Silencer® siRNA Transfection II Kit

(Part Number AM1631)

Protocol

P/N 1631M Revision C **Revision Date: June 4, 2009**

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I. Introduction

A. Background

RNA interference (RNAi), or gene silencing, is a technique for down-regulating the expression of a specific gene in living cells by introducing a double-stranded RNA (dsRNA) that is complementary to a target mRNA of interest. It has been demonstrated that 21 basepair (bp) RNA molecules (small interfering RNA or siRNA) can be a potent mediator of the RNAi effect in mammalian cells (Elbashir et al. 2001).

The choice of a transfection agent for delivery of siRNA is critical for gene silencing experiments. Without efficient transfection, siRNA will fail to elicit a cellular response. Most commercially available transfection agents were developed for plasmid transfections and many are limited in their efficacy to only a few cell types. The *Silencer*® siRNA Transfection II Kit contains two different transfection agents and controls for optimizing siRNA transfection.

B. Product Description

Transfection agents

Because different cell types respond differently to transfection conditions, the Ambion *Silencer* siRNA Transfection II Kit includes two transfection agents, siPORT™ *NeoFX*™ and siPORT *Amine*. Each transfection agent has different properties to support siRNA transfection of a broad range of cell types with high efficiency and reproducibility. siPORT *NeoFX* is a proprietary mixture of lipids and siPORT *Amine* is a proprietary blend of polyamines. These reagents function by complexing with siRNAs and facilitating their transfer into cells. Both reagents are easy to use and have minimal cytotoxic effects.

Controls

This kit includes positive and negative siRNA controls for use in optimization experiments. The positive siRNA control, targeting the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, has been verified to induce silencing in human, mouse, and rat cell lines. The Negative Control siRNA is a scrambled sequence that bears no homology to the human, mouse, or rat genomes.

Reverse transfection

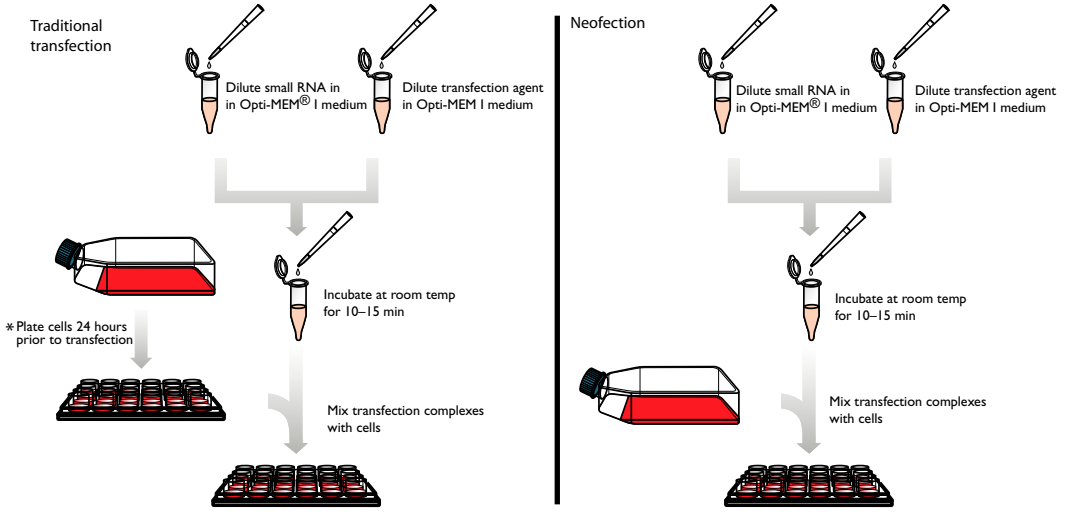
This Instruction Manual provides instructions for optimization by a newly developed transfection method—reverse transfection, or neofection. It is a transfection method in which cells are transfected as they adhere to a plate after trypsinization (Figure 1). This method bypasses several steps of the traditional "pre-plating" transfection method, making it faster and easier. We recommend neofection for convenience and ease. Furthermore, it can be used with both siPORT *NeoFX* and siPORT *Amine*, and it is effective in most cell types. Protocols for traditional transfection are provided in the Appendix (section IV.B on page 16).

Kit applications

The *Silencer* siRNA Transfection II Kit is designed for transfection of small RNAs, including siRNAs and miRNAs. This kit can also be used for co-transfection of DNA molecules, such as plasmids or PCR products (see *IV.C. Protocol Modification for Cotransfection* on page 18 for details).

