siPORT™ siRNA Electroporation Kit

(Part Number AM1629)

Protocol

P/N 1629M Revision B **Revision Date: November 4, 2008**

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I. Introduction

A. Background

RNA interference (RNAi) is a powerful experimental tool for reducing the expression of specific genes. Small interfering RNA (siRNA) introduced into the cellular cytoplasm are used by the RNA-induced silencing complex (RISC) to cleave target mRNA (Elbashir 2001). Experiments involving siRNAs have been mostly limited to immortalized cell lines, because these cells are relatively easy to grow, maintain, and transfect. However, primary cells are often a preferable model for studying gene function because they are more similar to their in vivo counterparts than immortalized cell lines. Under the proper conditions, electroporation provides a highly efficient method for direct transfer of siRNAs into primary cells.

Electroporation has long been used as a method for introducing plasmids into cells and is now being applied to siRNAs. Electroporation involves applying an electric field pulse to induce transient cell membrane permeability and the formation of microscopic pores in the cell membrane allowing molecules, ions, water and nucleic acids to traverse the membrane (Gehl 2003).

When electroporation is used to introduce a plasmid into cells, it is acceptable to use harsh conditions that cause significant cell damage and death, because the cells can then be allowed to recover for generations before experiments are performed on them, while maintaining the plasmid through selection. However, since the siRNAs do not replicate with the cell, as do plasmids, the effects of an siRNA need to be tested soon after transfection to avoid dilution of the siRNA by cell division. The cells must also be healthy so that any effects observed can be attributed to the siRNA and not to the electroporation process itself.

Fortunately, siRNAs are significantly more amenable to electroporation than plasmid DNA, allowing the use of mild conditions that maintain the health of the cell. This difference is due to the fact that siRNAs are small and merely need to enter the cellular cytoplasm to be functional, whereas plasmid DNA is much larger and must pass through the cytoplasm and enter the cell nucleus before it becomes active.

This product was designed only for electroporation of siRNA into cells and is not recommended for electroporation of DNA.

B. Product Description

The siPORT™ siRNA Electroporation Kit provides reagents and procedures for optimizing siRNA delivery conditions in primary, neuronal, and hard-to-transfect cell types.

The key component of the siPORT siRNA Electroporation Kit is an optimized siRNA Electroporation Buffer which is extremely effective for several reasons:

- It contains components that ensure high cell viability and enhanced delivery of siRNAs into cells.
- It is a low-conductivity pulse media that is designed to emulate the natural cytoplasmic composition. This minimizes changes in the composition of the cell when pores are opened in the cell membrane, and eliminates arcing while the electric field is applied.
- It was developed to resist temperature shifts and improve cell survival.
- It is nuclease-free, to prevent degradation of siRNAs prior to electroporation and to ensure delivery of intact siRNAs.

The siRNA Electroporation Buffer is compatible with most of the commercially available electroporators (see section I.D). Unlike many electroporation procedures that recommend using $\geq 10^6$ cells per reaction, the siRNA Electroporation Buffer can accommodate as few as 75,000 cells per reaction.

Since electroporation conditions need to be optimized for the particular cell type under study, the siPORT siRNA Electroporation Kit provides two siRNAs and one Negative Control siRNA that can be used to optimize electroporation conditions. The Cy™3 labeled Control siRNA is used for early-stage experiments to identify electroporation conditions for maximal uptake of siRNAs by cells while retaining high cell viability. The GAPDH and Negative Control siRNAs are used to fine-tune the electroporation conditions to ensure optimal silencing of target gene expression.

C. siPORT siRNA Electroporation Kit Components and Storage Conditions

Store kit components tightly capped.

The 4.5 mL of siRNA Electroporation Buffer is sufficient for up to 60 electroporation reactions in a standard 1 mm cuvette.

