Confirming Specificity of RNAi in Mammalian Cells

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INTRODUCTION

MATERIALS

- siRNAs
- Cell Culture
- Retroviral Transduction
- Transfection
- Mutagenesis

EQUIPMENT

RECIPES

INSTRUCTIONS

- Gene Silencing Using siRNA
- Rescue of RNAi with a cDNA Containing Silent Mutations

RELATED TECHNIQUES

- Rescue by Targeting the 3′ UTR

NOTES AND REMARKS

REFERENCES

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Abstract

RNA interference (RNAi) is a process of sequence-specific gene silencing. Recent advances in the understanding of RNAi have provided practical tools to silence gene expression in mammalian cells, opening new possibilities for studying the functions of genes and proteins. It is important to ensure that an observed effect of RNAi is due to silencing of the intended target. Indeed, it is possible that an siRNA may silence more than one messenger RNA that is homologous in the region complementary to the siRNA. Considering that we know little about how RNAi works in mammalian cells, other artifacts may be yet to be recognized. Thus, we suggest approaches to rescue the effect of RNAi by ectopically expressing the protein of interest. These approaches involve introducing silent mutations into the complementary DNA of the protein and targeting RNAi to the untranslated regions of the gene.

Introduction

Preventing expression of a protein is an effective way of learning what this protein actually does or does not do. Until recently, this approach required a substantial amount of effort and was largely limited to animal models. The advent of RNA interference (RNAi) allows one to silence gene expression in various types of cells, including human, which has opened new possibilities that biologists have only dreamed of. We refer the reader to a recent review (*1*) for a detailed description of RNAi technology and its underlying mechanisms. From the point of view of the practitioner, RNAi is a phenomenon that allows one to destroy an mRNA by introducing into a cell a double-stranded RNA that is the cognate of the target gene. Currently, the double-stranded RNA can be introduced by transfection as a short synthetic or in vitro transcribed RNA duplex (siRNA) (*2*), or expressed from an appropriate vector either as a hairpin RNA or as two separate strands (*3*, *4*). Because the field is relatively new and is developing rapidly, it is likely that, by the time you read this protocol, understanding of RNAi and the number of tools available for silencing genes will have expanded.

Whatever the tool, you would want to confirm that the observed effect of RNAi is indeed due to silencing of the intended target. A common way to test whether an effect is due to a deleted gene is to express the product of this gene ectopically. However, RNAi will silence expression of both endogenous and ectopic messenger RNA (mRNA). We suggest two ways to overcome this problem (Fig. 1). One method is to use a complementary DNA (cDNA) that has silent mutations in the region that is targeted by RNAi (Fig. 1A). These mutations prevent interaction of the RNAi machinery with the ectopic mRNA, but do not destroy the endogenous mRNA (*5*). The second approach (Fig. 1B) is to target RNAi to an untranslated region (UTR) of the gene of interest and to rescue the effect by expressing a cDNA that encodes the wild-type coding sequence but does not contain the native untranslated region (UTR).