This kit is primarily intended for transfection of adherent cells. In general, suspension cells are more difficult to transfect than adherent cells; we recommend electroporation using the Ambion siPORT™ siRNA Electroporation Buffer (P/N AM8990) for transfection of suspension and other hard-to-transfect cell types.

Figure 1. Overview of siRNA Transfection Protocol.



C. *Silencer*® siRNA Transfection II Kit Components and Storage

Amount	Component	Storage
400 µL	siPORT™ *NeoFX™* Transfection Agent	4°C
400 µL	siPORT *Amine* Transfection Agent	4°C*
50 µL	GAPDH siRNA, 20 µM	–20°C
30 µL	Negative Control siRNA, 20 µM	–20°C

* Do not freeze.

IMPORTANT

Keep the tubes of siPORT NeoFX and siPORT Amine tightly closed to prevent evaporation.

D. Required Materials Not Provided with the Kit

Cell culture material and equipment

- OPTI-MEM® I Reduced-Serum Medium (Gibco Invitrogen #31985-070)
- Routine tissue culture supplies and equipment

Detection of GAPDH silencing

Materials for measuring gene knockdown—knockdown can be assessed by measurement of GAPDH enzyme activity, Northern blot analysis, real-time RT-PCR, or indirectly by cell proliferation assays.

The Ambion KDalert™ GAPDH Assay Kit (P/N AM1639) provides a quick assay for both GAPDH gene knockdown and cell viability.

For silencing the target of interest

- siRNA to the gene of interest
- Reagents and equipment to detect gene silencing

E. Related Products Available from Applied Biosystems

siPORT™ *NeoFX™* Transfection Agent P/N AM4510, AM4511	siPORT *NeoFX* Transfection Agent was developed to streamline siRNA transfection procedures, cutting time and increasing reproducibility. This novel lipid-based formulation can be used to efficiently transfect adherent cells while subculturing, without increased cytotoxicity. This reagent is compatible with a wide range of cell lines and experimental designs, including high-throughput applications.
siPORT™ *Amine* Transfection Agent P/N AM4502, AM4503	siPORT *Amine* is an easy-to-use proprietary blend of polyamines that delivers siRNA into mammalian cells with minimal cytotoxicity.
siPORT™ siRNA Electroporation Buffer P/N AM8990	siPORT siRNA Electroporation Buffer is a low conductivity buffer optimized for delivery of siRNAs by electroporation. Designed to facilitate rapid resealing of pores induced by electroporation, siPORT siRNA Electroporation Buffer can be used with commonly available electro-pulse generators for highly efficient transfection with minimal cytotoxicity—even with primary cells, and other hard-to-transfect cell types.
Silencer® CellReady™ siRNA Transfection Optimization Kit P/N AM86050	The *Silencer*® CellReady™ siRNA Transfection Optimization Kit facilitates identification of optimal siRNA delivery conditions in a high-throughput format. Developed as a companion kit to the *Silencer* CellReady siRNA Libraries, the kit includes three 96-well *Silencer* CellReady Optimization Plates, each with 48 wells containing *Silencer* GAPDH siRNA and 48 wells plated with *Silencer* Negative Control #1 siRNA. It also includes Ambion's siPORT™ *NeoFX*™ lipid-based transfection agent, as well as step-by-step instructions for rapid and efficient optimization of transfection conditions. The conditions identified using the kit are generally applicable for siRNA transfections in 96-well plates.

KDalert™ GAPDH Assay Kit P/N AM1639	The KDalert GAPDH Assay Kit is a rapid, convenient, fluorescence-based method for measuring the enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cultured human, mouse, or rat cells. The KDalert GAPDH Assay Kit facilitates identification of optimal siRNA delivery conditions by assessment of GAPDH expression and knockdown at the protein level and integrates seamlessly with the *Silencer*® CellReady siRNA Transfection Optimization Kit (P/N AM86050) and *Silencer* GAPDH Control siRNAs (P/N AM4605, AM4624).
Silencer® siRNAs see our web or print catalog www.ambion.com/siRNA	Ambion's *Silencer* Pre-designed siRNAs, Validated siRNAs, and siRNA Libraries are designed with the most rigorously tested siRNA design algorithm in the industry. *Silencer* siRNAs are available for >100,000 human, mouse, and rat targets from our searchable online database. Because of their carefully optimized design, *Silencer* siRNAs are very effective, and they are guaranteed to reduce target mRNA levels by 70% or more. Furthermore, their exceptional potency means that *Silencer* siRNAs effectively induce RNAi at very low concentrations, minimizing off-target effects.
Silencer® siRNA Labeling Kits P/N AM1632, AM1634	The *Silencer* siRNA Labeling Kits are used for labeling siRNA synthesized with the *Silencer* siRNA Construction Kit or synthesized chemically. Labeled siRNA can be used to analyze the subcellular distribution of siRNA, in vivo stability, transfection efficiency, or the capability of the siRNA to attenuate target gene expression.
Silencer® siRNA Controls P/N AM4250–AM4639 see our web or print catalog www.ambion.com/siRNA	*Silencer* siRNA Controls are chemically synthesized siRNAs for genes commonly used as controls. Validated control siRNAs are available for genes such as GAPDH, β-actin, cyclophilin, KIF11 (Eg5), GFP, and luciferase. These siRNAs are ideal for developing and optimizing siRNA experiments and have been validated for use in human cells; many are also validated in mouse and rat cells.