Amount	Component	Storage
3 x 1.5 mL	siRNA Electroporation Buffer	4°C*
75 µL	50 µM GAPDH siRNA	–20°C
38 µL	50 µM Negative Control siRNA	–20°C
2.1 nmol	Cy3 labeled Control siRNA Resuspend in 42 µL Nuclease-free Water before use.	–20°C †
1.75 mL	Nuclease-free Water	any temp‡

* ***Do not freeze*** the siRNA Electroporation Buffer.
† Store Cy3 labeled Control siRNA away from light.
‡ Store Nuclease-free Water at –20°C, 4°C or room temp

GAPDH siRNA and Control siRNAs are available separately from Applied Biosystems.


NOTE

The siRNA Electroporation Buffer is susceptible to microbial growth; be careful to maintain sterility.

D. Materials Not Provided with the Kit

- Electropulse generator
  The following electroporators are fully compatible with the siPORT siRNA Electroporation Kit:
  – Gene Pulser Xcell™ (Bio-Rad®)
  – ECM830 (BTX®)
  – PA-4000S PulseAgile® (Cyto Pulse Sciences™, Inc.)
  The siRNA Electroporation Buffer is ***not*** compatible with the Nucleofector (Amaxa biosystems).
- Electroporation cuvettes: The procedures in this Protocol are written for standard 1 mm cuvettes.
- Cell culture equipment and supplies
- (Optional) Fluorescence microscopy supplies
- siRNA to the gene of interest
- (Optional) Supplies for Northern, Western, or real-time RT-PCR analysis. See section *II.B. Optimizing Electroporation* on page 7 for a discussion of analysis options.

E. Related Products Available from Applied Biosystems

Silencer® siRNAs see our web or print catalog www.ambion.com/siRNA	Ambion *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.
Control siRNAs see our web or print catalog	Ambion offers validated siRNAs to several common housekeeping genes for use as positive controls, as well as scrambled siRNA sequences that have no significant homology to mouse, rat, or human gene sequences for use as negative controls. For more information, see www.ambion.com/siRNA.
Silencer® siRNA Libraries see our web or print catalog	Ambion offers siRNA libraries to select gene groups. All of the siRNAs included in the Silencer siRNA Libraries have been designed using a proven algorithm developed by Ambion's partner, Cenix BioScience. This design algorithm yields a high percentage of active siRNAs. Multiple siRNAs per target are provided for enhanced confidence in gene silencing data. See our website for more information at: www.ambion.com/siRNA
NorthernMax® Kits P/N AM1940, AM1946	Ambion NorthernMax Kits: NorthernMax, and NorthernMax-Gly, combine ultrasensitive, reliable Northern blot protocols with unsurpassed quality control to ensure optimal results in less time.
RNAqueous® RNA Isolation Kits P/N AM1911, AM1912, AM1914, AM1920	RNAqueous Kits employ a simple and rapid procedure to purify total RNA from source material without using organic solvents (such as phenol). There are specialized RNAqueous Kits for several different applications. RNAqueous and RNAqueous®-Midi are designed for isolating RNA on a small or large scale respectively. RNAqueous®-4PCR is the kit of choice to isolate RNA that will be used in RT-PCR; it incorporates a DNase digestion and a novel reagent for rapid and safe removal of the DNase and divalent cations. RNAqueous®-96 and RNAqueous®-96 Automated bring the RNAqueous system to 96-well plate configuration.
Antibodies for siRNA Research see our web or print catalog	For select *Silencer* Control and Validated siRNAs, Ambion offers corresponding antibodies for protein detection. These antibodies are ideal for confirming mRNA knockdown results by analyzing concomitant protein levels.

II. Optimizing Transfection

A. Parameters That Impact Electroporation Efficiency

Electroporation conditions

Optimal electroporation conditions vary between different cell types; to ensure maximum delivery of siRNAs by electroporation, a few critical electroporation parameters (voltage, pulse length, number of pulses, and siRNA concentration) should be optimized for each cell type used.

Ambion scientists have optimized electroporation conditions for several cell types; these conditions are provided on our website. If you are working with one of these cell types, you can save time by using the electroporation conditions provided. Related cell types are expected to require similar electroporation conditions. See the website below:

www.ambion.com/techlib/resources/transfection

Voltage

The voltage applied to the cells over a given distance is referred to as the field strength (V/cm), and it has a strong influence on overall cell survival and transfection efficiency. When the field strength of the pulse is high enough, reversible permeation occurs in the cell membrane that allows outside molecules to enter the cell. This event is dependent on several factors including cell diameter, temperature, and the gap-width of the cuvette. In general, optimal transfection efficiency is achieved at higher voltages. On the other hand, increasing the voltage can cause cell damage and increase the temperature of the reaction, which combine to reduce cell viability.

