TECH NOTE

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Effective controls for RNA interference (RNAi) experiments using siRNA

Introduction

siRNA–mediated RNAi has become an indispensable tool in functional genomics research. Chemically synthesized siRNA reagents that target every gene in human, mouse and rat genome are available for convenient delivery *in vitro*. Data acquired from RNAi experiments are used to support important conclusions about how genes function.

How can you ensure that your conclusions are accurate?

A good way is to include proper controls in your experiments. Both the editors of Nature[1] and a leading researcher in RNAi, Dr. N. Caplen,[2] strongly advocate including appropriate controls to ensure the most accurate assessment of the true experimental outcome.

How do you decide which controls are essential for your experiment?

Our scientists recommend including at least three types of control samples in every RNAi experiment: positive control, negative control, and untreated control (Table 1). Positive controls monitor efficiency of siRNA delivery into cells and negative controls distinguish sequence–specific silencing from non-specific effects. Untreated samples determine the baseline level of cell viability, phenotype, and target gene level. This technical note discusses the function and features of a variety of control siRNAs.

Positive control siRNAs

To achieve successful gene silencing, efficient siRNA delivery into cells is paramount. siRNA delivery efficiency can vary between the cell types used and the delivery method used. All delivery methods can produce a negative effect on cell viability. A well–characterized positive control siRNA is an ideal reagent for establishing parameters that result in successful siRNA delivery without affecting cell viability. An siRNA that targets a housekeeping gene ensures that the target gene is expressed in all cell types at a level that does not fluctuate with cell cycle. An abundantly expressed gene allows easy and accurate assay of its mRNA and protein levels. However, silencing of the gene should not affect either the cell phenotype or its viability. Three popular target genes that meet these criteria are cyclophilin B (also known as PPIB), glyceraldehyde–3–phosphate dehydrogenase (GAPD), and Lamin. We offer siRNA reagents (Table 1) to target these well characterized genes. It is highly recommended to confirm that the expression of the targeted housekeeping gene is not affected by the experimental treatment (siRNA delivery), and is not involved in downstream pathway(s) of the targeted gene(s) of interest.

The ideal positive control siRNA is highly functional, as the level of silencing it produces needs to be correlated with the efficiency of delivery siRNA. We assign a threshold of 80% cell viability and 75% gene silencing for successful delivery of siRNA. As an example, Figure 1 shows the results for a transfection optimization experiment for 3×10^5 HeLa cells in 6-well plates. The positive control siRNA used in this experiment (siGENOME™) Cyclophilin B Control siRNA) has been characterized to silence the target mRNA 85% under optimal conditions. Therefore, the samples in which the silencing is lower than 85% were not optimally transfected. From this experiment, we concluded that 4 or 6 µL DharmaFECT™ 1 is best for this system, as they result in at least 85% silencing of cyclophilin B while maintaining at least 80% cell viability.

siRNA delivery efficiency may vary greatly from one experiment to another due to multiple factors such as variations in cell passage and batch of transfection reagent. Therefore, we recommend including a positive control in every RNAi experiment. The silencing level and cell viability of the positive control sample allows confirmation that the siRNA delivery process in each experiment is successful and non-toxic. Lower than expected silencing of the target gene indicates less than optimal delivery, but still allows normalization of the data to allow comparison with other experiments. However, lower than 80% cell viability may indicate compromised cell viability and usability of the data from this experiment should be reassessed.

Table 1. Recommended controls for RNAi experiments.

Positive control
Function
Optimizes and monitors efficiency of siRNA delivery into cells
Features
1. Targets a housekeeping gene that is constitutively and abundantly expressed in a wide variety of cell types
2. Silencing does not affect cell viability
3. Silencing does not affect cell phenotype
4. Silencing is convenient to assess
Products
1. Accell™ 2.0 GAPDH and Cyclophilin B Control siRNA
2. ON-TARGET*plus*™ 2.0 GAPD and Cyclophilin B Control siRNA
3. siGENOME™ Lamin A/C, GAPDH, Cyclophilin B Control siRNA
Negative control
Function
Distinguishes sequence–specific silencing from non–specific effects
Features
1. Designed to not target any known gene in the cell
2. Produces minimal effect on cell viability
3. Produces minimal effect on cell phenotype
Products
1. Accell 2.0, siGENOME and ON-TARGET*plus* 2.0 Non-targeting siRNA
Transfection control
Function
Determine optimal delivery conditions
Features
1. Fluorescent signal localizes to the nucleus as an unmistakable signal of efficient uptake
2. Maximum fluorescence correlates to optimal siRNA uptake conditions for high confidence RNAi results every time
Products
1. siGLO™ Transfection Indicators (siGLO Green and siGLO Red)
Untreated control
Function
Determines baseline cell viability, phenotype and target gene level
Features
1. Cells are cultured without any siRNA treatment