II. Optimizing Transfection

A. Parameters That Impact Transfection Efficiency

Transfection agent

siPORT *Amine* or siPORT *NeoFX*

It is important to select the appropriate siPORT transfection agent for the cell line under study. Different cell types vary in their response to different transfection agents; thus, the best transfection agent for a particular cell type must be determined experimentally. See our website for cell lines tested at Ambion:

www.ambion.com/techlib/resources/delivery

Volume of transfection agent

The volume of transfection agent used is a critical parameter to optimize; too little can limit transfection, but too much can be toxic. The overall transfection efficiency is influenced by the amount of transfection agent complexed to the siRNA. This critical volume should be determined empirically for each cell line. A recommended range and starting volume are provided in the protocol.

Exposure to transfection agent

Although the siPORT transfection agents were designed to minimize cytotoxicity, exposing cells to excessive amounts of transfection agent or for extended time periods can be detrimental to the overall health of the cell culture.

siRNA

siRNA quality

The quality of siRNA can significantly influence RNAi experiments. siRNA should be free of reagents carried over from synthesis, such as salts and proteins. Also, dsRNA contaminants longer than 30 bp are known to cause cytotoxicity. We recommend using column, HPLC, or gel purified, chemically synthesized siRNAs to ensure quality and purity. Ambion offers an extensive line of options for chemically synthesized siRNAs; for more details see our website at:

www.ambion.com/siRNA.

siRNA quantity

The optimal amount of siRNA and its capacity for gene silencing are influenced in part by properties of the target gene, including the following: mRNA localization, stability, and abundance, and target protein stability and abundance. If too much siRNA is used for transfection, it may lead to off-target effects. Conversely, if too little siRNA is transfected, reduction of target-gene expression may be undetectable. Because there are so many variables involved, it is important to optimize the siRNA amount for every cell line used, and in some cases, it may even be necessary to re-optimize for different targets.

Cell culture

Use healthy cells

In general, healthy cells transfect better than poorly maintained cells. Routinely subculturing cells before they become overcrowded or unhealthy will minimize instability in continuous cell lines from experiment to experiment. Information on basic cell culture technique can be found in *Culture of Animal Cells: A Manual of Basic Technique* (Freshney, 2000).

Transfect cells within 10 passages of optimization experiments

Since cells may gradually change in culture, we recommend transfecting cells within 10 passages of determining optimal transfection conditions. If transfection efficiency begins to drop, fresh cells should be thawed for subsequent experiments.

Presence of serum in the medium during transfection

Both siPORT *Amine* and siPORT *NeoFX* are compatible with transfection in serum-containing normal growth medium. No culture medium replacement or addition is required after transfection.

B. Optimization Overview

The point of optimization is to determine the conditions that will provide good gene knockdown while maintaining an acceptable level of cell viability for the particular cell type. Some of the conditions that improve gene knockdown (e.g., amount of transfection agent) also result in decreased cell viability. Therefore, both gene knockdown and cell viability should be considered when interpreting optimization experiments—with a balance between the two representing the ideal conditions for transfection. Once optimal conditions are established, they should be kept constant between experiments for a given cell type.

