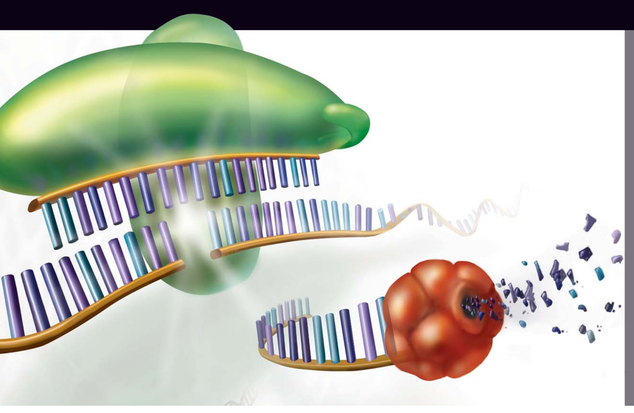

Parameters for Effective siRNA Transfection Using siLentFect™ Lipid Reagent

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Abstract

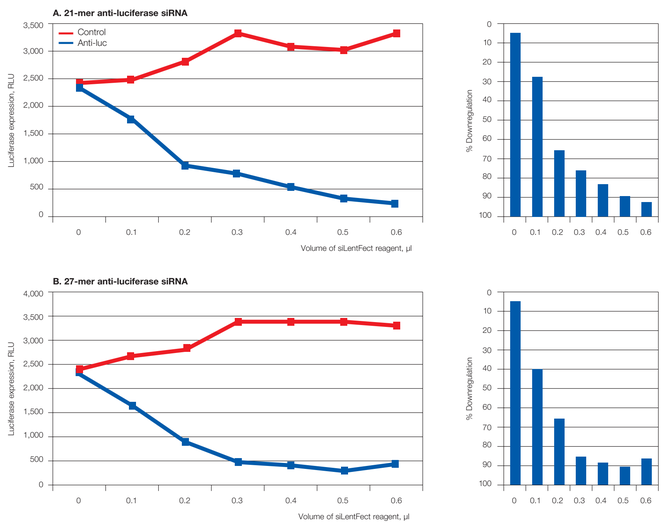
The ability to modulate gene expression and determine gene function in mammalian cells has benefited significantly from the use of RNA interference (RNAi). This well-conserved gene silencing pathway is found in a variety of eukaryotic organisms (reviewed by Meister and Tuschl, 2004). The activation of this pathway by double-stranded RNAs known as short interfering RNAs (siRNAs) stimulates sequence-specific degradation of the messenger RNAs with sequence homology to the siRNA used to activate the RNAi pathway.

The effector molecules that trigger RNAi are double-stranded siRNAs 21–23 nt long that get incorporated into the RISC complex to direct mRNA degradation. However, recently published studies demonstrate that 27 nt long siRNA duplexes (Dicer substrate siRNA) enter the RNAi pathway at an earlier step and may be more potent activators of the RNAi pathway in cultured mammalian cells (Kim et al. 2005).

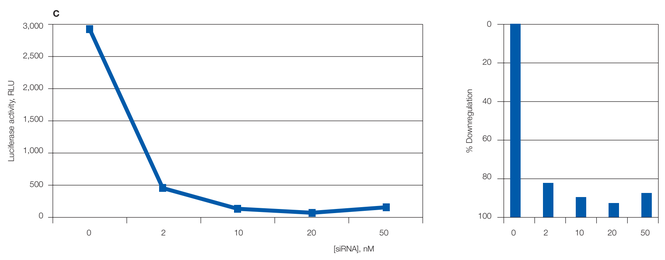
With either 21-mer or 27-mer siRNA, effective delivery of the siRNA is a key first step in RNAi experiments. Bio-Rad Laboratories has developed siLentFect lipid reagent for RNAi, a novel cationic lipid transfection reagent with a high affinity for siRNA. Designed to facilitate effective delivery using small amounts of lipid and low concentrations of siRNA, siLentFect reagent allows efficient transfection of siRNA into a wide variety of cultured mammalian cells with extremely low cytoxicity.