Pulse length

The duration of the electrical current applied is referred to as pulse length. The best pulse length is largely dependent on cell diameter. In general, larger cells require longer pulses for successful permeation of the membrane.

Number of pulses

For most mammalian cell types one pulse is usually sufficient for achieving at least 70% reduction of target gene expression using the GAPDH siRNA provided. Multiple pulses can be used to increase the level of siRNA-induced gene silencing, though it often reduces cell viability (see Figure 2 on page 9).

Cell cultures

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 the book *Culture of Animal Cells: A Manual of Basic Technique* (Freshney 2000).

Electroporate primary cells within 10 passages

Since cells may gradually change in culture, we recommend electroporating primary cells within 10 passages of determining optimal electroporation conditions (section II.B). If electroporation efficiency begins to drop, fresh cells should be thawed for subsequent experiments.

Purity, concentration, and size of siRNA to be transfected

Purity

The quality of siRNAs can significantly influence RNAi experiments. The siRNAs should be free of reagents carried over from synthesis including salts, proteins, etc. Also, dsRNA contaminants larger than approximately 30 bp are known to cause cytotoxicity. We recommend ordering standard desalted, or more highly purified siRNAs.

Concentration

The optimal siRNA concentration 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 in electroporation, it may be toxic. Conversely, if too little siRNA is transfected, reduction of target gene expression may be undetectable. The optimal amount must be determined empirically by varying the siRNA amount within a limited range (Figure 1 on page 6).

Size

Transfection efficiency is affected by the size of the nucleic acid electroporated; electroporator settings optimized for plasmids will usually not be effective in siRNA electroporation.

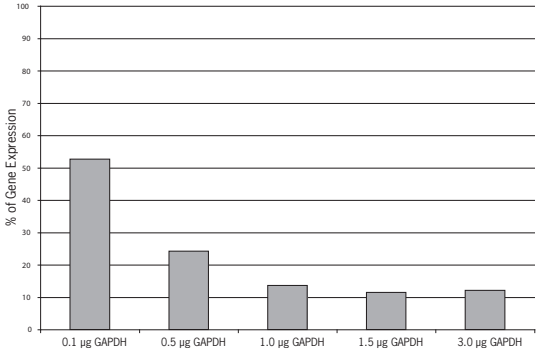

Figure 1. GAPDH Gene Silencing with Varying Amounts of siRNA.

Primary Human Mesenchymal Stem Cells (hMSC) were electroporated with varying amounts of siRNA (0.1–3 µg in 75 µL) targeting GAPDH or a scrambled sequence. 48 hours after transfection, cells were obtained and analyzed by real-time RT-PCR for both GAPDH mRNA and 18S rRNA levels (normalization control). "Percent gene expression" was calculated as a percentage of gene expression compared with the scrambled negative control siRNA.

B. Optimizing Electroporation

The goal of electroporation optimization is to identify the conditions that will provide high levels of nucleic acid transfer while maintaining an acceptable level of cell viability for the particular cell type. Some of the conditions that improve nucleic acid transfer (e.g., increasing voltage or pulse length) also result in decreased cell viability. Therefore, both nucleic acid transfer (evidenced as gene knockdown or Cy3 uptake) and cell viability must be considered when interpreting optimization experiments. A balance between the two represents the ideal conditions for transfection. Once optimal transfection conditions are established, keeping them constant between experiments with a given cell type will maximize the uniformity and reproducibility of results.

Optimization should be carried out in two stages: Initially, determine broad parameters by monitoring uptake of Cy3 labeled Control siRNA, quantitating cell viability, or both. Next, fine-tune the transfection conditions by testing the same conditions in a narrower range with smaller intervals, and comparing gene knockdown, using the GAPDH siRNA, to cell viability. Finally, test the siRNA of interest for optimal concentration. Each optimization stage should take about a week to complete, with only several hours of hands-on time. For all optimization steps follow the electroporation procedure in section III.A on page 10.

