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# Kinetic and Efficacy Analysis of RNA Interference in Stably and Transiently Expressing Cell Lines

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**Abstract:** RNA interference, particularly through the use of small interfering RNA (siRNA), has become an important laboratory tool for both fundamental and applied investigations. However, it is currently unknown whether siRNA-mediated knockdown of transiently expressed proteins is an acceptable quantitative surrogate for stably expressed proteins. Further, the best means by which to transfect cells with functionally active siRNA are poorly defined, and determination of the best reagent and transfection conditions for a particular cell line is a burdensome prerequisite for RNA interference studies. We therefore established the optimal transfection conditions for six commercial siRNA delivery reagents in three cell lines (HR5-CL11, HeLa, and NIH/3T3) transiently or stably expressing the firefly luciferase gene. The delivery efficiency, knockdown kinetics, and cytotoxicity of the reagents were evaluated. siPORT *Amine*, X-tremeGENE, and *Trans*IT-siQUEST achieved the best knockdown and consistency of performance among the three cell lines. Delivery efficiency varied and was cell line dependent in some cases. The knockdown kinetics were reagent-dependent, and knockdown was generally more rapid in the stably transfected cells. Cytotoxicity of the reagents was variable. GeneSilencer was the least cytotoxic reagent for all three cell lines, and *Trans*IT-siQUEST was the most cytotoxic to the HeLa and HR5-CL11 cell lines. These comparative results provide an initial basis for reagent selection and experimental design for RNA interference studies in HeLa, NIH/3T3, and their respective derivative cell lines.

**Keywords:** Gene knockdown; luciferase; RNA interference; siRNA

## Introduction

Since the finding of RNA interference (RNAi) in *Caenorhabditis elegans*[1] and its subsequent discovery in mammalian cells,[2] researchers have explored this cellular mechanism for a variety of potential uses ranging from fundamental cell biology to finding new treatments for disease. Emphasis has been placed on demonstrating specific and potent gene knockdown and exploring the value of RNAi as a tool for functional genomics.[3] However, the factors that govern the delivery of small interfering RNA (siRNA, one approach to initiate RNAi) have not been thoroughly elucidated. The efficacy of a siRNA delivery reagent may depend on its composition, transfection conditions, and the

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cell line in which it is used. Further, the assumption that optimal delivery reagents and conditions should be similar for both DNA and siRNA has recently been challenged in the literature.[4,5] The dilemma of which delivery reagent to use is compounded by the proprietary nature of many commercial siRNA delivery reagents and the limited comparative literature on siRNA delivery vectors.

An additional complication of RNAi studies is that the knockdown effect mediated by siRNA is transient, and it is unknown the degree to which the elapsed time between transfection and optimal knockdown depends on the reagent delivery mechanism, half-life of the protein of interest, cell line, transfection method, transfection time, and/or cell cycle.[6−8] Other factors such as the cell plating density, siRNA concentration, and general transfection conditions can also affect the degree of gene knockdown observed.[8] Additional complexity is introduced when the knockdown of transiently transfected exogenous genes is studied.[7] Luciferase is commonly used as a reporter gene to assess the kinetics of gene induction and repression[9,10] and the efficiency of various transient gene transfection and knockdown systems in vivo,[11] as well as a tool for in vivo imaging.[12] Luciferase expression kinetics in mammalian cell lines have been reported,[13−17] but little work has been done to elucidate whether the protein expression profile (transient or stable gene expression kinetics) may play a role in RNA interference studies.

***Table 1.*** Commercial siRNA Transfection Reagents Screened for Delivery Efficiency, Knockdown Kinetics, and Cytotoxicity

| reagent type[a] | name | source |
|---|---|---|
| lipid | siPORT *Lipid* | Ambion (Austin, TX) |
| | RNAiFect | Qiagen (Valencia, CA) |
| cationic lipid | GeneSilencer | Gene Therapy Systems (San Diego, CA) |
| lipopolyplex | *Trans*IT-siQUEST | Mirus Bio (Madison, WI) |
| polyamine | siPORT *Amine* | Ambion (Austin, TX) |
| lipid + other (proprietary) | X-tremeGENE | Roche Applied Sciences (Penzberg, Germany) |

[a] According to manufacturer Web site or product insert.