The results presented here demonstrate the delivery capabilities of siLentFect lipid reagent in a variety of cell types using both 21-mer and 27-mer siRNAs. Effective silencing was achieved at siRNA concentrations down to 100 pM. Data demonstrating effective delivery, low cytotoxicity, and efficient silencing are shown. These results were generated using fluorescently labeled siRNAs, siRNAs cotransfected with plasmid-based reporter genes, and functional siRNAs targeting a variety of endogenous genes.

Effect of Lipid Volume on Silencing



Effect of siRNA Concentration on Silencing



Effect of Cell Density on Silencing

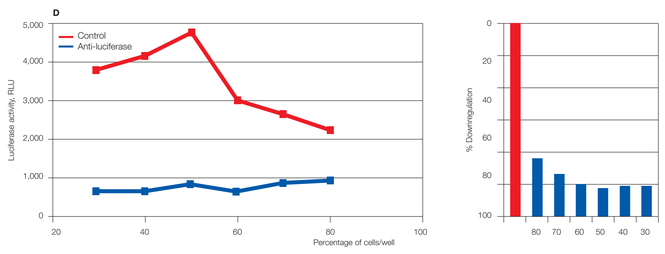


Fig. 1. Optimization of siRNA delivery using siLentFect lipid reagent. Efficient transfection of siRNAs is key to achieving successful gene silencing. A sample transfection optimization experiment is presented here. Stably transformed CHO cells expressing the luciferase gene (CHO-Luc cells) were transfected using various amounts of transfection reagent (siLentFect) at different siRNA concentrations and cell densities. **A** and **B**, optimization of amount of siLentFect. CHO-Luc cells were transfected in 96-well plates using either 5 nM of an anti-luciferase siRNA (—) or a nonspecific control (—) with increasing volumes of siLentFect reagent. Transfections were performed using a 21-mer siRNA specific to the luciferase gene (A) or a 27-mer siRNA specific to the luciferase gene (B). Downregulation of luciferase expression was monitored by a luciferase activity assay. At optimal siLentFect reagent volumes, over 90% downregulation of luciferase expression was attained by both the 21-mer and the 27-mer siRNAs. This experiment also provides an indication of the low cytotoxicity associated with siLentFect transfections, since the relative light units (RLU) for the nonspecific control remained steady with increasing lipid amount. **C**, optimization of siRNA concentration. CHO-Luc cells were transfected in 96-well plates using a constant amount of siLentFect reagent (0.3 µl) and different concentrations (2–50 nM) of a 21-mer anti-luciferase siRNA. Over 80% downregulation of the luciferase gene was achieved using only 2 nM of siRNA and over 90% downregulation was obtained using 10 nM of siRNA. The addition of siRNA at concentrations greater than 10 nM did not produce any further significant reduction in luciferase activity. Transfection with a lower amount of siRNA may help to minimize any potential off-target effects. **D**, optimization of cell density. CHO-Luc cells were transfected at different cell densities; lower cell densities provided better gene silencing than higher cell densities.

Efficient Delivery of Fluorescent siRNAs to Different Cell Types

Fig. 2. Visualization of intracellular delivery of fluorescent siRNAs to HeLa cells, human primary fibroblasts, and MCF-7 cells. Cy3-labeled 27-mer and 21-mer siRNAs were used to provide a direct assessment of transfection efficiency. Cells in a 24-well plate were transfected with 10 nM of the Cy3-labeled siRNAs (red) and 0.5 µl of siLentFect reagent. At 24 hr after transfection, cells were stained with a nuclear-specific stain, Hoechst 33342 dye (blue), and examined by fluorescence microscopy. The images show both perinuclear and cytoplasmic localization of the 21-mer and 27-mer fluorescent siRNAs labeled with Cy3, uniform uptake of these siRNAs, and high transfection efficiency in all cultures.