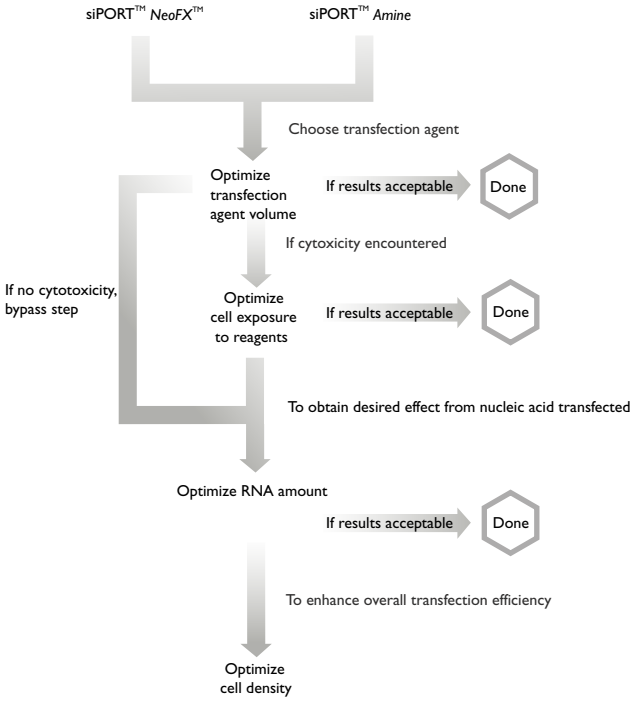


Figure 2. *Silencer*® siRNA Transfection II Kit Optimization.

First, follow the protocol in section *C. Initial Transfection Protocol to Select Transfection Agent* to evaluate the effectiveness of siPORT *Amine* and siPORT *NeoFX*. If one of the transfection agents yields acceptable results, no further optimization is needed. Otherwise, continue the optimizations outlined in section II.D using the transfection agent that performed the best in your initial screen. Once an acceptable level of knockdown and cell viability is obtained, no further optimization is needed.

Conduct optimization experiments with the control siRNAs

In each experiment, run controls in replicate using the GAPDH siRNA and the Negative Control siRNA.

When successfully transfected, the ***GAPDH siRNA*** reduces both the mRNA and protein levels of GAPDH in human, mouse, and rat cell lines. This slows the growth rate of the cells and reduces the rate of cell proliferation of most cell types.

The ***Negative Control siRNA*** is a scrambled sequence that has no significant homology to the human, mouse, or rat genome. The Negative Control siRNA should have no effect on the mRNA and protein levels of GAPDH; it serves as a baseline for measuring the effects of the GAPDH siRNA. The negative control can be used to identify

nonspecific effects such as nonsequence-specific siRNA effects, cytotoxicity of the transfection agent and/or the siRNA, or suboptimal transfection conditions.

Include a buffer-only control

To control for any nontransfection related phenomena, always include a sample well that is mock-transfected with OPTI-MEM medium and siRNA buffer, but lacks transfection agent and siRNA.

C. Initial Transfection Protocol to Select Transfection Agent

For the most rapid assay development, we provide suggested initial conditions. Use these conditions in your first experiments and optimize as needed based on the results.

1. Prepare cells

a. Trypsinize adherent cells.

Trypsinize healthy, growing, adherent cells using your routine procedure.

b. Resuspend cells to 1 x 10^5 cells/mL in normal growth medium.

Inactivate trypsin by resuspending the cells in normal growth medium to reach a concentration of 1 x 10^5 cells/mL. Keep the cells at 37°C while preparing the transfection complexes (below).


NOTE

Cells should be transfected before they re-adhere; proceed immediately with the following steps.

2. Prepare RNA/transfection agent complexes and distribute into culture plate wells

The instructions below give examples of reagent amounts to use per well for 96-, 24-, 12-, or 6-well plates.

- When possible, prepare master mixes to minimize variability.
- Bring the transfection agent and the OPTI-MEM I medium to room temperature before use. Briefly centrifuge the transfection agent before use to pool liquid at the bottom of the tube.
- For preparation of RNA and reagent dilutions, use a sterile polystyrene culture plate (round or V-bottom) or 12 x 75 mm tubes.

a. Dilute the transfection agent in OPTI-MEM I medium.

siPORT *NeoFX* Transfection Agent				
Reagent	96-well	24-well	12-well	6-well
siPORT *NeoFX* (µL)	0.5	1.5	3	5
OPTI-MEM I to (µL)	10	25	50	100

OR

siPORT *Amine* Transfection Agent				
Reagent	96-well	24-well	12-well	6-well
siPORT *Amine* (µL)	0.3	1.5	3	5
OPTI-MEM I to (µL)	10	25	50	100

b. Incubate 10 min at room temp.

c. Dilute the RNA in OPTI-MEM I medium for a final concentration of 30 nM in the transfection

Reagent	96-well	24-well	12-well	6-well
20 µM small RNA (µL)	0.15	0.75	1.5	3.75
OPTI-MEM I to (µL)	10	25	50	100

d. Mix diluted RNA and diluted transfection agent. Incubate at room temp for 10 min.
Combine the diluted transfection agent from step a with the diluted RNA from step c. Mix by pipetting up and down or flicking the tube. Incubate 10 min at room temp (this allows transfection complexes to form).

e. Dispense the RNA/transfection agent complexes into the empty wells of a culture plate.