Researchers often want to use RNAi as a tool (for functional genomics, for example). However, in the absence of a priori knowledge of the relative importance of reagent chemistry, cell line, and protein expression profile, it is difficult to rapidly determine which reagents and conditions are optimal. Here we present a comparative analysis of the delivery efficiency, knockdown kinetics, and cytotoxicity of six commercial siRNA delivery reagents (Table 1) using three cell lines that express luciferase as quantitative model systems, with the aims of determining the role of protein expression profiles (stable vs transient gene expression kinetics) and establishing optimized transfection conditions for RNA interference studies in these cell lines. Reagents of different classes (lipids and polymers, for example) were assessed to provide a broad comparison. Each reagent was evaluated in the HeLa, HeLa-derived HR5-CL11, and NIH/3T3 cell lines. HeLa and NIH/3T3 cells are commonly used in DNA and siRNA transfection studies, as well as in functional genomics.[18]

## Materials and Methods

**Cell Culture.** The HR5-CL11 cell line was purchased from the European Collection of Cell Cultures (ECACC, Health Protection Agency, Salisbury, U.K.). This cell line,


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a HeLa derivative, expresses luciferase via a tetracycline-controlled transcriptional transactivator.[19] The human cervical carcinoma HeLa cell line was generously provided by Dr. Bill Brown (Cornell University), and the murine embryonic fibroblast NIH/3T3 cell line was purchased from American Type Culture Collection (Manassas, VA). Cell lines were grown in monolayer culture at 37 °C and 5% $CO_2$. HR5-CL11 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing HEPES and 2 mM glutamine with 10% v/v fetal bovine serum (FBS), 100 units/mL penicillin, 100 $\mu$g/mL streptomycin, and the equivalent of 1 $\mu$g/mL doxycycline (prepared from doxycycline hyclate, Sigma-Aldrich, St. Louis, MO). HeLa cells were cultured in minimum essential medium α medium (MEM-α) with 10% v/v FBS, 100 units/mL of penicillin, and 100 $\mu$g/mL of streptomycin. NIH/3T3 cells were cultured in DMEM with 10% v/v calf serum (CS), 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin. Cells were passaged every 3−4 days. Cell culture reagents were from Gibco Cell Culture (Carlsbad, CA).

**Transfection. Plasmid DNA (pCMV-luc) Transfection.** HeLa and NIH/3T3 cells were transfected with luciferase plasmid DNA to initiate transient luciferase expression. Cells were grown in T-75 flasks until approximately 70% confluent. The DNA transfection complexes were made according to the manufacturer's directions as follows: *Trans*IT-LT1 delivery reagent (Mirus Bio Corporation, Madison, WI) was vortexed for 9 s and 60 $\mu$L was added to 1.5 mL of OPTI-MEM I (Gibco) in a sterile microcentrifuge tube. The tube was incubated at room temperature for 10 min. Luciferase plasmid DNA (pCMV-luc, 1 mg/mL, Elim Biopharmaceuticals, Hayward, CA) was vortexed for 7 s and 40 $\mu$L was added to the same microcentrifuge tube. While the mixture was incubated at room temperature for 20 min, the medium in the T-75 flask was replaced with 10 mL of warmed culture medium. The DNA solution was then added to the flask dropwise. The flask was rocked to distribute the transfection complexes, and incubated at 37 °C and 5% $CO_2$ for 4 h prior to siRNA transfection.

**siRNA Transfection.** T-75 flasks of luciferase-expressing cells were trypsinized, and solutions of 40 000, 50 000 and 100 000 cells/mL were made in 90% medium (no phenol red or antibiotics) with 10% v/v of the appropriate serum. Cells were plated in clear (for BCA assay) and opaque (for Bright-Glo assay) 96-well plates. The volume of cell stock solution and medium added to a well depended on the recommended conditions for the siRNA delivery reagent (see table in the Supporting Information for transfection conditions). Final cell density was 4000 cells/well with the exception of the HR5-CL11 knockdown kinetics study, in which 8000 cells were plated per well. This cell density was required to achieve luciferase expression that was measurably different from background at the concentration of doxycycline used to promote luciferase expression.

Plates were incubated at 37 °C and 5% $CO_2$ for approximately 2 h prior to siRNA transfection for the NIH/3T3 and HeLa cell lines, and 24 h for the HR5-CL11 cell line. Wells that received 8000 cells in the HR5-CL11 knockdown kinetics study were initially plated with 200 $\mu$L of 40 000 cells/mL stock solution. The medium was removed from the wells just before siRNA transfection and replaced with the appropriate amounts of cell culture medium (equal to the combined volume of cell stock and cell culture medium listed in the table in the Supporting Information) and OPTI-MEM I for each control and experimental condition.

