Protocol

RNAi in Cultured Mammalian Cells Using Synthetic siRNAs

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RNA interference (RNAi) enables sequence-specific, experimentally induced silencing of almost any gene by tapping into innate regulatory mechanisms that are conserved among virtually all eukaryotes. In a typical RNAi experiment, an artificial silencing trigger directs the RNAi pathway toward a target that it would not normally recognize. This is most often an endogenous protein-coding gene, although some noncoding RNAs can also be silenced effectively. The artificial silencing trigger varies; this protocol uses synthetic small interfering RNAs (siRNAs). Lipofectamine 2000 is used to deliver the siRNAs into HEK293 cells. This lipid reagent has proven to be effective for many different cultured mammalian cell lines.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Cell culture medium appropriate for cells used (e.g., Dulbecco's modified Eagle's medium [DMEM])

If performing Step 5, use DMEM containing 10% fetal calf serum (FCS) for HEK293 cells.

Lipofectamine 2000

Alternative transfection reagents can be used according to the manufacturer's instructions, with this protocol serving as a guide.

Mammalian cells in culture (e.g., HEK293)

Opti-MEM I Reduced Serum Medium (GIBCO-Invitrogen/Life Technologies 31985-062)

siGLO Red Transfection Indicator (Dharmacon/Thermo Scientific) for preparation of negative control siRNA

siRNAs, chemically synthesized

Synthetic siRNAs mimic the natural products of Dicer and comprise duplexes with ~19–20 paired bases and two-nucleotide 3′ overhangs (Elbashir et al. 2001a,b). These can be manufactured in a number of ways. The simplest is to contract the production of synthetic oligoribonucleotides using one of many available manufacturers (e.g., Integrated DNA Technologies, Dharmacon RNAi Technologies, or QIAGEN). Chemically synthesized RNAs most commonly arrive annealed and ready for use. These do not bear 5′-phosphate groups important for incorporation into RNA-induced silencing complexes (RISCs); however, cellular kinases correct this efficiently, making it unnecessary to enzymatically phosphorylate synthetic siRNAs before use (Weitzer and Martinez 2007). Pools of siRNAs can also be produced from long dsRNAs by exposing these to

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recombinant Dicer enzyme in vitro (e.g., using enzymes available from New England BioLabs or Gelantis) (Myers et al. 2003). In this case, it is critical that long dsRNA precursors be removed before introduction into cells with intact protein kinase R (PKR) responses. See Discussion.

Equipment

Centrifuge (low speed)

Hemocytometer for cell counting

Microscope

Platform rocker

Tissue culture incubator preset to 37°C, 5% CO_2

Tissue culture plates

METHOD

This procedure is optimized to transfect siRNA into mammalian cells in a 24-well format. All amounts and volumes are given on a per-well basis. For other formats, scale the reagent amounts based on the surface area of the culture vessel according to Table 1.

1. At 12–24 h before transfection, plate the cells in 500 µL of cell culture medium without antibiotics so that they will be 30%–50% confluent at the time of transfection.

   The precise number of cells depends on the doubling time and cell size and must be determined somewhat empirically for each cell type. About 5×10^4 to 10×10^4 cells serves as a good starting point.

2. For each transfection sample, prepare oligomer–Lipofectamine 2000 complexes as follows:

   i. Resuspend the siRNA in H_2O to a concentration of 100 µM. Dilute this to 20 µM in Opti-MEM I Reduced Serum Medium. Add 3 µL of the 20 µM dilution to 50 µL of Opti-MEM I Reduced Serum Medium to have a final RNA concentration of 100 nM when added to the cells (total volume will be ~600 µL). Mix gently.

   ii. Prepare a suitable negative control siRNA.

   This can vary by experiment, but a convenient control is siGLO Red Transfection Indicator. This permits both processing for biological assays and confirmation of transfection. A third control that is often desirable is a mock transfection with no small RNA added.

   iii. Before use, mix Lipofectamine 2000 gently and then dilute 1 µL in 50 µL of Opti-MEM I Reduced Serum Medium. Mix gently and incubate for 5 min at room temperature.

   It is critical to proceed to Step 2.iv within 25 min.

   iv. Combine the diluted siRNA with the diluted Lipofectamine 2000, mix gently, and incubate for 20 min at room temperature.

   The solution may appear cloudy.

3. Add the oligomer–Lipofectamine 2000 complexes (from Step 2.iv) drop by drop to each of the wells containing cells and medium (from Step 1). Mix gently by rocking the plate back and forth.

4. Incubate the cells in the presence of 5% CO_2 for 24–96 h at 37°C.

TABLE 1. Scaling up or down for different plate formats: Multiply all volumes and cell numbers by the surface area of the desired plate divided by the surface area of the six-well plate.