3. Mix cells with the RNA/transfection agent complexes

a. Transfer cells to the culture plate containing the RNA/transfection agent complexes.
Gently mix the cells prepared in step 1 to resuspend any that have settled, and pipet them into the culture plate containing RNA/transfection agent complexes.

Reagent	96-well	24-well	12-well	6-well
Cell Volume (µL)	80	450	900	2300
Cells per well	8×10^3	4.5×10^4	9×10^4	2.3×10^5
Transfection Complex (µL)	20	50	100	200
Final Transfection Volume (µL)	100	500	1000	2500

b. Gently mix the cells and RNA/transfection agent complexes.
Rock the plate gently back and forth to evenly distribute the complexes; avoid swirling, as this can cause the transfection complexes to aggregate in the center of the well.

4. Incubate at 37°C

Incubate the transfection mixture at 37°C until ready to assay.

5. Assay for transfection efficiency and cytotoxicity

a. Assay for target-gene activity and cell viability.
For initial experiments, we recommend analyzing mRNA ~48 hr after transfection or cell proliferation and protein ~72 hr after transfection. Assess both the knockdown and cell viability (see section *IV.A. Assessing Transfection Efficiency* on page 14).

b. Determine if further optimization is needed.
You should expect ≥70% knockdown with ≤15% cell death from a successful transfection. If neither siPORT *Amine* or siPORT *NeoFX* gives acceptable results, use the transfection agent that gave the best results in this initial screen for subsequent optimization (section *II.D. Optimizing siRNA Transfection Conditions* starting on page 10).

D. Optimizing siRNA Transfection Conditions

Table 1 gives suggested ranges of transfection agent volume, RNA amount, and cell number to evaluate in optimization experiments.

Table 1. Suggested reagent amounts per well for optimization.

Parameter for optimization	Culture Plate Type			
	96-well	24-well	12-well	6-well
Transfection Agent	0.15, 0.3, 0.6, 1.2 µL	0.5, 1, 2, 4 µL	1, 2, 4, 6 µL	3, 5, 7, 9 µL
RNA (20 µM)*	0.005–0.15 µL	0.025–0.75 µL	0.05–1.5 µL	0.125–3.75 µL
Cells per well	0.5–1 x 10^4	3–6 x 10^4	0.75–1.5 x 10^5	1.5–3 x 10^5
Replace medium	8–24 hr after transfection (if cytotoxicity is observed).			

* For ease of handling if you are not preparing master mixes, dilute the siRNA to 2 µM and use ten-fold the volume shown in the table above. Test 1–30 nM final concentration.

1. Amount of transfection agent

The most important parameter for optimization of siRNA delivery is the amount of transfection agent used.

a. Follow the protocol in section II.C (using 10 nM final concentration of siRNA) to test 4 different volumes of transfection agent in step II.C.2.

b. Assay for target knockdown and cytotoxicity.
   - If acceptable levels of knockdown and cell viability are obtained, no further optimization is necessary.
   - If excessive cytotoxicity is observed, proceed to *step 2*.
   - If acceptable levels of cell viability are obtained, but knockdown is insufficient, proceed to *step 3*.

2. Exposure time to transfection agent (if needed)

After determining the optimal volume of transfection agent for target knockdown, maximize cell viability by adjusting the time that cells are exposed to transfection complexes.

a. Replace the medium at 6 hr and 24 hr after transfection by carefully aspirating the old medium from the well and adding fresh medium. It is usually not necessary to wash cells.

b. Re-evaluate knockdown and cytotoxicity.
   - If knockdown and cell viability are acceptable, no further optimization is necessary.
   - If knockdown requires further optimization, proceed to *step 3*.

3. Amount of siRNA

To optimize the activity of transfected siRNAs, test 1, 3, 10, and 30 nM (final concentration) siRNA, using the transfection agent quantity and exposure time optimized in the experiments described above.

If knockdown levels are still not acceptable, proceed to *step 4*.

4. Cell density

For most adherent cells, the optimal confluency for transfection is 30–80%. The table below provides guidelines for seeding different sized culture plates to obtain 30–80% confluency after 24 hr of growth; these numbers are approximate because the exact number of cells required for seeding and transfection depends on cell type, size, and growth rate.

Plate Type	Number of Cells
96-well	0.2–2 x 10^4
24-well	0.2–1 x 10^5
12-well	0.5–2 x 10^5
6-well	1–5 x 10^5

- Follow the transfection protocol in section II.C, using the conditions optimized using the experiments described above while varying the cell plating density across the wells of the culture dish so that cells will reach between 30–80% confluency.
- Be sure to monitor cell viability during these experiments, as cell cultures can become unstable at low densities.
- Use the siRNA concentration optimized in *step 3*.

