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Targeting genes expressed in mammalian cells using siRNAs

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INTRODUCTION

RNA interference (RNAi) represents an evolutionarily conserved cellular mechanism for controlling the expression of genes in almost all eukaryotes, including humans. RNAi is triggered by double-stranded RNA (dsRNA) and results in sequence-specific degradation of homologous single-stranded target RNAs. The mediators of mRNA degradation are small interfering RNAs (siRNAs), which are produced from longer dsRNA by enzymatic cleavage in the cell. These short RNA duplexes are approximately 21 nucleotides (nt) in length and have a base-paired structure with 2-nt 3' overhangs. Although discovered only recently, siRNAs have revolutionized the analysis of mammalian gene function and are moving rapidly toward application in genome-wide systematic analysis of gene function in cultured cells. siRNA is becoming a valuable tool for target validation beyond classical tissue culture cell lines. The following protocol, modified from refs. 1,2, describes the use of siRNA for targeting endogenous genes in mammalian somatic cells. To target a specific mRNA for degradation, a portion of the mRNA target sequence must be known and a segment of the target mRNA must be selected that will be used for targeting by the cognate siRNA duplex. The design of siRNA duplexes that interfere with the expression of a specific gene requires accurate knowledge of at least a 20-nt segment of its encoded mRNA³ (see Fig. 1). More specific details of cell culture and manipulation may be found in ref. 4.

MATERIALS

Reagents

2× annealing buffer
200 mM potassium acetate
4 mM magnesium acetate ▲ CAUTION
60 mM HEPES-KOH (pH 7.4) ▲ CAUTION
NuSieve GTG agarose (BMA)
Dulbecco's modified Eagle medium (DMEM) (Life Technologies)
Fetal bovine serum (FBS; Life Technologies)
Mammalian cell lines (e.g., HeLa S3, HeLa SS6, COS-7, NIH/3T3, HEK 293, CHO, A431 and SKBR3)
Penicillin and streptomycin (BioChrom) ▲ CAUTION
Trypsin-EDTA solution (Life Technologies) ▲ CAUTION
Transfection reagents: Oligofectamine (Invitrogen) or TransIT-TKO siRNA Transfection Reagent (Mirus)
The quantities of reagents given below in steps 12–13 are calculated for the transfection of one well of a 24-well plate.
Opti-MEM 1 medium (Life Technologies) ▲ CAUTION
Hoechst 33342 (bisbenzimidazole, Serva) ▲ CAUTION
Methanol, chilled to –10 °C
Moviol mounting medium (Hoechst)
Specific primary and fluorescently labeled secondary antibodies (if necessary, dilute the antibodies with PBS buffer containing 0.5 mg/ml BSA (Sigma) and 0.02% Na₂S₂O₃)

Equipment

Upright light microscope (e.g., Zeiss Axiophot with an F Fluor 40×/1.30 oil objective and MetaMorph Imaging Software (Universal Imaging Corporation)) or laser-scanning microscope.

Selection of siRNA sequences

PROCEDURE

1| Select the target region from the open reading frame of a desired cDNA sequence, preferably 50–100 nt downstream of the start codon.

▲ **CRITICAL STEPS**

2| Search for sequences 5'-AA(N19)UU (where N is any nucleotide) in the mRNA sequence and, if possible, choose those with approximately 50% GC content (**Fig. 1a**). A range of GC content from 32% to 79% may be acceptable. Avoid highly G-rich sequences because they tend to form G-quartet structures. If there are no 5'-AA(N19)TT-motifs in the target mRNA, search for 5'-AA(N₂₁) or 5'-NA(N₂₁) sequences (**Fig. 1b**).

The siRNA selection process has recently been automated by Bingbing Yuan and Fran Lewitter at the Whitehead Institute and associated software has been made publicly available online (<http://jura.wi.mit.edu/bioc/siRNA/home.ph>). This software allows the user to define sequence motifs and G/C content, searches siRNAs against the human and mouse genome databases to prevent mistargeting, and excludes single-nucleotide polymorphic sites.

