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RNA Interference to Knock Down Gene Expression

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Abstract

RNA interference (RNAi) is a biological process by which double-stranded RNA (dsRNA) induces sequence-specific gene silencing by targeting mRNA for degradation. As a tool for knocking down the expression of individual genes post transcriptionally, RNAi has been widely used to study the cellular function of genes. In this chapter, I describe procedures for using gene-specific, synthetic, short interfering RNA (siRNA) to induce gene silencing in mammalian cells. Protocols for using lipid-based transfection reagents and electroporation techniques are provided. Potential challenges and problems associated with the siRNA technology are also discussed.

Keywords

RNA interference; RNAi; siRNA; Gene silencing; Transfection; Electroporation

1 Introduction

Specific inhibition or knockdown of gene expression in cultured cells has been widely used to study the effects of loss-of-function mutation in individual genes. Gene-specific degradation of mRNA is one way to silence individual gene expression post-transcriptionally. One of the most widely used technologies for induction of such gene-specific RNA degradation is the use of RNA interference (RNAi) technology. RNAi was first discovered in the nematode *C. elegans* as a response to small double-stranded RNA (dsRNA), which resulted in sequence-specific gene silencing [1].

RNAi is a multistep process. When dsRNA is introduced into cells, it is first recognized and processed into 21–23 base-pair small interfering RNAs (siRNA) by Dicer, a RNase III family ribonuclease. These short interfering RNAs are then incorporated into and direct the RNA-induced silencing complex (RISC) to the target RNA. RISC is a nuclease complex that is responsible for the ultimate destruction of the target RNA and gene silencing [2]. In 2001, Tuschl and colleagues [3] observed that transfection of synthetic 21 base-pair siRNA duplexes into mammalian cells effectively silences endogenous gene expression in a sequence-specific manner. This finding heralded the use of siRNA for gene silencing in mammalian systems. siRNA oligonucleotides (21–22 base pairs) can be generated by chemical synthesis [4] or by in vitro transcription using T7 RNA polymerase [5]. Alternatively, siRNAs can be endogenously expressed in the form of short hairpin RNA (shRNA), delivered to cells via plasmids or viral/bacterial vectors [6]. Chemically synthesized siRNAs are relatively simple and quick to generate. In recent years, a number of commercial manufacturers have started to offer siRNA oligonucleotide synthesis, which has greatly facilitated the use of synthetic siRNAs in research. In this chapter, I will focus on

procedures that utilize commercially synthesized siRNAs to knockdown gene expression in mammalian cells.

2 Materials

2.1 siRNA Oligonucleotides

1. Gene-specific siRNA oligonucleotides (*see Note 1*): siRNA sequences can be designed using freely available online tools and then custom-synthesized by commercial vendors (e.g., Integrated DNA Technologies or Thermo Fisher Scientific Inc.). Alternatively, predesigned or validated siRNA oligonucleotides for specific genes can be purchased from manufacturers (e.g., GE Healthcare Dharmacon Inc., QIAGEN, or Thermo Fisher Scientific Inc.).
2. Negative control (scrambled or non-targeting) siRNA oligonucleotides
Non-targeting siRNA oligonucleotides (*see Note 2*) are siRNAs that lack complementary RNA sequences in the targeting genome. These siRNAs serve as negative controls. They can be purchased from siRNA oligonucleotide manufacturers (e.g., GE Healthcare Dharmacon Inc., QIAGEN, or Thermo Fisher Scientific).
3. Positive control siRNA oligonucleotides
Positive control siRNA oligonucleotides (*see Note 3*) are siRNAs known to downregulate the expression of a specific gene.

2.2 Cell Culture Reagents

1. Mammalian cells.
Cells to be used to perform siRNA knockdown (*see Note 4*). Here, the pancreatic cancer cell line MIA PaCa-2 is used as an example.
2. Cell culture medium appropriate for the cells being cultured.
For MIA PaCa-2, we use RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (*see Note 5*).
3. Phosphate Buffered Saline (PBS), pH 7.4.
4. Gibco® Trypsin–EDTA solution.
This is a ready-to-use trypsin solution containing 0.025% trypsin and 0.01% EDTA in PBS.