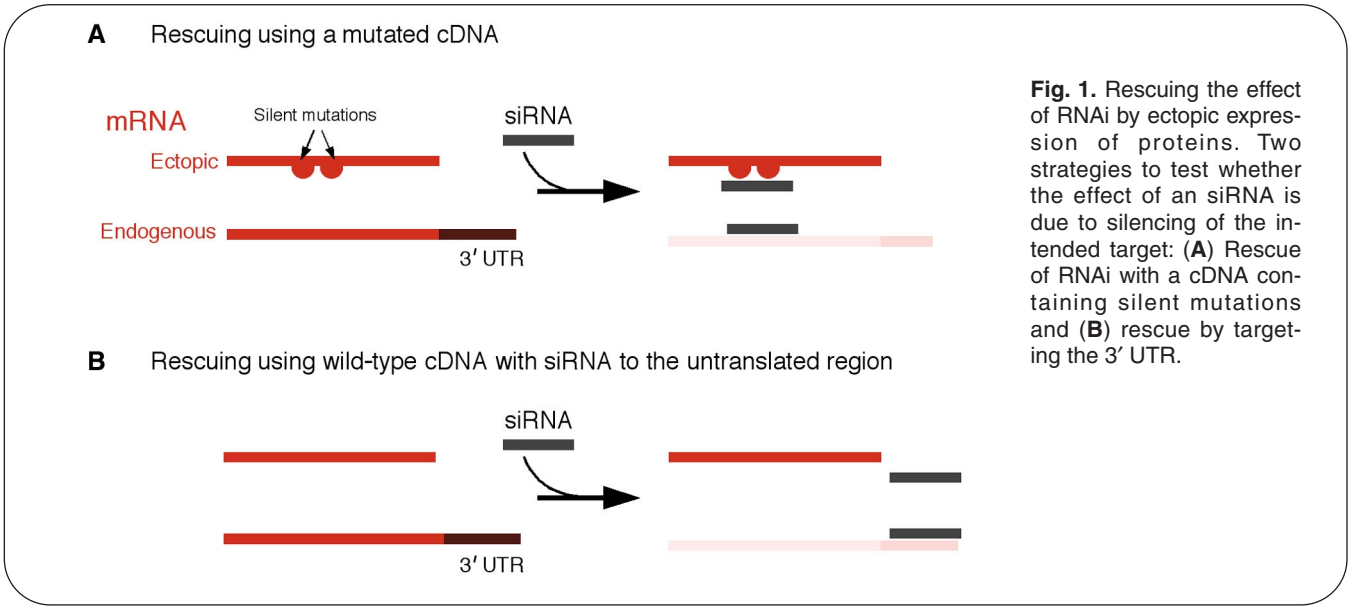

Fig. 1. Rescuing the effect of RNAi by ectopic expression of proteins. Two strategies to test whether the effect of an siRNA is due to silencing of the intended target: (**A**) Rescue of RNAi with a cDNA containing silent mutations and (**B**) rescue by targeting the 3′ UTR.

Materials

siRNAs

Control siRNAs; for example, siRNA complementary to lamin A [Dharmacon (http://www.dharmacon.com/)]

siRNAs complementary to the target gene (Dharmacon)

Cell Culture

3-ml syringes

Six-well tissue culture dishes (Falcon)

10-cm tissue culture dishes (Falcon)

Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine [Gibco (http://www.lifetech.com)]

Fetal bovine serum (FBS) (Gibco)

IMR90-E1A, which are human primary fibroblasts IMR90 [American Type Culture Collection (ATCC) #CCL-186] transformed with the adenoviral oncogene E1A (*6*)

Trypsin-EDTA (Gibco)

Retroviral Transduction

0.45-μm syringe filters (Millipore)

N,*N*-bis [2-hydroxyethyl]-2-aminoethane-sulfonic acid (BES) (Sigma-Aldrich)

$CaCl_2$

Deionized H_2O

Hepes

KCl

KH_2PO_4

MaRX vectors, such as pMaRx-hygro (provides resistance to hygromycin) or pMaRX-puro (provides resistance to puromycin) (*7*). The vectors are available from G. Hannon (hannon@cshl.org).

NaCl

Na_2HPO_4

Packaging cells [we use LinX, which are also available from G. Hannon (hannon@cshl.org)]

Pasteur pipettes

Polybrene [Sigma-Aldrich (http://www.sigma-aldrich.com)]

Siliconized 1.7-ml microcentrifuge tubes

Transducing cells (such as IMR90-E1A cells or other cell lines)

Transfection

Oligofectamine transfection reagent [Invitrogen (http://www.lifetech.com)] or Fugene [Roche (http://www.roche-applied-science.com)]

OptiMEM (Gibco)

Mutagenesis

QuikChange Site-Directed Mutagenesis Kit (Stratagene) (http://www.stratagene.com/manuals/200518.pdf)

Equipment

Automatic pipetter (such as PipetAid)

Microcentrifuge

Multiblock heater

PCR machine

Tabletop centrifuge with a rotor for six-well tissue culture plates

Tissue culture hood

Water bath

Recipes

Recipe 1: PBS

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.12 g
KH_2PO_4	0.24 g

Dissolve in 800 ml of dH_2O. Adjust pH to 7.3 with concentrated HCl if needed and adjust the volume to 1 liter. Autoclave the solution.

Recipe 2: Growth Medium

Under sterile conditions, add FBS to DMEM to 10% v/v. Do not add antibiotics.

Recipe 3: $CaCl_2$ Solution

$CaCl_2$	2.5 M
Hepes	10 mM

Adjust the pH to 5.5 with concentrated HCl.

Recipe 4: 2× BBS

BES	50 mM
NaCl	280 mM
Na_2HPO_4	1.5 M

Adjust the pH to 6.95 with 10 M NaOH.