3| Perform a BLAST search (www.ncbi.nlm.nih.gov/BLAST) using the selected siRNA sequences as the input against EST libraries or mRNA sequences of the respective organism to ensure that only a single gene is targeted.

4| Synthesize the sense siRNA as 5'-(N19)TT and the sequence of the antisense siRNA as 5'-(N'19)TT, where N'19 and N19 are ribonucleotides, N'19 is the reverse complement sequence to N19 and T indicates 2'-deoxythymidine.

Suspend sense and antisense siRNA in water at a concentration >80 μM.

▲ **CRITICAL STEPS**

5| Prepare a 20-μM siRNA duplex solution by combining:
 70 μl 2× annealing buffer
 Sense siRNA to 20 μM final concentration
 Antisense siRNA to 20 μM final concentration
 Aterile water to a final volume of 140 μl.

6| Incubate the reaction for 1 min at 90 °C followed by 1 h at 37 °C. The siRNA preparation may be frozen at this point and the protocol resumed at a later time.

Store unused siRNA duplex solution frozen at -20 °C. The siRNA duplex solution can be frozen and thawed many times and does not require any further heat-shock treatments. Always keep RNA solutions on ice as much as possible to reduce the rate of RNA hydrolysis.

7| Assess the completeness of the annealing reaction:
 Prepare three samples for electrophoresis: dilute 1 μl each of 20 μM sense and antisense siRNA and 0.5 μl of 20 μM siRNA duplex with a few microliters of 0.5× TBE buffer and sucrose loading buffer. Separately load each sample onto a 4% NuSieve GTG agarose gels (add the ethidium bromide to the 4% gel/0.5× TBE solution at a concentration of 0.04% w/v prior to casting of the gel). Run the gel in 0.5× TBE buffer at 80 V for 1 h. Detect the RNA bands under UV light. The annealed RNA should migrate more slowly than the single-stranded controls. Also, the RNA should form a compact band rather than a smear.

➡ **TROUBLESHOOTING**

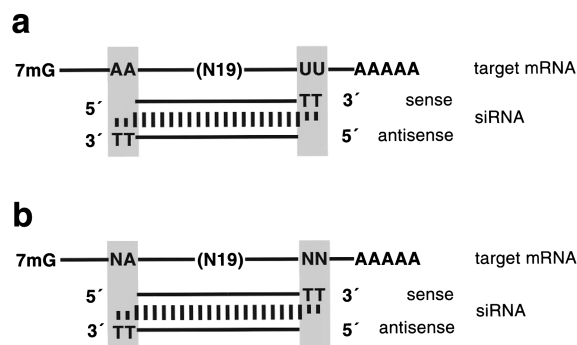


Figure 1 Selection of siRNA duplexes for mRNA targeting. (a) Design of siRNA duplexes for target mRNAs that contain the sequence AA(N19)UU. (b) Design of siRNA duplexes in the absence of AA(N19)UU target sequences. As long as one adenosine is present in the targeted region, siRNA duplexes with 3'-TT overhangs can be used without effect on specificity of target recognition or RNAi efficiency. (Figure adapted from ref. 2.)

Annealing siRNAs to produce siRNA duplexes

PROTOCOL

Cell culture and preparation of cells in 24-well plates

8| Grow mammalian cell lines in a 5% CO₂, humidified incubator at 37 °C in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Passage cells regularly to maintain exponential growth.

▲ CRITICAL STEPS

9| At 24 h before siRNA transfection, trypsinize 90% confluent cells grown in a 175-ml cell culture flask with 10 ml of trypsin-EDTA.

10| Dilute the cell suspension 1:10 with fresh DMEM without antibiotics and transfer 500-µl aliquots into each well of a 24-well plate.

If immunofluorescence assays are planned (steps 19–26), cells should be grown on coverslips placed at the bottom of the 24-well plates prior to addition of the cell suspension. At least 24 h after seeding the cells, a confluency of 50–80% should be reached, which corresponds to 3×10^4 – 1×10^5 cells per well, depending on the cell line and its doubling time.