Effective Silencing in Cotransfections

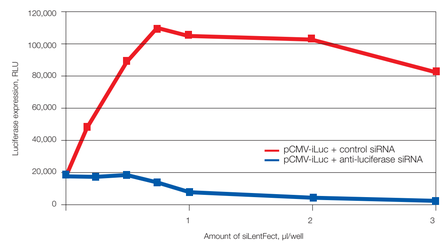


Fig. 3. Cotransfection of plasmid DNA and siRNA. To demonstrate that siLentFect reagent can be used for cotransfection experiments, we tested the siRNA-mediated downregulation of genes expressed by plasmid vectors. MCF-7 cells were transiently transfected in 24-well plates with different volumes of siLentFect reagent, 0.5 µg of a luciferase reporter gene expression vector, and 10 nM of either a 21-mer anti-luciferase siRNA (—) or a nonspecific siRNA control (—). siLentFect was allowed to first complex with the siRNAs and then with the plasmid. Luciferase activity was measured 24 hr after transfection. This experiment shows that siLentFect reagent may be used to deliver both plasmid and siRNA to cells to achieve silencing of a gene expressed by a cotransfected plasmid.

Effective Downregulation in Reporter Genes

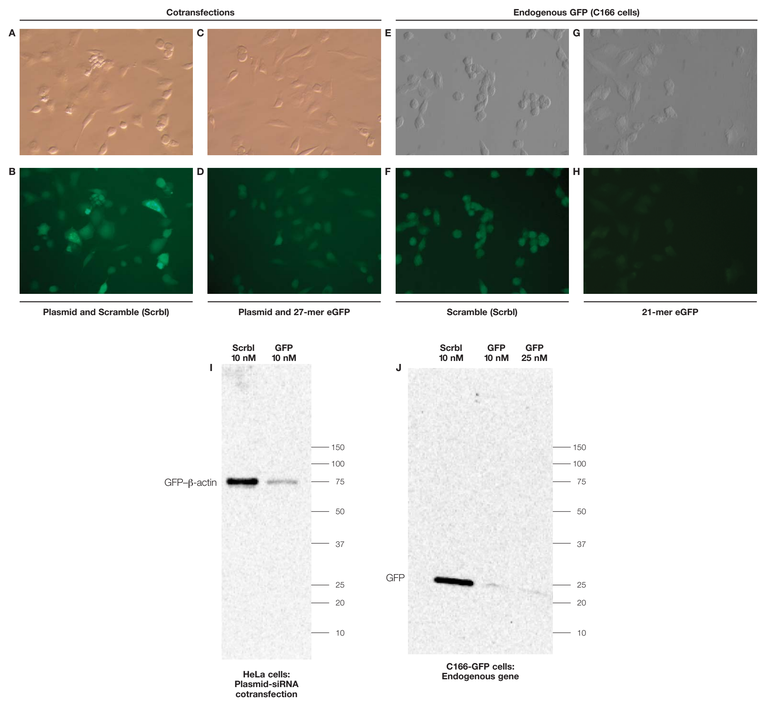


Fig. 4. Monitoring gene silencing by protein accumulation in siRNA transfection and plasmid-siRNA cotransfection experiments. Another way to measure gene silencing is to measure protein accumulation. Upper panels (**A–H**), brightfield images and corresponding fluorescent images show that when plasmids expressing GFP fused to the β-actin gene were cotransfected with a 27-mer eGFP siRNA, GFP expression was efficiently suppressed (compare panel B to D). Similarly, when endothelial C166 cells stably expressing the GFP gene (obtained from ATCC) were transfected with a 21-mer eGFP siRNA, GFP expression was reduced (compare panel F to H). Cells were examined by fluorescent microscopy 24 hr after transfection. Lower panels, immunoblot results show a significant reduction in the GFP protein level, both in cotransfection in HeLa cells (**I**) and in transfection of endothelial cells (**J**), which correlate well with the reduction observed by fluorescent microscopy.