Anti-luciferase GL2 (sense sequence, 5′-CGU ACG CGG AAU ACU UCG A dTdT-3′; antisense sequence, 3′-dTdT GCA UGC GCC UUA UGA AGC U-5′; targeting position 153−171 relative to first nucleotide of start codon; GenBank Accession number X65324) and Non-specific Control III (sense sequence, 5′-AUG UAU UGG CCU GUA UUA G UU-3′; antisense sequence, 3′-UU UAC AUA ACC GGA CAU AAU C P-5′) siRNA duplexes, 20 nmol each, were from Dharmacon, Inc. (Lafayette, CO). siRNA transfection complexes were made following the manufacturer-supplied protocols for each reagent and were added to cells (three wells per condition for each clear and opaque plate) prior to incubation at 37 °C and 5% $CO_2$. Final siRNA concentration was 50 nM for all conditions. Bright-Glo and BCA assays were performed at 48 h post-transfection, or approximately every 12 h for the knockdown kinetics studies. Positive controls were luciferase-expressing cells (HR5-CL11 cells cultured in doxycycline, or HeLa or NIH/3T3 cells transfected with luciferase plasmid) that received no siRNA or siRNA delivery reagent.

**Bright-Glo Luciferase Assay.** Bright-Glo working solution was prepared according to the manufacturer's directions (Promega, Madison, WI). After the plates were incubated for the desired duration, 60, 100, or 165 $\mu$L of Bright-Glo working solution was added to each well of the opaque 96-well plates containing treated cells in 60, 100, or 180 $\mu$L of total transfection volume, respectively. Plates were incubated for at least 1 min at room temperature before being read on a Molecular Devices SpectraMax Gemini EM (automatic calibration, 5 s of mixing prior to reading, automatic sensitivity of PMT detector, 10 readings per well in luminescence top-read mode, and 96-well standard opaque assay plate).

**BCA Protein Quantification Assay.** BCA working solution was prepared according to the manufacturer's directions (Pierce Biotechnology, Rockford, IL). After the plates were incubated for the desired duration, the culture medium was removed from the wells of the plates and each well was rinsed with 200 $\mu$L of phosphate buffered saline (PBS). To each well was then added an aliquot of 30 $\mu$L of RIPA buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate in 50 mM pH 8.0 Tris buffer with 0.5% protease inhibitor cocktail, Cat. No. P8340, all chemicals from Sigma-Aldrich). Plates were

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incubated at 4 °C for a minimum of 12 min to lyse the cells, and 10 μL of each sample lysate was pipetted into a new clear 96-well plate. To each well with lysate was then added 200 μL of BCA working solution. Plates were incubated at 37 °C and 5% $CO_2$ for 30 min before being read at 562 nm on a Molecular Devices SpectraMax Plus[384].

**Cytotoxicity.** Cells were grown in T-75 culture flasks to approximately 90% confluence. Flasks were trypsinized, and stock solutions of 44 444, 80 000, and 133 333 cells/mL were made. Volumes of 90, 50, or 30 μL were plated in clear 96-well plates to give 4000 cells/well, and the plates were then incubated at 37 °C and 5% $CO_2$ while siRNA transfection reagents were diluted in 90% medium (no phenol or antibiotics) and 10% of the appropriate serum. The diluted reagents were added to the wells to give 0−50% v/v of reagent in the appropriate total volume (180, 100, or 60 μL) per well, and the plates were incubated at 37 °C and 5% $CO_2$ for 48 h. The CellTiter 96 $AQ_{ueous}$ One Solution Cell Proliferation Assay (Promega) was performed by adding 20 μL of the CellTiter working solution per 100 μL of total cell culture volume to each well. Plates were incubated at 37 °C and 5% $CO_2$ for 2 h before the absorbance was read at 490 nm ($A_{490}$) on a Molecular Devices SpectraMax Plus[384]. Quadratic fits were performed to data points spanning 0−2.5 μL/well for *Trans*IT-siQUEST and 0−10 μL/well for all other reagents. Since reagent concentrations are not available from the manufacturers, $IC_{50}$ values were calculated using the Solver function in Excel as the concentration of reagent (% v/v) at which the calculated $A_{490}$ was one-half the measured $A_{490}$ for cells that received 0 μL of reagent. Data are presented in the Supporting Information as the measured absorbance normalized by the absorbance of wells with cells that received 0 μL/well reagent.