11| Incubate the plate in a 5% CO₂, humidified incubator at 37 °C until cells reach approximately 50% confluency.

50% is the confluency recommended for transfection of oligonucleotides.

12| Mix 3 µl of the 20 µM siRNA duplex (0.84 µg, 60 pmol) with 50 µl of Opti-MEM 1.

13| In a separate tube, add 3 µl of Oligofectamine (or 4.0 µl of Transit-TKO) to 12 µl of Opti-MEM 1. Mix gently and incubate it for 7–10 min at room temperature.

➔ TROUBLESHOOTING

14| Prepare the transfection solution:

Slowly add the siRNA solution (Step 12) to the solution prepared in Step 13 and mix gently by inversion, not by vortexing.

Incubate the tube for 20–25 min at room temperature to allow formation of lipid complexes; the solution will turn turbid.

Add 32 µl of fresh Opti-MEM 1 to obtain a final volume of 100 µl and mix gently by inversion.

15| Add the 100-µl lipid complexes from Step 4 to the well of cells (from Step 11) without replacing the growth medium and mix gently for 30 s by gently rocking the plate. Incubate the plate for 2–3 d at 37 °C in a 5% CO₂, humidified incubator.

➔ TROUBLESHOOTING

The knockdown effect may be determined by western blotting rather by immunofluorescence as described here.

16| Fix and permeabilize the knockdown cells.

Remove the coverslips carrying the knockdown cells (from Step 15) from the 24-well plate with tweezers.

Place the coverslips on a ceramic rack and incubate in methanol chilled to –10 °C for 6 min.

▲ CRITICAL STEPS

17| Wash the methanol-fixed coverslips three times in PBS and touch filter paper to the coverslips to remove excess PBS.

18| Prepare a wet chamber by soaking filter paper in water and placing it into a 13-cm-diameter Petri dish. Place the coverslips in a wet chamber with the cells side facing up.

It is important that the specimens do not dry out during this procedure.

19| Add 20 µl of appropriately diluted primary antibody on top of the coverslip without touching the cells. Make sure the solution is evenly spread out over the entire surface of the coverslip. Transfer the closed wet chamber into a 37 °C incubator and incubate for 45–60 min.

Transfection of siRNA duplexes

Immunofluorescence detection of protein knockdown

20| Place the coverslips again on the ceramic rack and wash them three times with PBS, each for 5 min. Touch filter paper to the coverslips to remove excess PBS, and transfer the coverslips back into the wet chamber.

21| Add 20 µl of appropriately diluted, fluorescently labeled secondary antibody to each coverslip. Incubate the cells in the closed wet chamber for 45 min at 37 °C.

22| Repeat Step 20.

23| Detect the cell nuclei by chromatin staining. Add 20 µl of 1 µM Hoechst 33342 solution in PBS on top of the coverslip and incubate for 4 min at room temperature.

24| Repeat Step 20.

25| Mount two coverslips per slide by placing the coverslips with the cells side facing downward on a drop of Moviol mounting medium. Place a piece of filter paper on top of the slide and press gently on top of the paper to remove excess mounting medium. Glue cover slips to the slide with nail polish.

26| Examine the immunofluorescence staining and take pictures using an upright light microscope or a laser-scanning microscope. Use identical exposure times for photographing the silenced and the control-treated cells.