1. Initial optimization using Cy3 labeled Control siRNA uptake or cell viability

Begin by performing an experiment to roughly determine the best transfection conditions. Follow the steps in Table 1, testing in the indicated ranges and intervals for each condition while keeping all other conditions the same. In other words, start by setting the pulse length to 100 µs and pulse number to 1, and test six aliquots of a culture with different voltages from 150–900 V (i.e., 150, 300...900 V). Next, use the voltage shown to be optimal (≤10% cell death) to test the indicated range of pulse lengths. Finally, use the optimal voltage and pulse length to determine how many electrical pulses give the best results.

Table 1. Initial Optimization Instructions

Determine optimal:	Test range	Test interval	Static settings
a. Voltage	150–900 V	150 V	Pulse length of 100 µs, 1 pulse
b. Pulse Length	100-400 µs	75 µs	Set to optimal voltage from previous experiment (1.a), use 1 pulse
c. Number of Pulses	1–5	1	Set to optimal voltage and pulse length from previous two experiments (1.a, 1.b)


NOTE

These recommendations assume the use of a standard 1 mm cuvette. Modifications may be needed if other cuvette sizes are used.

The results of these initial optimization experiments can be monitored either by fluorescence microscopy of the Cy3 labeled Control siRNA or by determining cell viability. Fluorescence microscopy allows for quick observation and a visual display of siRNA uptake, but it is not quantitative. The cell viability assay is more quantitative.

Fluorescence microscopy

Electroporate 2.25 µL of resuspended Cy3 labeled Control siRNA per reaction as described in section III.A on page 10, using mock transfections as a negative control. Observe cells 4–24 hours after transfection with a fluorescence microscope. Choose the condition that yields greatest fluorescence uptake by cells, with minimal cell death. See section *III.B. Fluorescence microscopy* on page 11.


NOTE

You must wait at least 48 hr after transfection before performing a Trypan Blue assay.

Cell viability assay

Mock transfections that do not include an siRNA can be used to assay cell viability. Observe cells ~48 hours after transfection. Choose the condition that allows for the highest voltage, pulse length, and pulse number, while maintaining 80–90% cell viability.

2. Fine-tune conditions using GAPDH siRNA to optimize gene knockdown

Using the conditions chosen in the initial optimization as a starting point, fine-tune the transfection according to Table 2. For example, if the optimal voltage was determined to be 450 V in the initial optimization experiments, then test between 350 V–550 V, in intervals of 50 V, in step *a* in Table 2. In this stage of optimization, optimum conditions must be determined by comparing gene knockdown, using the GAPDH siRNA, to cell viability—see section *III.B. Assessing Transfection Efficiency* on page 11 for more information.

Since different siRNA sequences have varying potency, the last step in this fine-tuning optimization procedure (step d in Table 2) is to determine the optimal concentration of the ***your experimental siRNA*** by electroporating three different siRNA amounts, ranging from 0.75–2.25 µg per reaction.

Whenever assessing siRNA-induced gene knockdown, a negative control must be included in order to provide a background reference. For each condition, transfect one sample with 2.25 µL (1.5 µg) of the GAPDH siRNA, and another with 2.25 µL (1.5 µg) of the Negative Control siRNA.

Table 2. Fine-tuning Optimization Instructions

Determine optimal:	Test range	Test interval	Static settings
a. Voltage	±100 from initial optimization	50 V	Set to optimal pulse length and number of pulses from initial optimization (1.b, 1.c); use 2.25 µL (1.5 µg) GAPDH siRNA and Negative Control siRNA
b. Pulse Length	±50 from initial optimization	25 µs	Set to optimal number of pulses from initial optimization (1.c); set the voltage to the optimal seen in the previous experiment (2.a); use 2.25 µL (1.5 µg) GAPDH siRNA and Negative Control siRNA
c. Number of Pulses	±1 from initial optimization	1	Set the voltage and pulse length to the optimal conditions seen in the previous two experiments (2.a and 2.b); use 2.25 µL (1.5 µg) GAPDH siRNA and Negative Control siRNA
d. siRNA Concentration	0.75–2.25 µg	0.75 µg	Test your experimental siRNA using optimized conditions from the previous three experiments (2.a, 2.b, 2.c); use 2.25 µL (1.5 µg) GAPDH siRNA and Negative Control siRNA

Assess gene knockdown and cell viability to generate a correlation as shown in Figure 2. Select the conditions that provide both effective gene knockdown and acceptable cell survival.