Culture vessel	96 well	48 well	24 well	12 well	6 well	35 mm	60 mm	100 mm	150 mm	T25	T75
Surface area (cm^2)	0.3	0.7	2	4	10	10	20	60	140	25	75

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5. (Optional) After 4–6 h, remove the transfection mixture and add the culture medium in which the cells are normally grown (DMEM/10% FCS for HEK293 cells).

   Most cells will tolerate culture in Opti-MEM for the duration of the assay; however, in many cases, medium exchange may be desirable.

Assessing Knockdown

6. Assess knockdown at the protein level by western blotting.

   This is more important in mammalian cells than in Drosophila *because only a fraction of mammalian RNA-induced silencing complexes (RISCs) are capable of carrying out small RNA-directed RNA cleavage, whereas the remainder suppress expression by interfering with protein synthesis. Thus, checking knockdown only by measuring RNA levels (e.g., by quantitative PCR or northern blotting) can overestimate knockdown for long-lived proteins or underestimate knockdown caused by contributions of noncatalytic RISCs. See Troubleshooting.*

TROUBLESHOOTING

Problem (Step 6): Knockdown does not occur or occurs at low levels.
Solution: Consider the following:

1. The most common reason for a failed siRNA experiment is the use of a small RNA that is not effective against the target. For this reason, it is critical to test several different siRNAs against any given target gene (see Discussion). Although design algorithms continuously improve, their predictive power is not yet sufficient to guarantee that every siRNA will potently knock down its target.
2. Occasionally, chemically synthesized siRNAs will arrive in poor condition or degrade from improper handling. Particularly if an experiment fails with previously validated tools, it is important to check the integrity of the siRNA dilution by gel electrophoresis or using a bioanalyzer.
3. It is critically important either that transfection be efficient or that transfected cells somehow be distinguished from untransfected cells in scoring knockdowns and their associated phenotypes. This can be accomplished through the use of a cotransfected green fluorescent protein (GFP) expression plasmid, by expression of ectopic cell surface markers that could facilitate selection on magnetic beads, or by fluorescence-activated cell sorting (FACS).

DISCUSSION

The ability to knock down genes in mammalian cells using RNAi has been nothing short of revolutionary. A key innovation came with the realization that one could deliver to mammalian cells synthetic mimics of the products of Dicer cleavage and that these would program RISC without triggering nonspecific suppression of gene expression via protein kinase R (PKR) (Elbashir et al. 2001a). The PKR response is particularly evident in most mammalian cell types. However, even in the presence of PKR responses, RNAi can be triggered by synthetic species that are sufficiently short. Typically, the 30-bp length is used as a rule of thumb for predicting what species will provoke a PKR response. But in reality, PKR is activated in a fashion that is graded with respect to length, depends on the subcellular localization of the dsRNA, and is effected by the precise structure adopted by the introduced element (Sadler and Williams 2007). Given these uncertainties, the most common synthetic RNAi trigger in mammalian cells is the siRNA.

Creating an siRNA is a simple matter of annealing two chemically synthesized oligoribonucleotides to generate a molecule with 19 paired bases and two overhanging nucleotides on the 3′ end of each strand. A number of vendors offer this configuration as a standard product, and the strands almost invariably come preannealed into a functional molecule. Some vendors have developed

proprietary variations on the standard siRNA configuration that are reputed to have improved properties. These include enhanced efficiency (e.g., dsiRNAs, IDT; stealth siRNA, Invitrogen/Life-Technologies), restriction of activity to only one of the two strands (e.g., ON-TARGET, Dharmacon; see below), or the ability to be taken up by mammalian cells passively, without the need for a transfection reagent (e.g., Accell, Dharmacon).

Many manufacturers offer predesigned and, in some cases, prevalidated siRNAs to certain genes or gene sets. Although these siRNAs eliminate one preparation phase of the siRNA-silencing experiment, it is important to understand the restrictions that accompany the use of stock tools. For example, some companies charge an additional fee to reveal sequences, structures, and chemical modifications (or refuse outright) and others place some restrictions on use. Many stock siRNAs were designed with proprietary algorithms that may or may not reflect the latest understanding of siRNA design rules. Therefore, one must always weigh the risks and benefits of using a predesigned tool.

Choosing a custom siRNA requires more effort but also affords flexibility. Sequences can be chosen to target specific alternatively spliced isoforms or even specific mutant or variant alleles. In all cases, the sequence of the siRNA itself is of critical importance to two properties: potency and specificity.

siRNAs are composed of two strands termed the guide and the passenger. The guide strand is complementary to the target RNA. The passenger strand has the same sequence as the target. The first goal is therefore to create a sequence that leads to predominant guide-strand incorporation into RISC, maximizing the concentration of active species and minimizing the possibility that RISC loaded with the passenger strand could recognize undesired targets (off-target effects). In fact, ON-TARGET from Dharmacon modifies the passenger strand to prevent its incorporation into active RISC.

