me:

1. Search GenBank to find all the mRNA sequences of the target gene (including all the different isoforms). Find the most conserved region if targeting all the isoforms, or the most specific region if only target the specific one (I go for the exon/intron junction region).
2. Use the procedure in Q2 (*see page 7*) to choose the siRNA sequence.
3. Check the sequence for non-specific targets following Q1 (*see page 5*).

— Ming Jiang

We use a couple. One of the systems that we really like is to use [Promega's] psiCHECK Vector system. Basically it is a plasmid that has two luciferases on it — firefly and *Renilla*. In the 3' end of the *Renilla* luciferase, we insert the target

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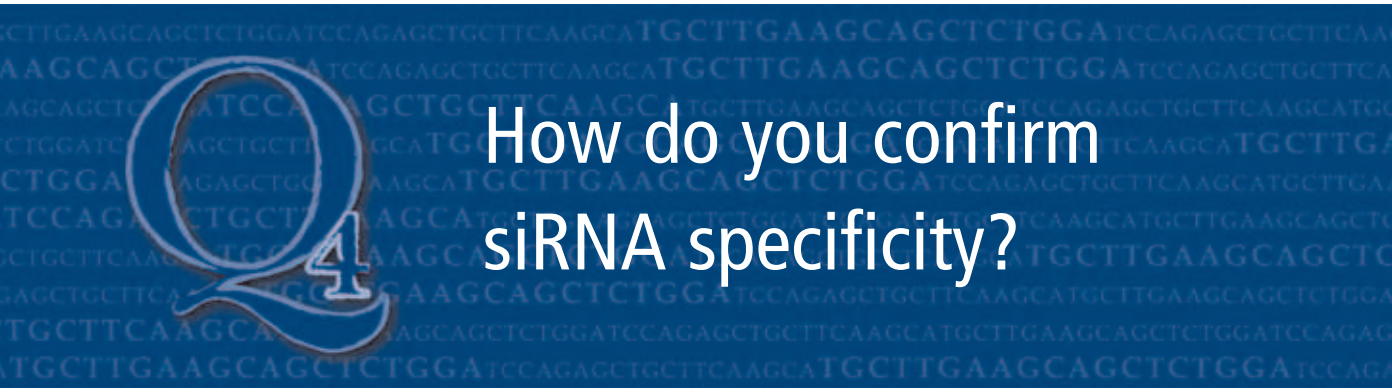

How do you confirm siRNA specificity?

As esiRNAs were used for the first time in a high-throughput manner in the report published by Kittler et al. (2004), it was more or less a must to check esiRNAs for their specificity. In the data set used were some genes with close paralogues, like the ribosomal proteins RPL10, RPL12 and the X-specific protein RPS4X. We analyzed the phenotypes of those genes and their closely related paralogues of these proteins, RPL10L, RPL12L, and RPS4Y. While RPL10, RPL12, and RPS4X showed a striking phenotype in terms of cell viability, the paralogues did not show any effect in our assay. Since the paralogous genes share between 79 percent and 89 percent identity on DNA level, we concluded that esiRNAs are highly target specific.

A similar strategy can be applied to siRNA-based RNA interference. In case there are closely related genes to either, design an siRNA against the paralogues of a target gene or analyze the effect on the expression of the paralogue by quantitative RT-PCR or quantitative western blotting when silencing the target gene.

A rescue experiment can also be a good control for siRNA specificity. In case the rescue construct is able to ablate the observed phenotype of the silenced gene, you have a good indication that your siRNA is indeed specific.

— Bianca Habermann

> "I always include positive and negative controls during the transfection."
>
> *— Ming Jiang*

Using immunoblotting to confirm the target gene is knocked down at the protein level and using Q-RTPCR to confirm the target mRNA is also knocked down. For some genes, the protein level doesn't show decrease (it may be due to the antibody quality/epitope recognition, or the half-life of the target protein). But the mRNA level of [the] target gene should always confirm the knockdown. To confirm the specificity, the housekeeping gene, such as actin, has to be included.

To confirm the siRNA specificity, I always include positive and negative controls during the transfection (a known siRNA that has a specific target in the host, a known siRNA that doesn't have a target in the host, and a non-siRNA transfection control).