Silencing of Endogenous mRNA

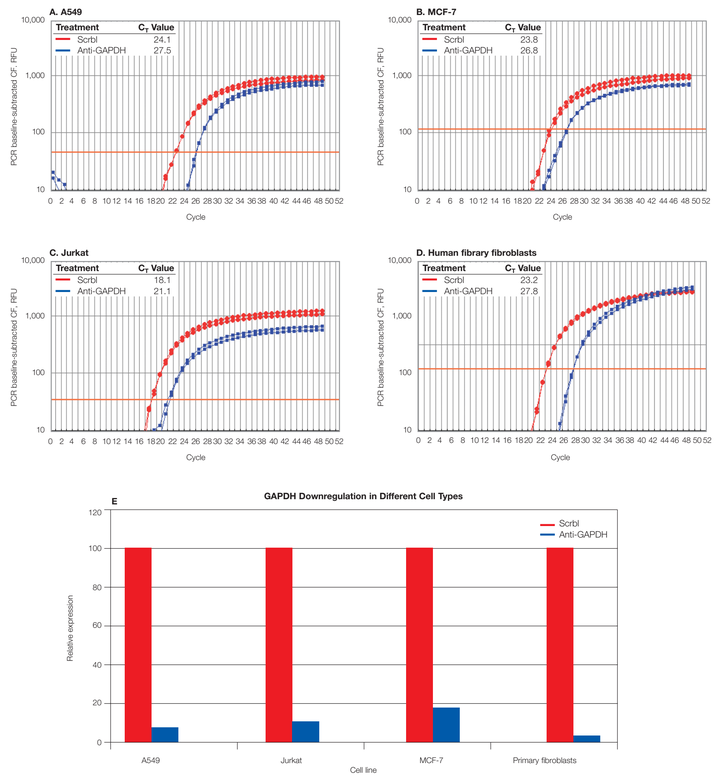


Fig. 5. Using siLentFect reagent to deliver siRNAs for downregulation of endogenous genes. A549 cells (**A**), MCF-7 cells (**B**), Jurkat cells (**C**), or human primary fibroblasts (**D**) were transfected in 6-well plates with 2 µl of siLentFect and 10 nM of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) SMARTpool siRNA (anti-GAPDH —, Dharmacon), or a nonspecific control siRNA (Scrb) —). Cells were harvested 48 hr after transfection and RNA was isolated using the Aurum™ total RNA kit. cDNA was produced using the iScript™ cDNA synthesis kit, and quantitative polymerase chain reaction (qPCR) was performed using the iCycler iQ® real-time PCR detection system. **E**, >85% reduction in transcript levels was observed in cells exposed to GAPDH siRNA compared to those exposed to nonspecific siRNA.