2.3 Transfection Reagent

1. siLenFect™ Lipid Reagent (Bio-Rad Laboratories Inc) (*see Note 6*).
2. Amaxa Nucleofector™ Kit V (Lonza Cologne AG) (*see Note 7*).

2.4 Other Reagents/Equipment

1. RNase-free water.

2. RNA Oligonucleotide Annealing Buffer (5 ×) (*see* Note 8).
Potassium Acetate: 100 mM.
HEPES–KOH: 30 mM, pH 7.4.
Magnesium Acetate: 2 mM
3. Nucleofector™ Device (Lonza Cologne AG).

3 Methods

3.1 Design of Gene-specific siRNA sequence

1. Designing a highly effective and specific siRNA sequence is the Specific siRNA first step for successful knockdown of a target gene. Various Sequences groups have developed specific guidelines for designing siRNAs [7–9] and a number of online design tools are freely available (e.g., <http://sirna.wi.mit.edu>; <https://rnaidesigner.thermofisher.com/rnaexpress>; and <http://dharmacon.gelifesciences.com/design-center>).
2. Several siRNA manufacturers such as Thermo Fisher Scientific, QIAGEN, and GE Dharmacon have predesigned (and sometimes validated) siRNA sequences for most known genes in the human genome. Researchers only need to enter the gene name or ID online to order siRNA oligonucleotides specifically designed to target the gene of interest (*see* Note 9).

3.2 Preparation of 1. siRNA Solution

1. siRNA oligonucleotides purchased from commercial vendors (e.g., QIAGEN and Thermo Fisher Scientific) are usually ready-to-use duplex RNAs and do not need to be desalted or annealed. Simply resuspend the lyophilized siRNA duplex powder in RNase-free water to a final concentration of 20 μM. However, if the RNA oligonucleotides come as single-stranded RNA, then an annealing step is needed.
2. To anneal single-stranded RNA, resuspend the lyophilized siRNA powder received from the vendor in RNase-free water at a final concentration of 100 μM. Mix the solution well by pipetting up and down a few times. Aliquot the solution into new tubes in small volumes (e.g., 20 μL) and store at –20 °C, if not to be used immediately. Combine 20 μL of each complementary single-stranded siRNA oligonucleotides, 20 μL of 5× Annealing Buffer, and 40 μL RNase-free water. Mix the solution by pipetting up and down a few times. Incubate the solution at 90 °C for 2 min, and then slowly cool to room temperature by placing the tube in a large beaker containing room temperature water for about 1 h. Briefly centrifuge the tube to bring down all droplets from the sides and lid of the tube. The final concentration of the annealed siRNA duplex is 20 μM.
3. Aliquot the resuspended/annealed siRNA into new tubes and store at –20 °C. Do not freeze-thaw siRNA solution more than five times.

3.3 Delivery of siRNA into the Cells

Two methods have been widely used to deliver chemically synthesized oligonucleotides into mammalian cells: transfection and electroporation. Transfection uses a lipid carrier to facilitate the cellular uptake of siRNA. Electroporation uses powerful electric pulses to generate transient hydrophilic pores on the cell membrane and by doing so, allows the uptake of macromolecules, such as siRNA oligonucleotides. Here I describe the general procedures for performing these two methods (*see* Note 10).

3.3.1 Transfection Using siLenFect™—A number of lipid carriers (transfection reagents) specifically developed for siRNA oligonucleotides are commercially available. They often have different delivery efficiency in different cell types. Choosing the optimal transfection reagents for the cell type of interest may necessitate comparing reagents from different vendors. Transfection protocols vary from reagent to reagent and often require optimization for different cell types (and sometimes even different cell lines of the same type). In general, the manufacturer's recommended procedures should be used as a starting point for optimization. Here, I describe the procedures for siLenFect™ from BioRad Laboratory as a guide. The procedures are based on the instruction manual provided by the manufacturer with some modifications.