The optimal cell plating density results in the greatest reduction in GAPDH expression without creating instability in the cell line.

5. Test siRNA of interest

Once an optimized protocol has been developed for a particular cell type, design and test siRNAs against your gene of interest. To identify an optimal siRNA and avoid off-target effects, we recommend testing 3 siRNAs per gene of interest. Perform transfections using the parameters established by these optimization experiments.

III. Troubleshooting

A. No Detectable Gene Silencing

The transfection protocol requires optimization

We strongly recommend that you optimize the transfection protocol for each cell type using the control siRNAs as described in section II.D starting on page 10.

Problems with siRNA/transfection agent complex formation

Follow the instructions for transfection complex formation closely; using the appropriate incubation times is important for good transfection efficiency.

Serum, polyanions, or other inhibitors were present during complexing.
Although both transfection agents are compatible with serum during transfection, neither is compatible with serum during complex formation. Use OPTI-MEM I reduced serum medium for siRNA/siPORT transfection agent complex formation.

Do not overmix.
It is important to ***gently*** mix the siRNA with the diluted transfection agent in step 2.d on page 9 of the reverse transfection protocol, or 2.d on page 17 of the traditional protocol.

Inactivated transfection agent

Store both siPORT *Amine* and siPORT *NeoFX* at 4°C. Do not allow siPORT *Amine* to freeze. Tightly cap tubes, because evaporation can significantly impact the activity of the transfection agents.

siRNA is degraded due to poor handling or storage

Check the integrity of the siRNA by running ~2.5 µg on a non-denaturing 15–20% acrylamide gel. Visualize the siRNA by staining with ethidium bromide, and verify that it is the expected size and intensity. The siRNA should migrate as a fairly tight band; smearing would indicate degradation.

Cells have been subcultured too many times or have undergone changes

Transfect cells within 10 passages of optimization experiments.

B. Transfection Causes Extensive Cell Death

Too much transfection agent was used

Titrate transfection agent over a broad dilution range, and choose the most dilute concentration that still gives good gene knockdown.

Cells were exposed to transfection agent/siRNA complex for too long

Sensitive cells may begin to die from exposure to the transfection agent after a few hours. If transfection causes excessive cell death with your cells, remove the transfection mixture, and replenish with fresh growth medium after 8–24 hours.

Cells are stressed

- Add fresh growth medium as early as 8 hr after transfection.
- Avoid using antibiotics when plating cells for transfection, and for at least 72 hr after transfection.
- Use healthy cells that have not been grown to the point of medium depletion between subculturing events.
- Avoid subjecting cells to frequent temperature and pH shifts.

The target gene is critical for cell survival

If the target gene is critical for cell survival, reducing its expression could cause cell death. If this is the case, assay mRNA levels at earlier time points (4–24 hr).

C. Gene Silencing Experiments Lack Reproducibility

Transfection complexes were not adequately mixed with cells

Distribute transfection agent/RNA complex by gently rocking the plate back and forth. Do not swirl plates to mix, because this could concentrate cells and/or reagents in the center of the wells.

There were differences in the experimental procedure

The time of transfection after cell plating, incubation times, master mix volumes, and the order of component addition can all affect transfection efficiency. To obtain reproducible results in experiments involving transfection, conduct experiments exactly the same way every time.

Cell density is too low

Optimize cell density as described in section II.D.4 on page 11. When cell density is too low, cell cultures can become unstable. This instability can vary from well to well because conditions (pH, temperature, etc.) may not be uniform across a multi-well plate, and can differentially influence unstable cultures.

Cells were passaged too many times

Repeat experiment using cells that have been subcultured fewer times.

IV. Appendix

A. Assessing Transfection Efficiency

To evaluate the results of optimization experiments, evaluate both the transfection efficiency (as measured by target knockdown) and cytotoxicity (as measured by cell death) of each transfection.

Target knockdown can be assessed by looking at either mRNA levels or protein levels. Determine the siRNA-induced suppression of GAPDH mRNA or protein levels, relative to the Negative Control siRNA. The Negative Control siRNA should have no effect on the mRNA and protein levels of GAPDH; its purpose is to serve as a baseline for measuring the effects of the GAPDH siRNA. In general, successful knockdown will cause a more dramatic reduction in target mRNA levels than in target protein levels.

Cytotoxicity can be evaluated by measuring the viability of Negative Control siRNA-transfected cells under each transfection condition.

Both target knockdown (at the protein level) and cytotoxicity can be assessed in the same experiment by measuring GAPDH enzyme activity using the Ambion KDalert GAPDH Assay Kit (P/N AM1639), described below. Other methods for separately determining target knockdown or cell viability are described as well.