— Ming Jiang

Basically, we make a second siRNA to the same target, and we should get the same phenotype.

— John Rossi

This is very difficult to test, although the effect on genes with close sequence similarities can be tested. Also, expression array assays may be incorporated, but these can be expensive.

— Muhammad Sohail

Because the siRNA specificity is sequence dependent, it may target some non-specific sites. To verify the specificity, several controls need to be performed to ensure the specificity. First, sequence non-relevant control. Then sequence mutant control. Based

(continued on page 12)

How do you assess transfection efficiency?

Different cell lines have different optimal transfection conditions, and different transfection reagents work best in a given cell line. Therefore it is important to test different transfection reagent under varying conditions when a new cell line is used. For a first pass we typically use fluorescently labeled siRNAs to evaluate which transfection reagents are able to efficiently deliver the cargo into the cells. Simple examination of the cells under the microscope identifies the reagents and conditions that worked 12 to 24 hours after transfection. However, this experiment does not deliver the information on how well a knockdown works with this transfection method.

An easy and straightforward method to measure the transfection efficiency is to use GFP or luciferase knockdown as a control. Ideally, you would have the identical cell-line stably transfected with GFP, but transient co-transfection of a plasmid encoding GFP or luciferase can also work. Using a functional siRNA against GFP or luciferase, you can then easily measure the efficiency looking at the reduction of GFP or luciferase expression in the cell line.

Alternatively, you can use a control known to produce a strong phenotype that is visible after a short time period, such as Eg5. The knockdown of Eg5 results in a mitotic arrest after 24 to 36 hours in most cell lines. In case you see the expected phenotype and a significant reduction of the expression levels of the positive control, you will have a good indication of the efficiency of transfection.

— Bianca Habermann

I use FITC-dextran (FD-150; Sigma-Aldrich) or fluorescence-labeled siRNA to assess the transfection efficiency.

— Ming Jiang

We usually co-transfect with something like an RFP or GFP plasmid to follow the transfection efficiency.

— John Rossi

When we start working with a new cell line, we tend to measure transfection efficiency of one or two fluoro-labeled siRNAs using lipids from two or three suppliers. We test different ratios of siRNA transfection reagent to see which one gives the best transfection efficiency. We find that once a set of conditions has been established for a particular cell line with one or two siRNAs, most others will transfect well under these conditions.

— Muhammad Sohail

There are several ways to measure the transfection efficiency. I prefer using GFP gene as marker as it is visible under microscopy and allowing easy measurement of the efficiency of transfection. The luciferase is also a frequently used quantifiable gene. In addition, beta-gar gene can also be used to evaluate the transfection efficiency as it is easily visible when the substrate of the beta-gar gene is added.

— Huifen Zhao

What quantitative and functional controls do you use?

In the cell cycle screen published by Kittler et al. (2004), several features were taken as functional controls:

1. A set of proteins was present in the esiRNA collection that were expected to have a phenotype in cell cycle regulation, like subunits of the anaphase promoting complex (APC) or the proteasome, as well as subunits of the mitotic spindle like Eg5. Those genes were considered as functional controls, since they were reliable targets expected to have a phenotype, as the cell cycle phenotype of the knock-down of those genes has been published before.
2. Another functional control that was carried out in the cell cycle RNAi screen were the secondary assays that were performed on selected positive hits. The spindle and DNA were stained to visualize the effects that knockdown of a target gene had on the progression of cell cycle, in this case on spindle formation and progression of mitosis.

In another type of RNA interference screen — in this case, the human kinome was analyzed for its effects in the endocytic pathway in the group of Marino Zerial (Pelkmans et al., 2005) — the secondary assays looked for the distribution and localization of cargo and endocytic marker proteins in the cell after target gene silencing.

More generally speaking, an assay that allows for the concrete analysis of a gene in a certain cellular process is a good strategy to pinpoint the specific effect a protein has within this cellular process.

— Bianca Habermann

For quantitative control, use Q-RTPCR to confirm the specific target knockdown, while the control siRNAs have no effects. I'm also using crook siRNA (Jiang et al., 2005) to quantify the siRNA uptake in the transfected cells by using Q-PCR. This also reflects the transfection efficiency as well.

All the control transfections [discussed] also will reveal the functional change by the specific siRNA.