**Statistical Analysis.** Two-tailed, heteroscedastic *t* tests were performed using the TTEST function in Excel to determine the statistical significance of results. Due to the 96-well format of these studies, statistical significance was defined as $p < 0.05$ or $p < 0.01$.

## Results

Figure 1 shows the relative luciferase expression at 48 h when siRNA was delivered using different volumes of each reagent to HR5-CL11 (stable luciferase expression), HeLa (transient luciferase expression), and NIH/3T3 (transient luciferase expression) cell lines. The volumes used for each reagent were chosen to span the ranges recommended by each manufacturer while keeping the siRNA concentration constant at 50 nM. Measured relative light units (RLU) were divided by the absorbance at 562 nm ($A_{562}$) for each well. The RLU/$A_{562}$ values were averaged for each experimental condition, and normalized by the average RLU/$A_{562}$ for the positive controls. Figures 2 and 3 show the kinetics of luciferase expression in HR5-CL11 and HeLa cells, respectively, over 72 h after transfection with a selected volume of each reagent, and allow a comparison of gene knockdown between stably and transiently transfected cells of the same cell lineage. The volume of each reagent used in the kinetics studies corresponds to the volume at which the greatest knockdown was achieved in our initial screening studies (Figure 1) except where noted in the text. Due to space limitations, the knockdown kinetics data for the NIH/3T3 cell line are provided in the Supporting Information. The time of maximum luciferase knockdown was determined by the percentage difference in average RLU/$A_{562}$ values between positive controls and cells transfected with anti-luciferase siRNA. The knockdown relative to the nonspecific siRNA controls was also assessed. The percentage difference was used to provide consistency with results previously reported for these reagents in the literature, as well as to decouple the knockdown results from the effect of waning luciferase expression over time in all three cell lines (in the HR5-CL11 cell line due to a reduced doxycycline concentration after transfection, and in the HeLa and NIH/3T3 cells due to the transient luciferase expression following plasmid DNA transfection). The $IC_{50}$ values are summarized in Table 2 (see the Supporting Information for data), and the recommended optimal transfection conditions are listed in Table 3.

**siPORT *Amine*.** The siPORT *Amine* showed maximal knockdown of luciferase expression (81% knockdown, to 19% of the expression measured in the positive controls, and 48% less than the nonspecific siRNA controls) at a volume of 1 μL/well for the HR5-CL11 cells in the initial screening study (Figure 1a). In comparison, the maximum observed reduction in luciferase expression (86%) in the HeLa cell line was observed when 2 μL of siPORT *Amine* were used to deliver anti-luciferase siRNA. However, the largest knockdown in luciferase expression in the HeLa cells relative to the nonspecific siRNA controls occurred at a volume of 1 μL/well siPORT *Amine*. When siPORT *Amine* was used to examine the knockdown kinetics in these two cell lines (Figures 2a and 3a, respectively), the maximum knockdown was observed at 12 h for the HR5-CL11 cell line (68% when they were treated with 1 μL/well) and at 24 h for the HeLa cell line (98% when they were treated with 2 μL/well). The siPORT *Amine* was the second most cytotoxic reagent in both the HR5-CL11 and HeLa cells, and the calculated $IC_{50}$ values in both cell lines were less than 1% v/v, or 1 μL/well (Table 2).

The efficacy and cytotoxicity of the siPORT *Amine* were quite different in the NIH/3T3 cell line than in the HR5-CL11 and HeLa cell lines. While the observed minimum luciferase expression (21% of the expression of the positive controls at 2 μL/well siPORT *Amine*, Figure 1a) was similar to that observed in the other two cell lines, this result was not statistically significant for the NIH/3T3 cells. However, the largest reduction in luciferase expression relative to the nonspecific siRNA controls (64%) occurred at a volume of 1 μL/well siPORT *Amine* and was statistically significant. The maximum luciferase knockdown (76%) occurred in the kinetics study at 48 h when 2 μL/well siPORT *Amine* was used, later than the observed minima for the other two cell lines (see the Supporting Information). Additionally, the siPORT *Amine* was one of the least cytotoxic reagents for