Using the KDalert Kit to monitor cytotoxicity and GAPDH knockdown

The KDalert GAPDH Assay Kit streamlines determination of optimal transfection conditions by providing a rapid, convenient, fluorescence-based or colorimetric method for measuring the enzymatic activity of GAPDH in cultured cells derived from human, mouse, and rat. The general strategy for using the KDalert GAPDH Assay is provided below; detailed instructions are provided with the KDalert Kit.

GAPDH activity as a measure of cytotoxicity

GAPDH enzyme levels per cell are fairly constant in untransfected cells or in cells transfected with Negative Control siRNA. GAPDH activity in Negative Control siRNA-transfected cells can be used as an indicator of cell number, or of relative viability, under the transfection conditions being tested.

GAPDH activity as a measure of gene knockdown

The GAPDH protein knockdown for a given transfection condition is determined from the ratio of GAPDH activity in cells transfected with GAPDH siRNA to that in cells transfected with Negative Control siRNA.

Determining optimal transfection conditions with the KDalert Kit

Optimal transfection conditions are those which maximize specific target knockdown while minimizing transfection-associated toxicity. Target knockdown and cell viability can be measured using the KDalert GAPDH Assay, as described above, and then mathematically evaluated to easily determine the best transfection conditions; see the KDalert Protocol for details.

Other cell viability assays

There are many ways to assess cell viability; any established method that is appropriate for the cells in the experiment can be used. For cell viability assays to be valid, it is important to accurately count cells prior to transfection to establish a baseline viable-cell number.

a. Check visual appearance of cells for evidence of cell necrosis and/or apoptosis.

b. Measure cell viability (or total cell number) using any of the following methods:
   - Trypan blue exclusion assay
   - Alamar Blue assay
   - Acid Phosphatase or Alkaline Phosphatase assay
   - Flow cytometry
   - Fluorescence microscopy

Other assays for gene knockdown

Quantitating mRNA levels by Northern analysis or RT-PCR

Determine the siRNA-induced suppression of GAPDH mRNA or protein levels, relative to the Negative Control siRNA. We recommend analyzing 18S rRNA levels as a means to normalize the GAPDH mRNA data. The GAPDH siRNA typically reduces GAPDH mRNA levels 70–90%, 48 hr after transfection. Either of the following methods can be used to determine mRNA levels:

a. Northern blot: Follow standard Northern procedures. Alternatively, Ambion offers kits and reagents for probe-labeling and Northern blotting (see our catalog for details). We recommend using the Ambion RNAqueous® Kit (P/N AM1912) or RiboPure™ Kit (P/N AM1924) for RNA isolation.

b. Real-time PCR: We recommend the following products to facilitate real-time PCR analysis of RNAi.
   - The Ambion RNAqueous® Kit (P/N AM1912) or RiboPure™ Kit (P/N AM1924) for RNA isolation followed by the RETROscript® (P/N AM1710) procedure to produce cDNA.
   - The Ambion Cells-to-Signal™ Kit (P/N AM1724, AM1726) for synthesis of cDNA directly from cell lysates without RNA isolation—the cDNA or the lysate itself can then be used in real-time PCR.

- Applied Biosystems' TaqMan® Gene Expression Assays, an extensive collection of gene-specific primer-probe sets for real-time PCR (www.allgenes.com).

Quantitate protein levels by Western blot, immunohistochemistry, or immunofluorescence

The knockdown can also be determined from protein levels, by Western blot or immunostaining. In a successful transfection with the GAPDH siRNA, protein levels are typically reduced by 70–90%, 72 hr after transfection. Ambion offers select antibodies for siRNA research (anti-GAPDH, mouse monoclonal, P/N AM4300).

B. Traditional "Pre-plating" Transfection Protocol

The following protocol is a traditional "pre-plating" method. It requires more time than reverse transfection, but may be more effective with some cell types.

Table 2. Approximate Reagent Amounts per Well.