Recipe 5: Polybrene Solution

Polybrene	8 mg/ml in deionized H_2O

Instructions

We will describe how to rescue the effect of RNAi using silencing by synthetic siRNA as an example. However, the principles of the rescue are likely to be applicable to other RNAi techniques. The instructions explain in detail how to use siRNA in mammalian cells and describe how to test whether the observed effect of siRNA is due to silencing of the intended target.

Gene Silencing Using siRNA

Application of siRNA in mammalian cells involves the following steps: (i) choosing the siRNA sequence, (ii) obtaining the siRNA from a supplier, (iii) transfecting the siRNA, (iv) testing efficiency of gene silencing, and (v) determining whether the silencing causes any changes in the experimental system.

Selection of siRNA sequences

We follow the guide (*8*) provided by T. Tuschl (http://www.mpibpc.gwdg.de/abteilungen/100/105/index.html), who pioneered the siRNA approach. A general rule is that the sequence should be AA(19N)TT (where N is any nucleotide) with a GC content of 30 to 70%. A computer program developed by Lin (*9*) in our laboratory uses this rule to select potential siRNA sequences and determine whether the selected sequences match cDNA sequences other than those of the intended target. In addition, it is important to consider that a guanine base in siRNA and the target RNA may pair not only with a cytosine, but also with a uracil. In principle, both coding and UTR of the mRNA can be targeted. However, targeting siRNA to UTR is not recommended, because regulatory protein complexes that bind to these regions may interfere with the binding of the RNAi machinery. Despite this potential problem, we found that five out of six siRNA that target the 3′ UTR of six genes are very efficient, and we suggest using this approach if specificity of silencing is confirmed (Fig. 2).

Although the available rules for siRNA selection are a reliable general guide, they do not guarantee that each of the selected siRNAs will work. Therefore, you will need to decide how many siRNAs to order, a choice that will be determined by your budget and the time that you can wait for the results. If you are on a tight budget, ordering siRNA one at a time may be an acceptable approach, because there is about a 50% chance that the first siRNA you order will work well. If money is not an issue, we suggest ordering four siRNAs, two for the coding sequence and two for the 3′ UTR. The companies that provide synthetic siRNA and descriptions of their products can be found at the Tuschl laboratory Web site (http://www.mpibpc.gwdg.de/abteilungen/100/105/index.html). We have used Dharmacon, because we have found their service reliable.

We also suggest using two siRNAs as controls: one that has no effect on the cells being tested (for example, an siRNA targeting a gene that is not expressed in these cells), and another that has a known and an easily tested effect, such as the siRNA to lamin A. The siRNA that is targeted to lamin A is available commercially.