Fluorescent–labeled control siRNAs

Fluorescently–labeled RNAi controls are popular as transfection efficiency controls, as they provide a rapid visual evaluation. The fluorescence may be detected by either fluorescence microscopy or flow cytometry. We offer two transfection indicators, siGLO Red and Green, to provide reliable visual assessment of transfection success. The fluorescent signal localizes to the nucleus as an unmistakable signal of efficient uptake. While the maximum fluorescence correlates to the optimal siRNA uptake conditions, the fluorescence may not correlate quantitatively with gene silencing.

Negative control siRNAs

Negative control siRNAs are designed to have no known target in the cells being used. They are important for distinguishing sequence-specific silencing from non–specific effects in the RNAi experiment. Samples that are treated with a negative control siRNA are analyzed similarly to samples that are treated with the siRNA targeting your gene of interest. Neither the mRNA nor protein level of the experimental gene should be affected by the negative control siRNA. For example, Figure 1 shows that samples treated with a negative control siRNA have comparable cyclophilin B mRNA level as the untreated sample. The lack of cyclophilin B silencing by the negative control siRNA confirms that the experimental conditions and siRNA delivery alone are not contributing to the gene silencing of cyclophilin B.

Cell viability as well as cell phenotype of samples treated with a negative control siRNA should also remain comparable to those of untreated samples. When these conditions are met, it can be concluded that silencing by the test siRNA is specific, and any downstream effect of gene knockdown can be attributed to the specific silencing of the target gene.

There are a variety of negative control siRNAs commercially available. The most common group of negative control siRNAs are designed to have no known mRNA targets in the cells used, and are described as non-targeting siRNAs. We offer many options for negative control siRNAs, including the ON–TARGET*plus* 2.0 Non–targeting siRNA (Table 1).

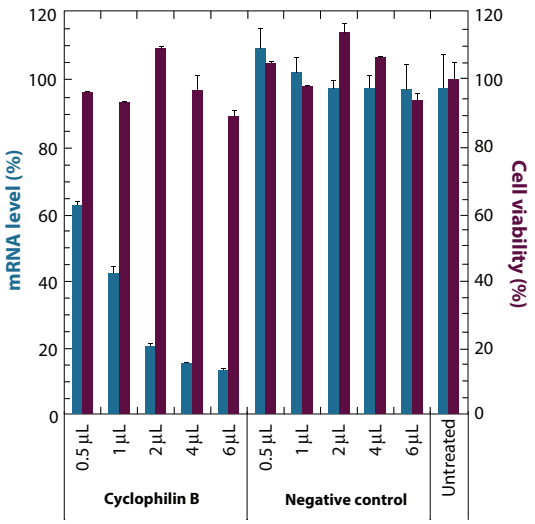

Figure 1. Using a positive control siRNA to optimize transfection efficiency. Cyclophilin B mRNA was measured in HeLa cells 24 hours after transfection with 100 nM siRNA targeting cyclophilin B (Cat. #D–001136–01) or negative control siRNA (Cat. #D–001210–01) using 0.5–6 µL/well DharmaFECT 1 in a 6-well plate. mRNA level was measured using bDNA [Panomics (Freemont, CA)]. Cell viability was measured by the alamarBlue™ Assay. All values are expressed as percent of untreated sample (mean ± SD, n=3).

Characterization of the microRNA seed region, nucleotides 2–6 of the siRNA antisense strand, predicts that nucleotide identity of this region may allow the siRNA to act through the microRNA pathway.[3–5] Thus, a negative control siRNA should have minimal overall sequence identity between the negative control siRNA and any known gene, but also have no sequence identity between the microRNA seed region and any known gene in the cell. Consequently, an siRNA with only a few nucleotide mismatches to the target mRNA that are considered essential for siRNA binding and activity may not serve as a good negative control. ON–TARGET*plus* 2.0 Non-targeting siRNA is not only designed and chemically modified to not target known genes in human, mouse and rat cells, but microarray-confirmed to have no detectable silencing in human cells.