The design of effective siRNAs also relies both on the understanding of RNAi biochemistry and on large-scale studies of effective and ineffective siRNAs. The former has led to the realization that the siRNA strand with the less stable 5′ end has a greater probability of being incorporated as the guide strand into RISC. Thus, so-called "thermodynamic asymmetry" became an important consideration in effective small RNA design. Improvements were subsequently made by a simple brute-force measurement of the silencing potential of >2000 siRNAs. Feeding these data into an artificial neural network, Biopredsi (Huesken et al. 2005), which is no longer available online, or a linear regression model (dsir; Vert et al. 2006; http://biodev.extra.cea.fr/DSIR/DSIR.html) led to what may be the most effective algorithms at the time of the preparation of this article. However, RNAi is a fast-moving field, and it is critical that investigators keep abreast of new developments that will undoubtedly occur in the future. Among these, one would predict the development of algorithms that take into account features not only of the small RNA but also of its target.

Once siRNAs have been designed and purchased, one must consider the best way to introducing them into mammalian cells. Each mammalian line is most efficiently transfected with a different reagent or protocol. The best approach is to follow the guidance of the literature where possible and to test the panoply of available transfection methods where necessary. If using reagents suitable for the transfection of DNA and RNA, transfection of a plasmid that encodes GFP is a convenient way to test and calibrate efficiencies. As a rule of thumb, small RNAs will give greater effective transfection than the GFP plasmid, so even if 50% of cells become GFP positive, it is still possible to achieve 80%–90% knockdown with an effective siRNA. A number of reagents have been developed specifically for small RNA delivery. Although most will also deliver plasmids, one can calibrate small RNA transfection using fluorescently labeled siRNAs that can be obtained from a number of commercial vendors (e.g., siGLO Red, Dharmacon).

When considering the biological consequences of a gene knockdown, it is critical to consider that the specificity of any given siRNA reagent is an unknown. Even if a target is knocked down effectively, other genes may also be silenced (so-called off-target effects). Off-target effects can lead to misinterpretation of results and to incorrect biological inferences being drawn. A few rules of thumb should be considered as good practice in the use of RNAi methodologies. First, it is essential to use multiple, independent RNAi triggers, either long dsRNAs or siRNAs, and to assess both the phenotypic effects and the degree of knockdown caused by each trigger. The phenotypic effect should not be produced by negative controls and should correlate with the degree of silencing seen with the targeted agents.

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Ideally, any phenotype caused by silencing should be rescued by expression of an RNAi-resistant version of the target gene. This last suggestion is, of course, much more straightforward if siRNAs are used to elicit silencing, because the binding site of the siRNA can be altered by mutation or by eliminating those sequences from the rescue construct (e.g., if the original target site was in a 3′- or 5′-untranslated region [UTR]). If the binding site cannot be eliminated, it is a good idea to introduce multiple mutations into the resistant target, focusing on alterations in the seed region and in the RISC cleavage site (opposite guide nucleotides 10–11).

Ultimately, the specificity of the RNAi process is intrinsically limited. Endogenous triggers of RNAi and their associated RISC components have evolved to recognize and regulate targets with remarkably little complementarity (Bartel 2009). Only a seed match is sufficient in some cases to reduce expression of a complementary target. As we come to understand the biochemistry of RNAi in more detail, rules may emerge that minimize undesired side effects. However, at present, the best practice is to draw conclusions on the basis of multiple independent RNAi alleles that all behave in a manner consistent with the hypothesis being tested.

REFERENCES

Bartel DP. 2009. microRNAs: Target recognition and regulatory function. *Cell* **136:** 215–233.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. 2001a. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411:** 494–498.

Elbashir SM, Lendeckel W, Tuschl T. 2001b. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* **15:** 188–200.

Huesken D, Lange J, Mickanin C, Weiler J, Asselbergs F, Warner J, Meloon B, Engel S, Rosenberg A, Cohen D, et al. 2005. Design of a genome-wide siRNA library using an artificial neural network. *Nat Biotechnol* **23:** 995–1001.

Myers JW, Jones JT, Meyer T, Ferrel JE Jr. 2003. Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing. *Nat Biotechnol* **21:** 324–328.

Sadler AJ, Williams BR. 2007. Structure and function of the protein kinase R. *Curr Top Microbiol Immunol* **316:** 253–292.

Vert JP, Foveau N, Lajaunie C, Vandenbrouck Y. 2006. An accurate and interpretable model for siRNA efficacy prediction. *BMC Bioinformatics* **7:** 520.

Weitzer S, Martinez J. 2007. The human RNA kinase hClp1 is active on 3′ transfer RNA exons and short interfering RNAs. *Nature* **447:** 222–226.

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