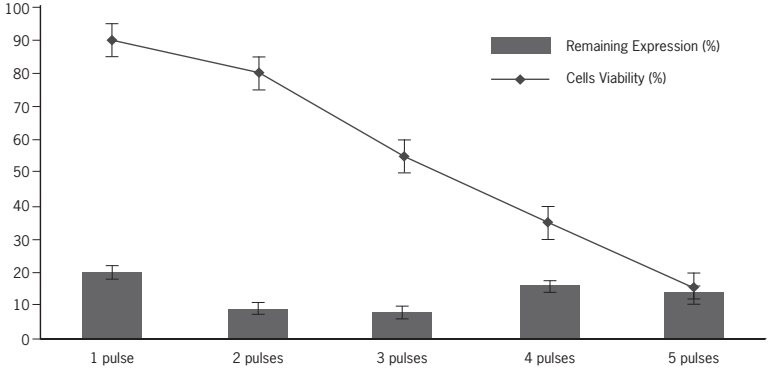

Figure 2. Varying Number of Electropulses.

NHDF-neo primary cells were electroporated with a GAPDH siRNA (1.5 µg), using a Gene Pulser Xcell (Bio-Rad) electroporator. A varying number (1–5) of electroporation pulses were delivered. The cells were obtained 48 hr post-transfection, and analyzed by real-time RT-PCR for both GAPDH mRNA and 18S rRNA levels. The 18S rRNA levels were used to normalize the GAPDH data. Remaining expression was calculated as a percentage of gene expression compared with the Negative Control siRNA. All samples were performed in duplicate.

III. Transfection Procedure and Analysis

A. Procedure for siRNA Electroporation

Before beginning, have the following prepared for each reaction:

- ≥7.5 x 10^4 adherent cells, or ≥1.5 x 10^5 suspension cells
- 0.75–2.25 µg of your experimental siRNA or control siRNA, as appropriate. siRNA must be in ≤8 µL of water or low conductivity buffer (see step A.2.a on page 10.)
- 75 µL siRNA Electroporation Buffer
- 1 mm electroporation cuvette (1 mm gap)
- Growth medium prewarmed to 37°C
- Set the electroporator to the optimized voltage, pulse length, and number of pulses. These electroporation conditions should be determined empirically for every cell type. See section *II. Optimizing Transfection* for a complete discussion.

IMPORTANT

If obtaining adherent cells, be aware that excessive exposure to trypsin can lead to significantly reduced cell viability and electroporation efficiency. Trypsinize for 2–5 min only.

1. Obtain cells

This procedure can be scaled up if the experiment will include multiple transfections.

a. Count cells and collect cells.
Collect ≥7.5 x 10^4 adherent cells or ≥1.5 x 10^5 suspension cells per electroporation sample.

b. Pellet cells at 200 x g for 5–7 min at room temp.

c. Remove media and resuspend cells in 75 µL of siRNA Electroporation Buffer.
Aspirate the media, removing as much as possible, then resuspend cell pellets in 75 µL of siRNA Electroporation Buffer per reaction. Avoid storing the cell suspension longer than 1 hr at room temp in the siRNA Electroporation Buffer, as this may reduce cell viability and transfection efficiency.

2. Electroporate

These steps cannot be scaled up.

a. Mix 75 µL of cells with siRNA.
In an electroporation cuvette, mix 75 µL of cells (in siRNA Electroporation Buffer) with the appropriate siRNA. Avoid creating air bubbles while pipetting. Mix the solution gently and immediately proceed to the next step.