Protocol step	Reagent	96-well	24-well	12-well	6-well
1. Cell plating	Plate cells (per well)	0.2–1 x 10^4	2–10 x 10^4	0.5–2 x 10^5	1–5 x 10^5
2. Prepare siRNA/transfection agent complexes	Dilute Transfection agent	0.15–1.2 µL	0.5–4 µL	1–6 µL	3–9 µL
	in OPTI-MEM I to:	to 10 µL	to 25 µL	to 50 µL	to 100 µL
	Dilute small RNA (20 µM)*	0.005–0.15 µL	0.025–0.75 µL	0.05–1.5 µL	0.125–3.75 µL
	in OPTI-MEM I to:	to 10 µL	to 25 µL	to 50 µL	to 100 µL
3. Transfect cells	Adjust medium in wells to:	80 µL	450 µL	900 µL	2300 µL
	Final transfection volume	~100 µL	~500 µL	~1000 µL	~2500 µL
	Add fresh normal growth medium after 8–48 hr	100 µL	0.5–1 mL	1–2 mL	1–3 mL

* This gives a final concentration of 1–30 nM. If not preparing a master mix, we recommend diluting the stock siRNA to 1–2 µM using nuclease-free water or OPTI-MEM I for easier handling.

IMPORTANT

The volumes and amounts in the following protocol are for transfection in a 24-well plate.

1. Cell plating

a. Approximately 24 hr before transfection, plate cells in normal growth medium (e.g., DMEM, 10% FBS) so that they will be 30–80% confluent after 24 hr. (see Table 2 on page 16 for an estimate of how many cells to plate).

b. Incubate the cells overnight under normal cell culture conditions.

2. Prepare siRNA/transfection agent complexes

a. Briefly vortex the transfection agent before use.

b. Dilute the transfection agent into OPTI-MEM I reduced serum medium.

   i. In a sterile, round-bottom (or V-bottom) 96 well tissue culture dish or in sterile polystyrene tube, dilute 1–3 µL of transfection agent dropwise into OPTI-MEM I reduced serum medium for a final volume of 25 µL.

   ii. Vortex well, and then incubate at room temp 10–15 min.

c. Dilute 0.025–0.75 µL of 20 µM siRNA (for a final concentration of 1–30 nM) into OPTI-MEM I for a final volume of 25 µL.

d. Add diluted siRNA to diluted transfection agent; mix by gently flicking the tube or pipetting.

e. Incubate at room temp for 10–15 min.

3. Transfect cells

a. Adjust the volume of normal growth medium (e.g., DMEM, 10% FBS) in a well containing cells to 450 µL.

b. Add the transfection agent/siRNA complex from step 2.e dropwise to the cells (the final transfection volume will be 500 µL).

c. Without swirling, gently rock the dish back and forth to evenly distribute the complexes.

d. Incubate cells under normal cell culture conditions for 48 hr.

e. 0.5–1 mL fresh normal growth medium may be added to each well after 8–48 hr to maximize cell growth and prevent potential cytotoxicity.

4. Assay for target gene activity 8–72 hr after transfection

For initial experiments, we recommend analyzing mRNA ~48 hr after transfection or protein ~72 hr after transfection. Assess both knockdown and cell viability (see section *IV.A. Assessing Transfection Efficiency* on page 14).

Plasmid DNA can be cotransfected with small RNAs using the *Silencer* siRNA Transfection II Kit. Typically, both circular and linearized plasmids less than 10 kb can be effectively transfected. Plasmids larger than 10 kb must be linearized for efficient transfection.

C. Protocol Modification for Cotransfection

To cotransfect plasmid DNA and siRNA using the *Silencer* siRNA Transfection II Kit, modify the standard protocol as follows:

Add DNA with the RNA in step 2.c on page 9 of the protocol. Continue the protocol as described.

Optimization

It is important to optimize the amount of DNA used in transfection. Transfection efficiency depends more on the ratio of DNA to transfection agent (µg/µL) than on the overall amount of DNA. For more information on optimizing DNA transfection see the siPORT *XP-1* manual at:
www.ambion.com/catalog/CatNum.php?4506

D. References

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

Freshney RI (2000) *Culture of Animal Cells: A Manual of Basic Technique*, 4th Edition, New York (NY):Wiley-Liss.

E. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chem-

ical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com), telephone (650-554-2756; USA), or fax (650-554-2252; USA) your request, specifying the catalog or part number(s) and the name of the product(s). The associated MSDSs will be e-mailed unless you request fax or postal delivery. Requests for postal delivery require 1 to 2 weeks for processing.

Note: For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

F. Quality Control

Functional testing

Cells are transfected with gene-specific siRNA or Negative Control siRNA using siPORT *NeoFX* or siPORT *Amine* transfection reagent. The percent knockdown of the target genes (GAPDH for siPORT *NeoFX*, luciferase for siPORT *Amine*) is evaluated to ensure that each lot of siPORT *NeoFX* or siPORT *Amine* Transfection Reagent can efficiently deliver siRNAs.

RNase activity testing

The siPORT *NeoFX* and siPORT *Amine* transfection agents are tested for RNase using the Ambion RNaseAlert® assay.

The GAPDH siRNA and Negative control siRNA are tested for RNase activity: Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.