➔ TROUBLESHOOTING

➔ TROUBLESHOOTING

PROBLEM	SOLUTION
My gel seems to melt during electrophoresis. [Step 7]	Note that NuSieve agarose is a low-melting-temperature agarose, which may melt if electrophoresis is performed with excessive electric current.
We used a standard transfection technique known to work well in our lab, but obtained low efficiencies. [Step 13]	siRNAs are best delivered with transfection reagents developed for delivery of antisense oligodeoxynucleotides. Such reagents are sometimes less toxic than plasmid delivery reagents and may produce higher transfection efficiencies than conventional transfection reagents.
My cells appear not to survive (or do not remain healthy) during the transfection procedure using Transit-TKO. [Step 15]	Transit-TKO reagent is more difficult to handle than Oligofectamine, because the concentrations required for effective transfection also cause cytotoxic effects. Typical side effects of Transit-TKO siRNA transfection are formation of extended lamellipodia as well as oval-shaped nuclei that appear ~2 d after transfection. These effects are observed using between 4.0 and 4.5 µl of Transit-TKO reagent. If these effects are observed, it is advisable to transfect cells using the Oligofectamine reagent instead.
My siRNA procedure did not work properly—there appeared to be no detectable effect upon gene expression. [Step 26]	a. If the siRNA does not work, first verify that the target sequence and the cell line used are derived from the same organism. According to our study, many cell lines are misidentified or contaminated with cells from other species ⁶ . b. Also, make sure that the mRNA sequence used for selection of the siRNA duplexes is reliable; it could contain sequencing errors, mutations (e.g., such as occur in cancer cell lines) or polymorphisms.

▲ CRITICAL STEPS

Step 1 It is conceivable that 5′- or 3′-untranslated regions (UTR) or regions close to the start codon are less effectively targeted by siRNAs, as these may be richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes could interfere with binding of RISC to the target RNA.

Step 4 Select and synthesize several different (discrete) sequence pairs along the target gene to determine which may provide the strongest knockdown effect and to control for the specificity of the knockdown experiments; those siRNA duplexes that are effective for silencing should produce exactly the same phenotype

Furthermore, a nonspecific siRNA duplex should be used as a control. It is possible to reverse the sequence of an effective siRNA duplex or to use a siRNA duplex that targets a gene absent from the selected model organism, such as the genes encoding GFP or luciferase. The author's laboratory has used a siRNA duplex targeting firefly luciferase as a control for targeting endogenous genes in mammalian cells because the firefly luciferase gene is not present in the targeted cells¹.

Step 8 Do not exceed a passage number of 30 after unfreezing the stock culture. The number of passages may affect DNA and siRNA transfection efficiencies. Aliquots of cells with low passage number may be stored frozen and can be thawed as needed.

Step 16 The preferred method for analyzing a protein knockdown is immunofluorescence detection using a specific antibody that recognizes the targeted gene product. Depending upon the system under study, however, western blotting may be a more suitable alternative.

Step 16b Methanol fixation is suitable for the detection of many cellular proteins; however, it may be necessary to establish the optimal fixation procedure experimentally for each individual protein⁴. It is reasonable to begin with methanol fixation, which preserves the ultrastructure of the cell and sufficiently permeabilizes the cells for penetration of the antibody.

COMMENTS

Mammalian gene function has traditionally been determined by methods such as disruption of murine genes, introduction of transgenes, molecular characterization of human hereditary diseases, and targeting of genes by antisense or ribozyme techniques. In addition, microinjection of specific antibodies into cultured cells or binding of antibodies to cell surface-exposed receptors may provide information on the function of the targeted protein. siRNAs can target genes as effectively as long dsRNAs^{3,5} and are widely used today for assessing gene function in cultured mammalian cells or early developing vertebrate embryos^{1,6,7,8}. Standard tissue culture cell lines provide starting points for mammalian functional screens because siRNAs can be effectively delivered by classical gene transfer methodologies such as classical electroporation or cationic liposome-mediated transfection. Transfection efficiencies greater than 90% are commonly achieved in standard laboratory cell lines provided that transfection reagents are used that were specially designed for siRNA or antisense oligonucleotide applications¹. For small scale-applications, microinjection of siRNAs may represent an alternative. Technical problems resulting from low transfection efficiencies may also be partially overcome by including cell-sorting protocols after cotransfecting siRNAs together with sorting markers such as GFP-expression plasmids.

SOURCE

This protocol was adapted from *RNAi: A Guide to Gene Silencing* (ed. Hannon, G.) pp. 265–295 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2003).

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