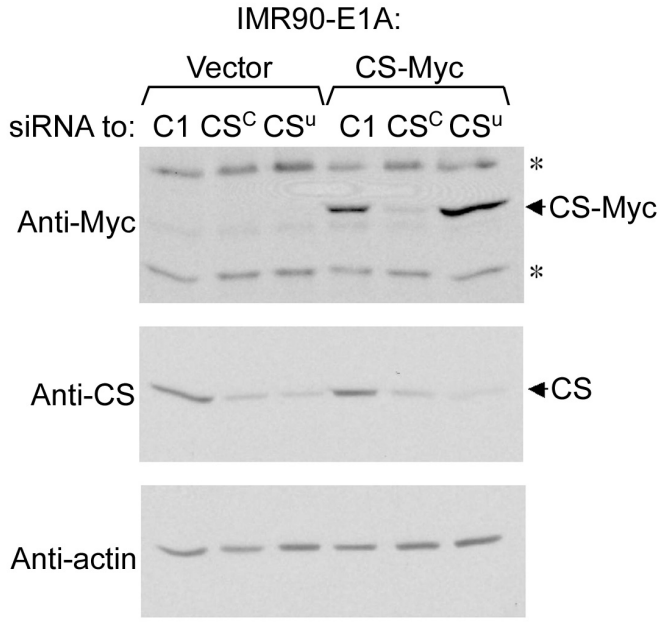

Fig. 2. Rescue of RNAi silencing by ectopically expressing the target gene that is silenced by an siRNA to the gene's 3′ UTR. This experiment was designed to test for silencing of an abundant protein, citrate synthase (CS). Human fibroblasts transformed with adenovirus oncogene E1A were transfected with one of the following siRNAs: an siRNA to caspase-1 (negative control), an siRNA to the coding region of the citrate synthase gene (CS^c), or an siRNA to 3′ UTR (CS^u) of the gene. One day later, the cells were transfected with either an empty vector (pMaRX) or the plasmid expressing a fusion of CS with a Myc epitope tag (pMaRX-CS-Myc). One day after transfection, expression of CS and CS-Myc was analyzed by immunoblotting with antibodies to either CS or the tag. Note that expression of endogenous CS is repressed by siRNAs to the coding and untranslated region, whereas the expression of the ectopic protein is affected only by the siRNA to the coding region. The amount of CS-Myc expressed by the cells in this experiment was only a fraction of the amount of endogenous CS. The concentration of β-actin and of the proteins cross-reacting with the antibody to the tag (indicated by asterisks) were used as controls for equivalent sample loading.

Preparation of siRNA for transfection

The supplier we use, Dharmacon, provides three options for purchasing siRNA. The most expensive option provides RNA duplexes that are ready for transfection; with this option, the three steps below are unnecessary. However, we usually use the less expensive option B, which provides purified, lyophilized, single-stranded oligonucleotides that are ready to anneal according to the following steps.

1. Resuspend the oligonucleotides in the water provided by the supplier.
2. Anneal the oligonucleotides according to the manufacturer's instructions.
3. Store the resulting 20-μM siRNA solutions in 50 μl aliquots at −70°C.

Transfection of siRNA

Transfection of siRNA is the most critical factor in the success of gene silencing. Transfection conditions depend on the cells and the transfection reagents. The procedure that we describe below has been optimized for human fibroblasts, such as IMR90, transformed with the adenoviral oncogene E1A [(*6*) and the Oligofectamine transfection reagent]. We have found that this procedure is also effective with at least some other cell lines, such as Hela, MCF7, U2-OS, and A549.

Note: All procedures are should be performed in a tissue culture hood at room temperature. PBS (Recipe 1) and Growth Medium (Recipe 2) should be warmed to 37°C before use.

1. Plate cells in 6-well plates such that the cells will be 25 to 30% confluent at the time of transfection.

   Note: Cell density is very important. Not enough cells per plate will result in high background toxicity; too many cells will lower transfection efficiency. The right cell density will have to be determined empirically, but a good starting point is to initiate cultures with about 10^5 cells per well.

2. Incubate cells overnight in an incubator set at 37°C and 3% CO_2.
3. Set up a siliconized microcentrifuge tube for each 20-μM siRNA (including controls). This will be Tube 1 for each siRNA. Gently mix together 10 μl of the siRNA duplex with 200 μl of OptiMEM.

   Note: Mixing can be done by pipetting the solution or inverting the tube. Do not vortex to mix.

4. In a second siliconized microcentrifuge tube (Tube 2) for each siRNA (including controls), gently mix 10 μl of Oligofectamine with 50 μl of OptiMEM.
5. Allow the samples to sit for 5 min at room temperature.
6. For each siRNA, combine the contents of Tube 1 and Tube 2, and mix gently by pipetting or inverting. This is the transfection mixture.

   Note: Do not vortex to mix.

7. Allow the transfection mixtures to sit 20 to 25 min at room temperature.
8. Meanwhile, rinse cells with 2 ml of PBS (Recipe 1), and add 2 ml of Growth Medium (Recipe 2) per well.
9. Add the transfection mixture dropwise to the cells while gently agitating the plate.
10. Incubate the cells overnight in an incubator set at 37°C and 3% CO_2 with a humidified atmosphere.

    ***Note:** If the transfection is toxic to the cells, the cells can be rinsed with PBS (Recipe 1) after 6 hours and Growth Medium (Recipe 2) added.*

11. Replace the transfection medium with 2 ml of Growth Medium (Recipe 2) per well.
12. Incubate the cells in an incubator set at 37°C and 3% CO_2 with a humidified atmosphere until the target protein has disappeared from the cells.

    Note: It may take several days for the siRNA to be effective, depending on the efficiency of siRNA and the stability of the protein. We found that 2 days after siRNA transfection was optimal to decrease the concentration of some proteins, whereas others required 4 days. The timing for each experimental system should be determined empirically.