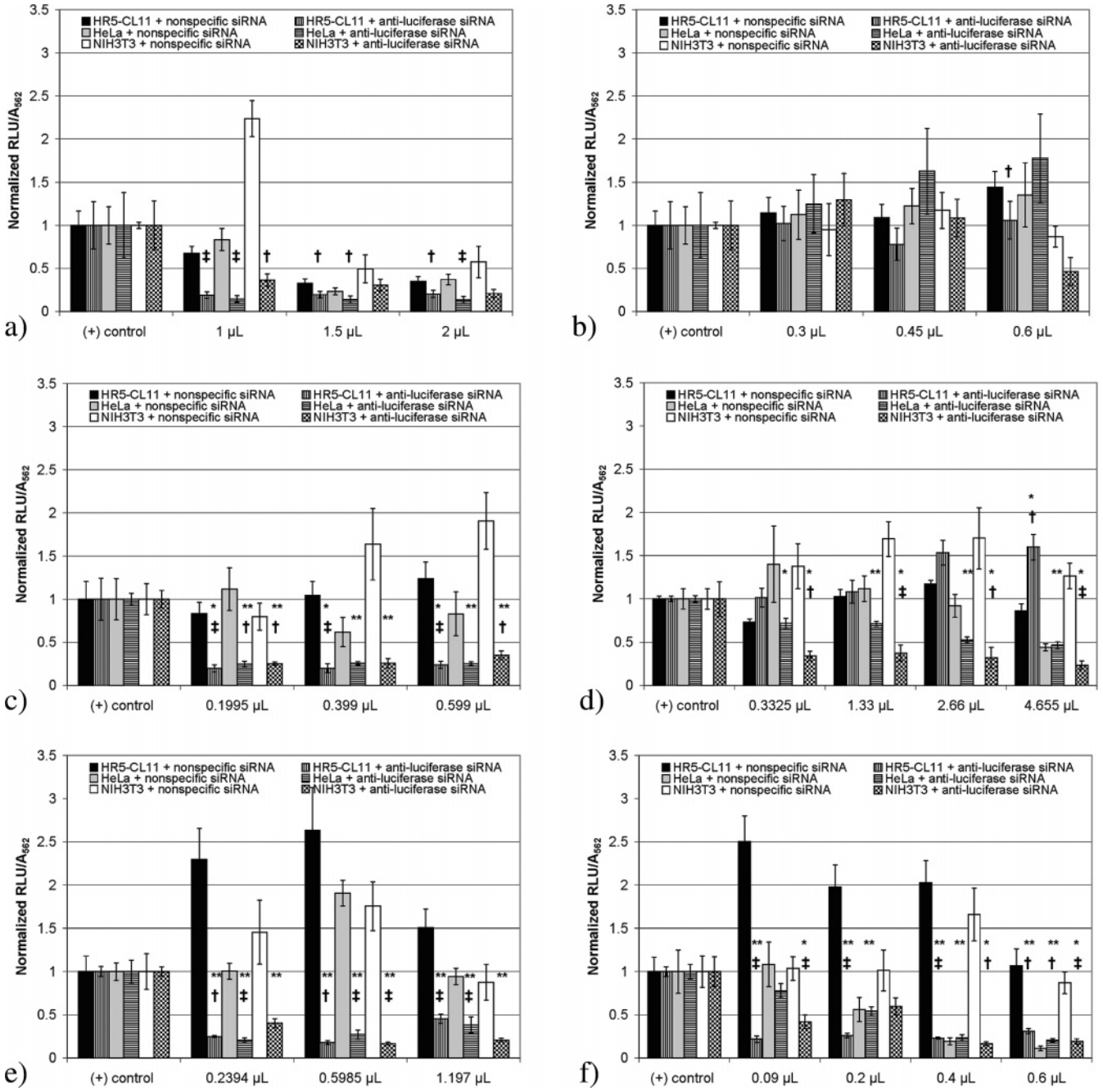


***Figure 1.*** Relative luciferase expression 48 h post-transfection. Luciferase expression (RLU) normalized by protein content ($A_{562}$) over time for cells transfected with different volumes of various reagents and 50 nM siRNA. On each panel, solid bars indicate HR5-CL11 cells treated with nonspecific control siRNA, vertically striped bars indicate HR5-CL11 cells treated with anti-luciferase siRNA, shaded bars indicate HeLa cells treated with nonspecific control siRNA, horizontally striped bars indicate HeLa cells treated with anti-luciferase siRNA, open bars indicate NIH/3T3 cells treated with nonspecific control siRNA, and checkered bars indicate NIH/3T3 cells treated with anti-luciferase siRNA. Statistical significance between positive controls and cells that received anti-luciferase siRNA indicated by * ($p < 0.05$) or ** ($p < 0.01$). Statistical significance between cells that received nonspecific control and anti-luciferase siRNAs indicated by † ($p < 0.05$) or ‡ ($p < 0.01$). Error bars are the standard error of the mean, $n = 3$. (a) siPORT *Amine*, (b) siPORT *Lipid*, (c) RNAiFect, (d) GeneSilencer, (e) X-tremeGENE, (f) *Trans*IT-siQUEST.

the NIH/3T3 cells, with a calculated $IC_{50}$ value of almost 30% v/v (Table 2).