1. Grow MIA PaCa-2 cells in a T75 cell culture flask to 70–90% confluency. Wash the cells with 5 mL PBS twice. Add 1 mL of trypsin solution to the cells and incubate in a humidified CO₂ incubator for 5 min. Stop trypsinization by adding 10 mL of cell growth medium (RPMI-1640) containing 10% FBS. Transfer the cells and the media into a 15 mL conical tube and centrifuge the tube at 200 × g for 5 min to pellet the cells. Wash cell pellets twice with 5 mL PBS, and then resuspend cells in 10 mL serum-containing growth media. Count cells in a cell counter (e.g., the Cellometer by Nexcelom Bioscience).
2. Seed 0.5 to 1 × 10⁶ cells / flask (*see* Note 11) in 5 mL growth media containing 10% FBS in T25 cell culture flasks (*see* Note 12). Allow cells to grow overnight at 37 °C in a humidified 5% CO₂ incubator.
3. On the second day, 15–60 min before transfection, aspirate medium from the flask and add 2.5 mL fresh serum-containing growth medium to the cells.
4. For each T25 flask to be transfected, prepare 250 µL of transfection reagent solution in a 1.5 mL Eppendorf tube by adding 7.5 µL of siLenFect™ to 242.5 µL of serum-free medium (*see* Note 13).
5. For each T25 flask to be transfected, prepare 120 nM siRNA solution in 250 µL serum-free medium in a 1.5 mL Eppendorf tube (*see* Note 14). This can be done by first diluting the stock siRNA from 20 to 1 µM using serum-free medium (e.g., 5 µL of 20 µM siRNA plus 95 µL medium), and then further diluting it to 120 nM by taking 30 µL of the diluted siRNA (1 µM) and adding to 200 µL of cell-free medium (*see* Note 15).

6. Add the siRNA solution to the diluted siLenFect™ solution (*see* Note 16). Mix by tapping the tube or pipetting up and down. Incubate the mixed solution for 20 min at room temperature.
7. Add 500 µL of the siRNA/siLenfect™ complexes to the cells. Mix by rocking the flasks back and forth several times. Incubate the cells at 37 °C in a humidified 5% CO₂ incubator.
8. Twenty-four to seventy-two hours following transfection, harvest cells by trypsinization as described above (*see* Note 17) to assess knockdown efficiency or examine functional effects of gene knockdown.

3.3.2 siRNA Delivery Using Electroporation—Lipid-based transfection methods work efficiently for many cell lines. However, for some cell lines and cell types, particularly primary cells and suspension cells, these methods yield low efficiency. For those hard-to-transfect cells, electroporation-based methods are often used to deliver nucleic acids. However, electroporation can induce high cell mortality, and often requires careful optimization of electroporation parameters (voltage, electric pulse length, and pulse number) to achieve high efficiency and low cell mortality. Amaxa's Nucleofector™ (Lonza Cologne AG) technology is an advanced electroporation technology that has been widely used for delivery of siRNA and other nucleic acids to hard-to-transfect cells. The company has developed an extensive database of cell type-specific electroporation programs and solutions, which has minimized the optimization process for end users. Here I describe the general protocol for using the Amaxa Nucleofector™ device and kit to deliver siRNA, using MIA PaCa-2 cells as an example. This protocol is modified from the manual provided by the kit and device manufacturer (Lonza Cologne AG).