Another negative control strategy is the "scramble" siRNA that has the same nucleotide composition, but not the same sequence, as the test siRNA. This is achieved in two ways: randomizing (also known as scrambling) the nucleotides in the siRNA or reversing the sequence of the siRNA. When their sequences are confirmed to not target a known gene or have an miRNA seed region match with a known gene, these siRNAs are appropriate as negative control siRNAs. However, it is not practical and often impossible, to identify a scramble control for every gene-specific siRNA that is used, therefore a universal Non-targeting control is more practical. There is a wide selection of Dharmacon Non-targeting control siRNAs and pools available to suit your experiment. (Table 1).

Other control siRNAs

Chemically modified control siRNAs

Does a control siRNA need to have the same modification as the test siRNA? Yes. A control siRNA should be as similar to the test siRNA as possible. For example, if the test siRNA is chemically modified to improve specificity of target binding, then the control siRNA should also have the same chemical modification. This allows us to conclude that effects observed with the test siRNA are due to specific silencing of the target gene, and not due to non–specific effects related to the chemical modification. We offer two different sets of chemically modified control siRNAs suitable for use with similarly modified experimental siRNAs, siSTABLE and ON–TARGET*plus* 2.0. siSTABLE control reagents are stability-enhanced to extend siRNA stability in nuclease–rich environments, while the ON–TARGET*plus* 2.0 control siRNAs are chemically modified for greater specificity, and significant reduction in potential off–targeting events.

Pooled control siRNAs

Does a control siRNA need to be pooled when experimental siRNAs are pooled for silencing? Yes, we recommend using the same format for the control siRNA and the experimental siRNA(s). Additionally, it is important to keep siRNA concentration constant between the experimental and control siRNA conditions. We offer pooled controls including the positive and negative control reagents for ON-TARGET*plus* 2.0, siGENOME and Accell 2.0 (see Table 1 for more details).

Product solutions

We offer a wide selection of positive and negative control siRNAs to meet many experimental setups (Figure 2). They are available unmodified or chemically modified to improve targeting specificity (ON–TARGET*plus* 2.0 Control siRNAs) or nuclease stability (siSTABLE siRNAs) for use with test siRNA with similar modifications (Figure 2). For qualitative estimation of siRNA uptake, we also offer fluorescent-labeled siRNAs (siGLO).

Please visit dharmacon.horizondiscovery.com or more information.

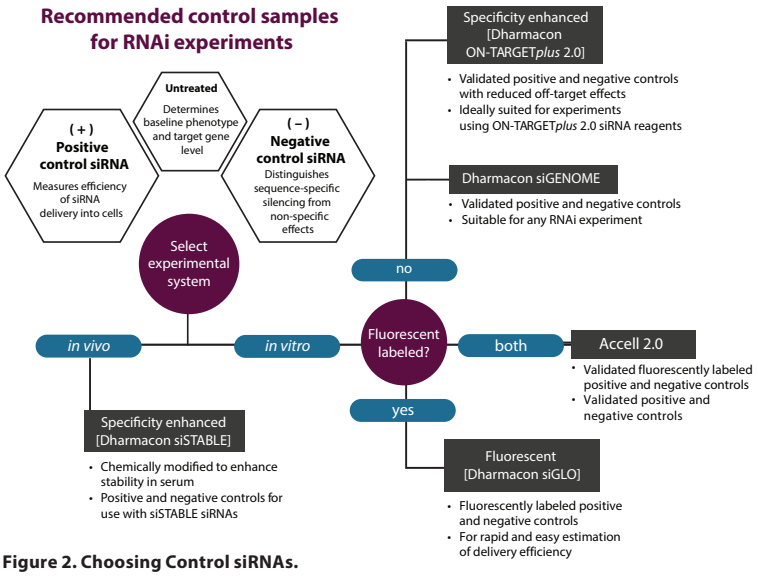

Figure 2. Choosing Control siRNAs.

References

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