Evaluating the efficiency of siRNA

If the functional product of the gene is a protein, two direct ways of measuring the effectiveness of siRNA are immunoblotting or immunofluorescence. If a specific antibody is not available, one possibility is to ectopically express the protein as a fusion with an epitope tag, such as hemagglutinin (HA) or Myc, and use a commercial tag-specific antibody to determine protein expression. This approach is based on a reasonable assumption that expression of the endogenous protein will be as efficiently silenced as that of the ectopic. If an epitope-tagged version of the protein is used to test for expression levels, then the sequence targeted by RNAi should also be part of the ectopically expressed mRNA.

Rescue of RNAi with a cDNA Containing Silent Mutations

This method uses ectopic expression of the target protein from a cDNA that contains silent mutations in the sequence complementary to the siRNA (Fig. 1A). siRNA should silence expression of the endogenous protein and the protein that is expressed from the native, but not from the mutated, cDNA. If this is the case, determine whether the phenotype being investigated is observed only in cells that do not express the protein. For an example, see our study that describes silencing of caspase-2 (*5*). This next section of the protocol describes how to mutate a cDNA and presents methods for either retroviral transduction or transfection of the wild-type and mutated cDNAs.

There are no rules for choosing the bases to mutate except that the mutations must be silent. Consistent with published reports (*10*), we found that two mutations were sufficient to prevent the destruction of the mRNA by the siRNA, although introducing more mutations would probably only help. The mutations are more efficient if placed together and as close as possible to the middle of the sequence complementary to the siRNA, a region that is required for siRNA silencing (*10*). The mutations can be introduced by any of several methods. We follow the protocol provided with the QuikChange Site-Directed Mutagenesis Kit by Stratagene, which gives consistently reliable results. To aid in the screening of mutants, it is useful to combine silent mutations with a restriction site. The Webcutter program (*11*) is helpful for this purpose.

Once you have generated a mutant cDNA, you can attempt to rescue the phenotype obtained with the siRNA by expressing the cDNA in cells. Two methods we describe in detail below are retroviral transduction and transient transfection. We prefer the former because it provides a population of cells rather than single clones, and it allows one to obtain a sufficient amount of cells within 2 weeks. Regardless of the approach you choose, you will need to confirm that siRNA results in silencing of expression of the endogenous, but not of the ectopic, gene. A convenient way of distinguishing the endogenously and ectopically expressed proteins is to express the ectopic protein as a fusion with an epitope tag. The tag may change the mobility of the protein in gel electrophoresis, which will separate the ectopic and endogenous proteins, and allow for detection of the ectopic protein independently of the endogenous (Fig. 2).

Retroviral transduction

The first step is to clone the mutated, as well as the native, cDNA into a retroviral vector containing a drug selection gene. Various systems for retroviral transduction are available. We often use the MaRX set of vectors (*7*), which has been consistently efficient with our experimental systems. We also use pBABE retroviral vectors (*12*). When planning the number of cells needed for retroviral transduction, consider that you will need cells that express the mutated or native cDNA, as well as cells that are not transfected by the retroviral vector ("mock-transduced") as a control for selection.

Remember that even a retrovirus deficient in proliferation should be handled with caution. You may not want to express your favorite protein in yourself. Follow exactly your institution's rules governing the use of retroviruses and use common sense.

Note: All solutions should be sterile.

1. Split the packaging line (LinX) into six-well plates so that they will be 70 to 80% confluent the next day.

   Note: Plan to have enough packaging cells to include nontransfected controls in addition to those for transfection with the mutant and wild-type cDNA.

2. On the day of transfection, replace the media with 2 ml of fresh Growth Medium (Recipe 2) per well.
3. Return the cells to the incubator set at 37°C and 3% CO_2 for at least 1 hour.
4. For each well containing cells to be transfected, aliquot 6 μg of plasmid (mutated cDNA or wild-type cDNA in a pMaRX vector) into separate 1.7-ml microcentrifuge tubes, adjust the volume to 225 μl with deionized H_2O, and add 25 μl of $CaCl_2$ Solution (Recipe 3).
5. Add dropwise 250 μl of 2× BBS (Recipe 4) while bubbling air through the solution with a Pasteur pipette connected to a PipetAid.