1. Grow MIA PaCa-2 cells in T75 cell culture flasks as described above.
2. On the day of transfection, preincubate 6-well plates containing 1.5 mL/well of serum-containing media at 37 °C in a humidified CO₂ incubator.
3. Harvest cells by trypsinization and count cells as described above.
4. Transfer cells to 15 mL conical tubes (1×10^6 cells per tube) and centrifuge at $100 \times g$ for 10 min at room temperature. Remove media by aspiration.
5. Resuspend the cells carefully in 100 µL room temperature Nucleofector® Solution V per sample. Do not leave the cells in the Nucleofector® Solution longer than 15 min (*see* Note 18).
6. Add 1.5 µL of siRNA (20 µM) to the cell suspension (for a final siRNA concentration of 300 nM) (*see* Note 19). Mix by pipetting up and down.
7. Transfer cell/siRNA mixture into a certified cuvette (included in the Nucleofector™ kit). Make sure the solution covers the bottom of the cuvette. Close the cuvette with the cap.
8. Insert the cuvette containing the cell/siRNA solution into the Nucleofector® Cuvette Holder. Select the Nucleofector® Program T-020 (*see* Note 20) and apply the program.

9. Remove the cuvette from the holder once the program is finished. Add 500 μ L of the preequilibrated culture media to the cuvette. Gently mix and transfer the solution to the pre-incubated 6-well plate. Use the pipettes supplied by the kit and avoid repeated aspiration of the solutions.
10. Incubate the cells at 37 °C in a humidified 5% CO₂ incubator.
11. Assess knockdown efficiency or examine functional effects of the knockdown 24–72 h following electroporation.

3.4 Assessment of Gene Knockdown Using Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Western Blotting

Because siRNA oligonucleotides target mRNA for degradation, Gene Knockdown RT-PCR can be used to measure effects on gene expression using Using Reverse negative control siRNA-treated cells and gene-specific, siRNA-trea-Transcription ted cells. Readers are referred to other literature for RT-PCR pro-Polymerase Chain tocols [10–12].

Although reduction in transcript expression usually results in Western Blotting decreased protein abundance, mRNA levels do not always correlate with protein levels. For example, mRNA measurement can overestimate knockdown of genes whose protein products have long half-lives. Therefore, it is necessary to assess protein levels to ensure efficient knockdown of gene expression and to determine the optimal time point for assessing cellular effects of siRNA knockdown. Western blotting is the most widely used technique for detecting proteins (*see* Note 21). Protocols for Western blotting can be readily found in the literature; for example, “Western Blotting: A guide to current methods” (edited by Hicklin T, 2015), found in a supplement to *Science magazine*.

3.5 Examination of the Functional Effects of siRNA Knockdown

Once knockdown of the siRNA-targeted gene is confirmed, assays can then be carried out to investigate resulting functional effects. Depending on the known or predicted functions of the target gene, a variety of assays (cell growth and survival, migration, apoptosis, effects on downstream signaling, etc.) can be used.

4 Notes

1. siRNA oligonucleotides designed to target different regions of a gene can have different knockdown efficiencies [13]. Although the current siRNA design algorithms are getting better at selecting efficient siRNA sequences, only about one in four siRNAs produces a knockdown efficiency of >80%. Therefore, it is imperative that multiple (usually 2–4) siRNA sequences for each target gene are obtained and optimized individually. Alternatively, multiple siRNAs can be pooled and used in a single transfection (e.g., GE Dharmacon offers pre-designed/pooled siRNA for human and other species).
2. A negative control siRNA is included in the experiment to distinguish sequence (or gene)-specific effects from non-sequence specific effects in the siRNA-treated cells. The negative control siRNAs can be siRNA sequences that have the

same nucleotide composition as the gene-specific siRNA, but lack significant sequence homology to the human genome or siRNAs that have been designed to have no known homology to the human genome (often called non-targeting siRNA). Non-targeting siRNAs are commercially available from a variety of vendors (e.g., QIAGEN or GE Dharmacon).