— Ming Jiang

For quantitative controls, we'll look at a non-targeted RNA. We usually use GAPDH or beta-actin — some housekeeping gene — and make sure the levels of expression of that gene are not affected by our siRNAs. We've given up using mutant siRNAs because sometimes they still cause knockdowns. The best control is really to have two siRNAs at the same target, get the same phenotype, and then monitor two non-targeted mRNAs to make sure we're not getting any non-specific interferon-type responses.

— John Rossi

Normally the luciferase gene is often used for quantitative analysis; however, the GFP gene [can] also be used as a marker for quantitative analysis. To evaluate the activity of sequence to design shRNA, we used the GFP-target system to quantify the efficiency of the selected sequence. In addition, we often use immunocytochemistry and western blotting to quantify or semi-quantify the effect of the siRNA. To see the phenotype caused by siRNA, we normally observe the cell migration pattern in developing mouse brain, or cell apoptosis, defect of cell development, etc.

— Huifen Zhao

Q1: What are your tips for reducing off-target silencing effects? (continued from p.5)

siRNAs with any chemical modifications of normal nucleotides, as the incorporation of chemical modifications in siRNA strands are likely to have their own problems.

— Muhammad Sohail

As siRNA silencing target gene is sequence specific, when we select sequence to design shRNA or synthesize dsRNA, we use the Blast search method to reduce any possible sequence homologue of sequence selected for siRNA experiment.

— Huifen Zhao

Q2: How do you select highly effective siRNA sequences? (continued from p.7)

this thermodynamic asymmetry rule.

4. has at least two AT bonds in the central region.
5. include a Blast homology search and discard any sequences that show more than 15nt homology with non-targeted gene, especially at the termini. We generally test four to five sequences.

We find that commercially available computer programs do not particularly increase the probability of finding effective siRNAs, especially those programs that are freely available. Also, we find that the computer programs from different suppliers almost never return the same siRNAs sequences.

— Muhammad Sohail

Randomly select several sequences using an online program. To select "good" sequences, first I design a fusion-target system by which the fusion plasmid DNA is co-transfected with either shRNA or dsRNA. As the target gene (located at 3') is fused (not necessary) with either GFP or luciferase, once co-transfection is performed, lower GFP signal or weaker activity of luciferase indicate the high effectiveness of siRNA sequence selected.

— Huifen Zhao

Q3: What design parameters increase your confidence in target validation? (continued from p.8)

sequences, and usually a big chunk of the target sequence, so it's actually in the 3'-UTR of the *Renilla* luciferase. We just co-transfect that with our siRNAs, our 27mer, and we look for the knockdown of *Renilla* luciferase versus firefly luciferase, which is our standardized control. That turns out to be a good, quick way of screening. We can do this assay basically overnight and make sure that it's working well at knocking down that target. That seems to correlate with good knockdown of the endogenous target.

For the endogenous target you've got to develop PCR primers or do northern gel or have some way of monitoring your knockdown of the endogenous or viral target. So that's more work, but once you've gone through all of your screening, it almost never fails that the endogenous or the viral target will be knocked down pretty effectively.

— John Rossi

Before an experiment, try to consider all possibilities, such as non-specificity, quality of material, right controls, and possibilities of technologies [to use at] all steps of the method to effectively achieve the best possible result.

— Huifen Zhao

Q4: How do you confirm siRNA specificity? (continued from p.9)

on my experience using 21 base pair siRNA or shRNA, a two- to three-base pair mutant needs to be designed. More mutant leads to less silencing of target gene. The efficiency of the mutant varies from gene to gene. For some genes, only a three-base pair mutant may greatly reduce the activity of siRNA, while others may need more base pair mutations. The third control is called rescue control. The target gene is inserted into an expression vector, such as pcDNA3, and co-transfected with shRNA or siRNA. If the siRNA is very specific, the efficiency of the siRNA in the rescue experiment will be abolished.

— Huifen Zhao

List of resources

Our RNAi experts referred to a number of products and papers, which can be found on this page and the next. We've included additional resources to round out your information sources for siRNA design. Contributor references are noted in bold blue text.

Publications

Editors of Nature Cell Biology (Jun 2003).
Whither RNAi?
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Henschel A, Buchholz F, Habermann B (2004).
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Nature, 436: 78-86.