Silencing of Endogenous mRNAs at Low siRNA Concentrations

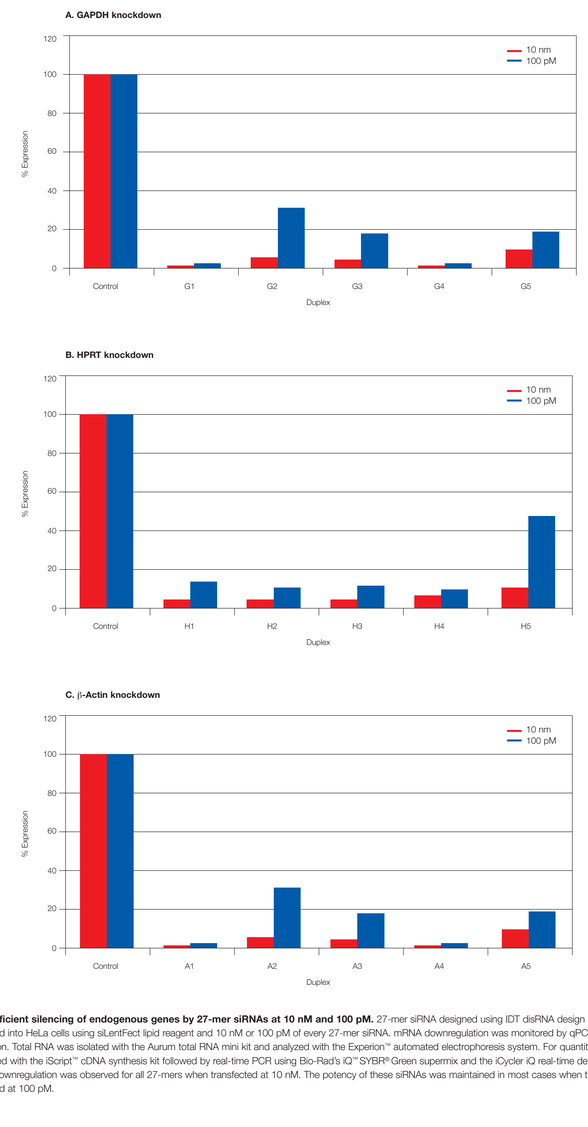


Fig. 6. Efficient silencing of endogenous genes by 27-mer siRNAs at 10 nM and 100 pM. 27-mer siRNA designed using IDT disRNA design software was transfected into HeLa cells using siLentFect lipid reagent and 10 nM or 100 pM of every 27-mer siRNA. mRNA downregulation was monitored by qPCR 24 hr after transfection. Total RNA was isolated with the Aurum total RNA mini kit and analyzed with the Experion™ automated electrophoresis system. For quantitation, cDNA was synthesized with the iScript™ cDNA synthesis kit followed by real-time PCR using Bio-Rad's iQ™ SYBR® Green supermix and the iCycler iQ real-time detection system. Efficient downregulation was observed for all 27-mers when transfected at 10 nM. The potency of these siRNAs was maintained in most cases when they were transfected at 100 pM.

Cell lines, siRNAs, and Gene Targets Used in This Study

Cell Line	Cell Type	21-mer	27-mer	Endogenous Gene	Reporter Gene	Cotransfections
HeLa	Human cervical adenocarcinoma	•	•	•		•
HEK293	Human kidney epithelial	•		•	•	•
A549	Human lung carcinoma	•		•		
MCF-7	Human breast adenocarcinoma	•		•		•
Jurkat	Human T lymphoblast	•		•		•
FS	Human foreskin fibroblast	•		•		
CHO-K1	Hamster ovary epithelial	•	•	•	•	
C166-GFP	Mouse yolk sac endothelial	•		•	•	

Conclusions

- siLentFect lipid reagent effectively delivers siRNA to a variety of cell lines using conditions and strategies common to RNAi experiments
- This effective lipid reagent has extremely low toxicity and is compatible with a variety of adherent and suspension cell lines
- siLentFect reagent allows effective delivery and silencing with subnanomolar concentrations of traditional 21-mer siRNAs as well as the recently described 27-mer siRNAs
- siLentFect reagent can be used for effective silencing of endogenous targets or cotransfected plasmid-based reporter genes

siLentFect Lipid Reagent Key Features

- Developed specifically for siRNA, increasing delivery efficiency
- Low cytotoxicity reduces experimental bias
- Compatible with cotransfection applications
- Validated for standard-, rapid-, and reverse-transfection strategies

In addition to siLentFect lipid reagent, the following Bio-Rad products were used in this work: iCycler iQ real-time PCR detection system, Aurum total RNA kit, iScript cDNA synthesis kit, iQ SYBR Green supermix.

For more information on these Bio-Rad products, visit us on the Web at **discover.bio-rad.com**

References and Notes

Kim D-H et al., Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy, Nat Biotechnol 23, 222–226 (2005)

Meister G and Tuschl T, Mechanisms of gene silencing by double-stranded RNA, Nature 431, 343-349 (2004)

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