3. Positive control siRNAs are used to monitor the efficiency of siRNA delivery to cells. These are siRNA sequences known to induce reproducible knockdown of a gene in vitro. If the gene targeted by the positive control siRNA is essential for cell survival then knockdown of that particular gene will result in rapid cell death, and the efficiency of siRNA delivery can be evaluated under a microscope. We have found that siRNAs targeting the Ubiquitin B (UBB) gene or the AllStars Cell Death Control siRNA from QIAGEN are good positive controls that produce rapid cell death.
4. Cells should be evaluated for the expression level of the gene of interest. To optimize siRNA knockdown conditions, a cell line expressing relatively high levels of the target gene should be used.
5. The antibiotics penicillin and streptomycin are often added to culture medium to prevent bacterial contamination. However, because transfection reagents increase cell permeability, the delivery of antibiotics may also be increased, which could result in increased cytotoxicity. Therefore, adding antibiotics to the transfection medium is not recommended.
6. A number of lipid-based transfection reagents are commercially available. Their delivery efficiency varies and can be cell line-dependent. It is advisable to first consult the literature to determine if any other groups have reported siRNA transfection in the same cell lines/types, and then start with the same transfection reagents and conditions.
7. Lonza has developed five different Nucleofector™ Solutions designed to work for different cell lines/types. The manufacturer has also developed optimized protocols for a number of cell lines and primary cell types. Kits containing the optimized Nucleofector™ Solution recommended by the manufacturer should be purchased. If the cell line or type is not on the list with an optimized protocol, then an optimization kit should be obtained.
8. Other annealing buffers have also been reported in the literature, e.g., 50 mM Tris, pH 7.5–8.0, 100 mM NaCl, and 5 mM EDTA (5 ×).
9. Two to four siRNA sequences that target different regions of a gene of interest should be tested (*see* Note 1).
10. The two delivery methods have their own advantages and disadvantages [14]. The transfection method is simple and requires no specialized equipment, but it does not work well with primary cells and suspension cells. The electroporation method can achieve very high delivery efficiency, even in hard-to-transfect cells, although it often causes high cell death. It also requires specialized equipment (i.e., an electroporator). Selecting the right method will depend on the

- experimental conditions, such as the cells being used and the assays to be run after transfection.
11. The exact cell number to seed must be optimized for different cell lines. The goal is to achieve 50–70% confluency on the following day.
 12. Depending on the assays to be run after transfection, other culture vessels can be used. Depending on the surface area of the vessel, the amount of reagents may need to be scaled up or down. Refer to the manufacturer's manual for recommended medium volumes and the amount of reagents for different culture vessels.
 13. The amount of siLentfect™ may need to be optimized using a range of volumes from 2.5 to 20 μ L.
 14. The concentration of siRNA needed for efficient knockdown may vary depending on cell lines used and the gene target itself. It is advisable to optimize the concentration of siRNA by carrying out transfections using different siRNA concentrations ranging from 5 to 20 nM (final concentration).
 15. Master mix can be prepared if replicates of the same siRNA concentrations are being carried out.
 16. It is recommended that a mock transfection with only siLentfect™ (no siRNA added) be included as a control.
 17. Gene knockdown can be detected as early as 4 h and could last up to 5 days, and even 7 days in some cases [15]. However, in general, 24–96 h is the ideal time periods for accessing gene knockdown and investigating functional effects of the siRNA knockdown in cell culture. Cells can be retransfected with the siRNA to extend the duration of gene knockdown.
 18. Do not leave cells in the Nucleofector® Solution for longer than 15 min, as longer exposure may lead to reduced transfection efficiency and cell viability.
 19. The optimal siRNA concentration may vary from cell line to cell line. A range (30–300 nM) of concentrations should be used if an optimal concentration is not known.
 20. The manufacturer has optimized programs for a number of cell lines. Please refer to the manufacturer's website for details (<http://www.lonza.com/research/>).
 21. In addition to Western blotting, other methods such as immunofluorescence staining and ELISA (enzyme-linked immuno-sorbent assay) can be used to monitor the knockdown of gene expression by siRNA.

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