Khvorova A, Reynolds A, Jayasena SD (2003).
Functional siRNAs and miRNAs exhibit strand bias.
Cell, 115: 209-16.

Schwarz DS, Hutvagner G, Du T, et al. (2003).
Asymmetry in the assembly of the RNAi enzyme complex. *Cell*, 115: 199-208.

siRNA Vendor Websites

Ambion
http://www.ambion.com

Dharmacon
http://www.dharmacon.com

Invitrogen
http://www.invitrogen.com

Promega
http://www.promega.com

Qiagen
http://www1.qiagen.com/siRNA

Stratagene
http://www.stratagene.com/

General Information

Alnylam Pharmaceuticals publications:
http://www.alnylam.com/news-events/publications.asp

Karolinska Institute siRNA Resources:
http://sirna.cgb.ki.se/

Recent siRNA Publications:
http://www.si-rna.com/

RNAi Glossary:
http://www.rnaiweb.com/RNAi/RNAi_Glossary/

Sirna Therapeutics publications:
http://www.sirna.com/sirnascience/publications.html

Databases

esiRNA library: http://www.mpi-cbg.de/esiRNA/

HuSiDa (Charité and Humboldt Universities):
http://itb.biologie.hu-berlin.de/~nebulus/sirna/v2/

Protein Lounge siRNA database:
http://www.proteinlounge.com/sirna_home.asp

RNAi Codex (Cold Spring Harbor Laboratory):
http://rnaicodex.cshl.edu/scripts/newmain.pl

siRNAdb (Karolinska Institute):
http://gemini.cgb.ki.se:8080/sirnadb/index.jsp

Acknowledgments

We thank Gwen Fewell, Kerry E. Lowrie, Carl Novina, David Root, David Sabatini, and Kristin Wiederholt for helpful advice when formulating the questions for this guide.

Many thanks also to Frank Buchholz for his input to the answers submitted by Bianca Habermann.

Silencing Toolbox

Dharmacon siDESIGN Center:
http://www.dharmacon.com/sidesign/

DEQOR:
http://cluster-1.mpi-cbg.de/Deqor/deqor.html

EnergyCalculator:
http://sirna.cgb.ki.se/cgi-bin/EnergyCalculator

GenScript siRNA Target Finder:
https://www.genscript.com/ssl-bin/app/rnai

Invitrogen BLOCK-iT RNAi Designer:
https://rnaidesigner.invitrogen.com/rnaiexpress/

Emboss:
http://emboss.sourceforge.net/apps/sirna.html

Promega's siRNA Target Designer:
http://www.promega.com/siRNADesigner/default.htm

Qiagen siRNA Design Tool:
http://www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx

Sfold (Wadsworth Center):
http://sfold.wadsworth.org/index.pl

Sigma Proligo Design Service:
http://www.proligo.com/pro_primprobes/PP_07-1_DS-siRNA.html

siDirect (University of Tokyo):
http://design.rnai.jp/index.html

siSearch (Karolinska Institute):
http://sonnhammer.cgb.ki.se/siSearch/siSearch_1.7.html

siRNA Wizard (InvivoGen):
http://www.sirnawizard.com/design_advanced.php

siRNA Selection Program (Whitehead Institute):
http://jura.wi.mit.edu/bioc/siRNAext/

SVM RNAi 2.0 (Chang Bioscience)
http://www.changbioscience.com/stat.sirna.html

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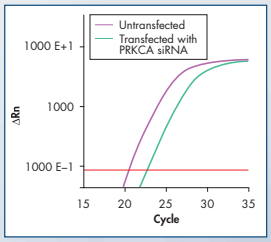
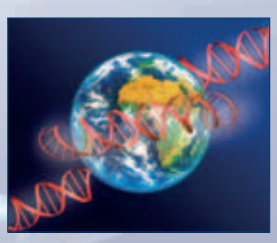


Reliable quantification after knockdown



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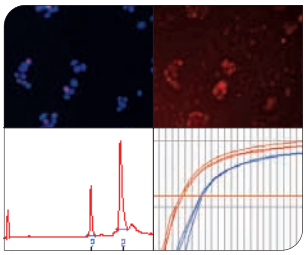

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