**siPORT *Lipid.*** In the initial screening study (Figure 1b), only a 22% reduction in luciferase expression relative to the positive controls and a 31% reduction relative to the nonspecific siRNA controls were observed in the HR5-CL11 cells when they were treated with 0.45 $\mu$L/well siPORT *Lipid* and anti-luciferase siRNA, and these results were not statistically significant. The luciferase expression for HR5-CL11 cells treated with 0.6 $\mu$L/well siPORT Lipid and anti-

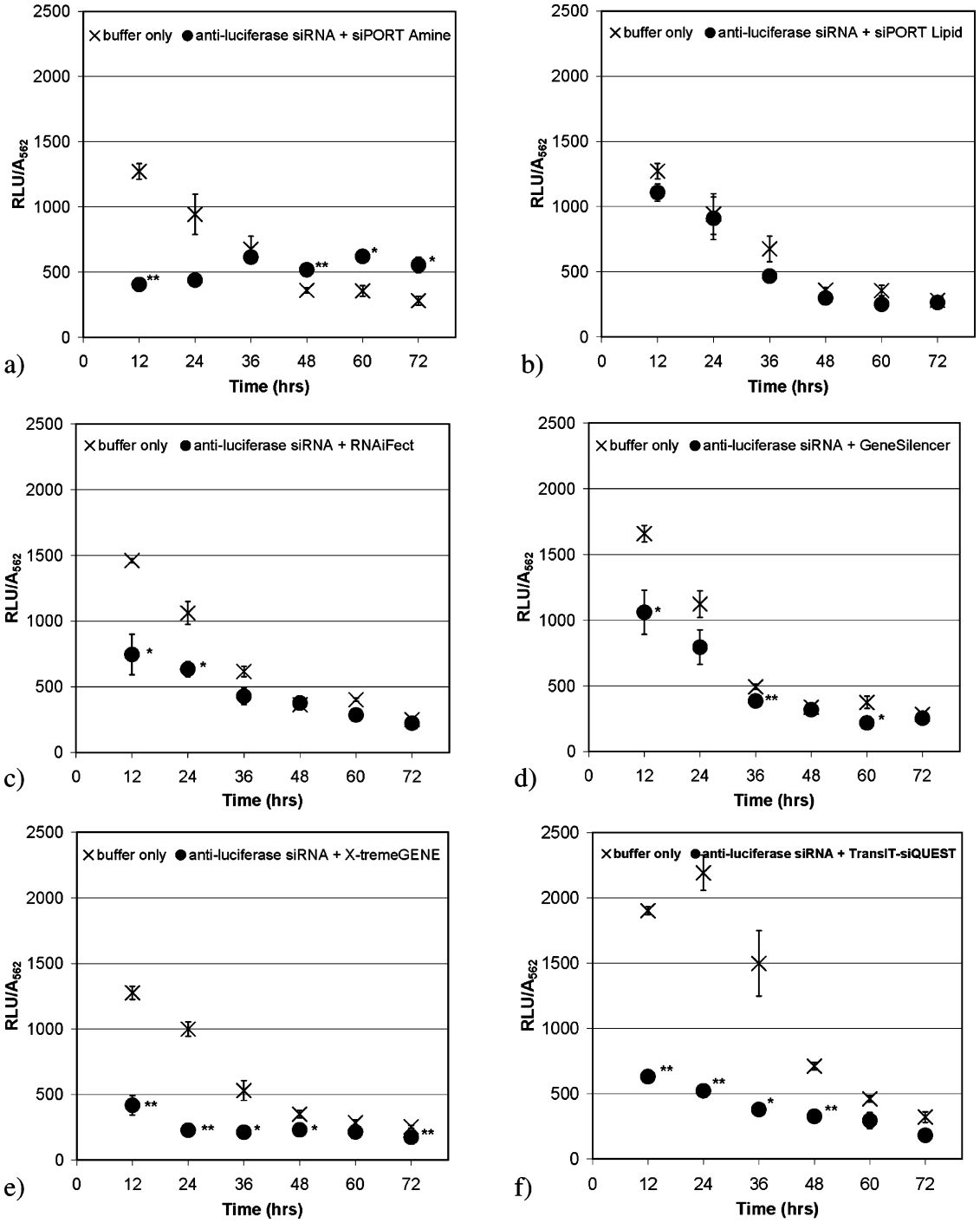


***Figure 2.*** Luciferase expression knockdown kinetics for HR5-CL11 cells. Luciferase expression (RLU) normalized by protein content ($A_{562}$) over time for HR5-CL11 cells treated with buffer only (positive controls, ×), or transfected with anti-luciferase siRNA and a selected volume of each reagent (●). Statistical significance between sample groups indicated by * ($p < 0.05$) or ** ($p < 0.01$). Error bars are the standard error of the mean, $n = 3$. (a) 1 μL/well siPORT *Amine*, (b) 0.45 μL/well siPORT *Lipid*, (c) 0.1995 μL/well RNAiFect, (d) 0.3325 μL/well GeneSilencer, (e) 0.5985 μL/well X-tremeGENE, (f) 0.09 μL/well *Trans*IT-siQUEST.

luciferase siRNA was 38% less than that of the cells that received nonspecific siRNA, and this result was statistically significant. siPORT *Lipid* appeared ineffective in mediating RNAi in the HeLa cell line, and statistically insignificant 54% and 41% reductions in luciferase expression relative to the positive controls and nonspecific siRNA controls, respectively, were observed in the NIH/3T3 cells when they were treated with 0.6 μL/well siPORT *Lipid* and anti-luciferase siRNA. When 0.45 μL/well siPORT *Lipid* was used to deliver anti-luciferase siRNA to HR5-CL11 cells in the knockdown kinetics study (Figure 2b), the largest reduction in luciferase expression (31%) was observed at