During ***optimization assays***, use a control or experimental siRNA, as appropriate:

- 2.25 µL Cy3 labeled Control siRNA
- 2.25 µL (1.5 µg) GAPDH siRNA
- 2.25 µL (1.5 µg) Negative Control siRNA
- 0.75–2.25 µg of your experimental RNA in ≤8 µL RNase-free water or low-conductivity solution

For ***further experiments***, use the optimum amount of your experimental siRNA, as determined in Table 2 step d, and 1.5 µg Negative Control siRNA or another appropriate negative control siRNA.

b. Electroporate the sample using the conditions identified in optimization experiments.

3. Incubate cells at 37°C

a. Incubate the sample in the cuvette for 8–12 min at 37°C.

b. Transfer cells to a culture dish containing an appropriate volume of prewarmed growth medium.

Gently remove the sample from the cuvette and transfer to a fresh container containing growth medium prewarmed to 37°C.

c. Incubate the cells at 37°C in a humidified 5% CO_2 incubator until they are analyzed.

B. Assessing Transfection Efficiency

Fluorescence microscopy

We recommend plating cells transfected with the Cy3 labeled Control siRNA on glass coverslips immersed in media. Fluorescence can be measured as early as 4 hr after electroporation following the directions below.

A rough estimation of cell death caused by the various electroporation conditions should be made by microscopic observation of cells while the cells are still in media. To view the Cy3 labeled Control siRNA in transfected cells, fix cells (if necessary), and stain with DAPI if nuclear staining is desired. Estimate siRNA uptake by visual inspection with fluorescence microscopy (Ex = 550 nm, Em = 570 nm).

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.

Gene knockdown

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. We also recommend analyzing 18S rRNA levels as a means to normalize the GAPDH data.

Quantitate mRNA levels by Northern analysis or RT-PCR

In a successful transfection with the GAPDH siRNA, GAPDH mRNA levels are typically reduced 70–90%, 24–48 hr after electroporation. Either of the following methods should 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 one of the Ambion RNAqueous® line of products for RNA isolation.

b. Real-time PCR
   We recommend the following products to facilitate real-time PCR analysis of RNAi:
   - The Ambion RNAqueous® line of products for RNA isolation followed by the RETROscript® procedure to produce cRNA
   - Ambion Cells-to-cDNA™ II Kit 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%, 48–72 hr after electroporation. Ambion offers select antibodies for siRNA research (anti-GAPDH, mouse monoclonal, P/N AM4300).

* This product is compatible with the 5' nuclease detection and dsDNA-binding dye processes covered by patents owned or licensable by Applied Biosystems. No license under these patents is conveyed expressly, by implication, or by estoppel to the purchaser by the purchase of this product. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

IV. Troubleshooting

A. Using the Controls

Three controls are provided with this kit, the GAPDH siRNA, Negative Control siRNA, and Cy3 labeled Control siRNA; they can be used for procedure optimization, troubleshooting, and as experimental controls.

GAPDH siRNA

The GAPDH siRNA has been validated by Ambion to reduce GAPDH mRNA levels by at least 70% in transfected HeLa cells. This control can be used to confirm that all kit reagents are functioning properly. If you suspect that there is a problem with the reagents, follow the procedure in III.A for electroporation using electroporation conditions known to be effective for a particular cell type. If conditions have not been optimized for the cell type under study, use one of the cell types for which Ambion provides optimized conditions (www.ambion.com/techlib/resources/transfection).

Negative Control siRNA

The Negative Control siRNA is a scrambled sequence that has no known homology to the human, mouse, or rat genome; it should therefore have no RNAi effect. The Negative Control siRNA serves as a baseline for measuring the effects of the GAPDH siRNA, and can be used to identify nonspecific effects such as non-sequence-specific siRNA effects, and cytotoxicity of the siRNA Electroporation Buffer.

Cy3 labeled Control siRNA

The Cy3 labeled Control siRNA is a scrambled sequence that has no known homology to the human, mouse, or rat genome; it should therefore have no RNAi effect. It is labeled with Cy3 fluorescent dye making it possible to track siRNAs in electroporated cells by direct observation. To visualize the Cy3 labeled Control siRNA, view cells using fluorescence microscopy (Ex = 550 nm, Em = 570 nm).