6. Add the resulting solution dropwise to the cells while swirling the plate.
7. Incubate at 37° and 3% CO_2 overnight in an incubator.
8. Replace the medium with 2 ml of fresh Growth Medium (Recipe 2) and incubate at 32° and 3% CO_2 for 60 hours (2.5 days).
9. On the day before transduction, split the cells to be transduced into a six-well plate such that they will be 50 to 60% confluent at the time of transduction. Use 2 ml of Growth Medium (Recipe 2) media per well.
10. Collect the medium containing retrovirus from the packaging line with a 3-ml syringe.
11. Attach a 0.45-μm filter to the syringe and pass the retrovirus-containing medium through the filter onto cells to transduce. This will result in a volume of 4 ml of medium per well (2 ml of medium with the virus added to 2 ml of medium already in the well).
12. Add supernatant from untransfected packaging cells to one of the wells. These cells will be called "mock-transduced."
13. Add 4 μl of 8 mg/ml Polybrene Solution (Recipe 5) to each well (final concentration of 8 μg/ml).
14. Spin the plate in a tabletop centrifuge at 1700 rpm for 1 hour at room temperature.
15. Incubate at 32°C and 3% CO_2 overnight.
16. Change the medium and incubate at 37°C and 3% CO_2 for 2 days (or until confluent).
17. Transfer the cells from each well into a 10-cm tissue culture dish.
18. Incubate at 32°C and 3% CO_2 overnight.
19. Add the appropriate selection drug, such as puromycin or hygromycin, which will depend on the vector used.

    Note: The appropriate drug concentration will depend on the cells used and must be determined empirically.
20. Maintain cells in this selection medium until all mock-transduced cells die.

    Note: If selection requires longer than one week, the medium should be replaced with fresh selection medium every 4 days.
21. Test for protein expression by immunoblotting or immunofluorescence.

    Note: We recommend preparing a stock of frozen cells from cells that are positive for protein expression before doing any experiments.
22. Transfect these cells with the siRNA and determine whether the mutated cDNA rescued the phenotype of interest.

Transient transfection

If the cell line being tested can be transfected with a high efficiency (more than 80%), or if the phenotype can be studied at the single cell level (for example, by immunofluorescence), then transient transfection may be suitable. An example of this approach is given in Fig. 2. The conditions resulting in high efficiency with low toxicity must be determined empirically by varying cell density and transfection reagents. We routinely use Fugene from Roche or Lipofectamine 2000 from Invitrogen and follow the instructions provided by the manufacturers. We recommend that the cDNA be transfected at least one day after transfection of the siRNA. The following typical procedure is performed at room temperature in a laminar flow hood to transfect cells plated in six-well tissue culture plates.

1. Label one 1.7-ml siliconized microcentrifuge tube for each plasmid.
2. Add 100 μl of OptiMEM into each tube.
3. Add 5 μl of Fugene and mix gently by pipetting.
4. Add 2 to 3 μg of plasmid (mutated cDNA or wild-type cDNA) and mix gently by pipetting.
5. Wait 20 to 25 min.
6. Rinse the cells previously transfected with the siRNA with 2 ml of PBS.
7. Add 2 ml of fresh Growth Medium (Recipe 2) per well.
8. Add the transfection mixture dropwise to the cells while gently agitating the plate.
9. Incubate the cells at 37°C and 3% CO_2 overnight in an incubator.

   Note: *If the transfection is toxic to the cells, then the transfection medium can be replaced with Growth Medium after 4 to 8 hours.*

10. Replace the medium with 2 ml of Growth Medium (Recipe 2) per well.
11. Incubate the cells at 37°C and 3% CO_2 for 1 to 2 days before collecting the cells.
12. Test for protein expression by immunoblotting or immunofluorescence. If the protein is not detectable, optimize the transfection conditions or consider retroviral transduction.

Related Techniques

Rescue by Targeting the 3′ UTR

If the siRNA is directed to the 3′ UTR of the gene, the effect of the siRNA can be rescued by ectopically expressing the protein using the wild-type cDNA. You would need to make sure, however, that the expression plasmid does not contain the targeted sequence of the 3′ UTR of the gene of interest. This rescue method is more practical if you have a collection of plasmids that express the gene and various mutants. Thus, an siRNA directed against the 3′ UTR would allow the function of all of these mutants to be analyzed. The steps required to apply this approach are the same as those described above, except the cDNA of the protein being tested does not need to be mutated. An example of this approach is provided in Fig. 2.

Notes and Remarks

Applications of RNAi in mammalian cells are likely to expand in scope and variety in the near future, which may likely affect how particular steps of this protocol should be implemented. We would appreciate comments and suggestions.

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