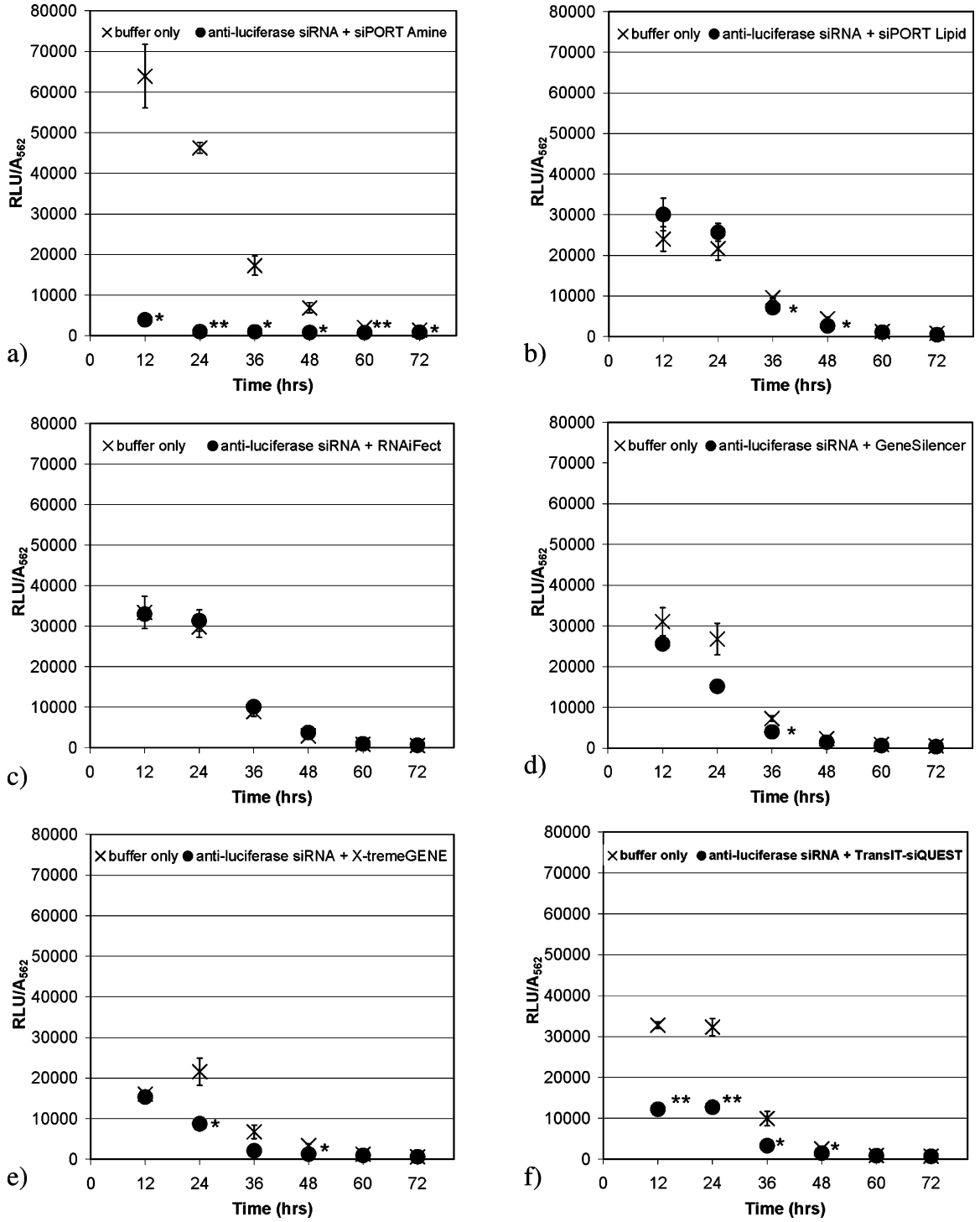


***Figure 3.*** Luciferase expression knockdown kinetics for HeLa cells. Luciferase expression (RLU) normalized by protein content ($A_{562}$) over time for HeLa cells. On each panel, symbols indicate cells transfected with pCMV-luc and *Trans*IT-LT1 (positive controls, ×), and transfected with pCMV-luc and *Trans*IT-LT1 followed by anti-luciferase siRNA and a selected volume of each reagent (●). Statistical significance between sample groups indicated by * ($p < 0.05$) or ** ($p < 0.01$). Error bars are the standard error of the mean, $n = 3$. (a) 2 $\mu$L/well siPORT *Amine*, (b) 0.6 $\mu$L/well siPORT *Lipid*, (c) 0.399 $\mu$L/well RNAiFect, (d) 4.655 $\mu$L/well GeneSilencer, (e) 0.5985 $\mu$L/well X-tremeGENE, (f) 0.2 $\mu$L/well *Trans*IT-siQUEST.

36 h. While smaller reductions in luciferase expression were also observed up to 72 h, none of the results at any of these time points were statistically significant. Although no knockdown was observed in the HeLa cell line at any of the reagent volumes investigated in the initial screening study, the knockdown kinetics in HeLa cells treated with 0.6 $\mu$L/well siPORT *Lipid* and anti-luciferase siRNA were similar to those observed in the HR5-CL11 cell line. Small to moderate reductions in luciferase expression were observed from 36 to 72 h (Figure 3b). The largest reduction in