B. No Detectable Gene Silencing

The electroporation procedure requires optimization

We strongly recommend that you optimize the electroporation procedure for each cell type using the Cy3 labeled Control siRNA, or GAPDH control siRNA as described in section II.B.

The siRNA concentration is too low

If too little siRNA is used in the electroporation reaction, reduction of target gene expression may be undetectable. It is important to optimize the siRNA amount for every cell type used.

The siRNA is degraded

Check the integrity of the siRNA by running ~2.5 µg on a nondenaturing 15–20% acrylamide gel. Visualize the RNA by staining with ethidium bromide, and verify that it is intact. Intact siRNA should migrate as a fairly tight band; smearing would indicate degradation.

Cells have undergone changes

Use primary cells for electroporation within 10 passages of optimization experiments. If you do not have frozen stocks to reculture from, it may be necessary to reoptimize the transfection conditions.

Cell density in the cuvette is too high or too low

Cell density can influence electroporation efficiency dramatically. We recommend using ≥7.5 x 10^4 cells per sample for adherent cells and 1.5 x 10^5 cells per sample for suspension cells. If too many cells are used, and the amount of siRNA is not increased proportionally, the concentration of siRNA in the sample may be too low to detect gene silencing.

Cells are contaminated with *Mycoplasma*

Test cells for *Mycoplasma* contamination. Discard contaminated cells, and start a new culture.

C. Electroporation Causes Extensive Cell Death

The electroporation protocol requires optimization

We strongly recommend that you optimize the electroporation protocol for each cell type using the Cy3 labeled Control siRNA, or GAPDH control siRNA as described in section II.B.

Too much siRNA was used

High concentrations of siRNA can be toxic to cells. Test a titration of varying siRNA amounts from 0.75–2.25 µg/75 µl of electroporation reaction.

Cells are damaged by harvesting or handling

Cells can be easily damaged if they are subjected to severe conditions such as centrifugation at high speed, overexposure to trypsin (longer than 10 min), temperature shock, or vigorous pipetting; all of these should be avoided to ensure maximal cell recovery.

Cells are stressed

Many factors can stress cells. Below we offer some suggestions for avoiding cell stress.

- Avoid using antibiotics for at least 48 hr after electroporation.
- Freshly thawed cells should not be used for electroporation (wait at least 48 hr after plating).
- Use healthy cells that have not been grown to the point of medium depletion between passages.
- Avoid subjecting cells to frequent temperature 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 cells at earlier time points (4–12 hr).
Cells are contaminated with *Mycoplasma*	Test cells for *Mycoplasma* contamination. Discard contaminated cells, and start a new culture.

D. Gene Silencing Experiments Lack Reproducibility

Cell passage number is too high	Repeat experiment using cells that have been subcultured fewer times.
There were differences in the experimental procedures	Incubation times, master mix volumes, siRNA amount, and cell number per sample can all affect electroporation efficiency. To obtain reproducible results across multiple experiments, be very careful to conduct experiments the same way every time.
Cell density in the cuvette is too high or too low	Cell density can dramatically influence transfection efficiency. We recommend using 7.5×10^4 cells per sample for adherent cells, and 1.5×10^5 cells per sample for suspension cells. If too many cells are used, and the amount of siRNA is not increased proportionally, the ratio of siRNA to cells may be reduced to the point that experimental reproducibility is affected.
Cell confluence is inconsistent	Different levels of cell confluence at the time of harvest may affect the transfection efficiency. Harvest cells at comparable confluencies.

V. Appendix

A. References

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, and Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, *Nature* **411**:494–98.

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

Gehl J (2003) Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research, *Acta Physiol Scand* **177**:437–47.

B. Quality Control

Functional testing

Cells were electroporated with GAPDH siRNA or GAPDH Negative Control siRNA. Cells transfected with the GAPDH siRNA are shown to have at least 70% reduction in GAPDH RNA signal compared to cells transfected with the Negative Control siRNA by qRT-PCR.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

C. 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 chemical 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) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

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