***Table 2.*** $IC_{50}$ Values (%, v/v) of Commercial siRNA Transfection Reagents for HR5-CL11, HeLa, and NIH/3T3 Cell Lines[a]

| | siPORT *Amine* | siPORT *Lipid* | RNAiFect | GeneSilencer | X-tremeGENE | *Trans*IT-siQUEST |
|---|---|---|---|---|---|---|
| HR5-CL11 | 0.74 | 11.27 | 2.41 | 15.76 | 6.94 | 0.63 |
| HeLa | 0.98 | 4.04 | 2.72 | 11.85 | 4.94 | 0.35 |
| NIH/3T3 | 29.61 | nd | nd | 12.53 | 9.18 | 1.06 |

[a] $IC_{50}$ values calculated from quadratic fit to experimental results (data available in the Supporting Information); nd = could not be determined.

***Table 3.*** Recommended Optimal Transfection Conditions (Volume of Reagent per Well of 96-Well Plate and Assay Time Post-Transfection) for Commercial siRNA Transfection Reagents Used with 50 nM siRNA To Obtain Maximum Luciferase Knockdown while Avoiding Cytotoxicity and Nonspecific Knockdown[a]

| | siPORT *Amine* | | | siPORT *Lipid* | | | RNAiFect | | | GeneSilencer | | | X-tremeGENE | | | *Trans*IT-siQUEST | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | vol ($\mu$L) | time (h) | KD (%) | vol ($\mu$L) | time (h) | KD (%) | vol ($\mu$L) | time (h) | KD (%) | vol ($\mu$L) | time (h) | KD (%) | vol ($\mu$L) | time (h) | KD (%) | vol ($\mu$L) | time (h) | KD (%) |
| HR5-CL11 | <1 | 12 | 68[b] | 0.6 | 36 | 31[d] | 0.1995 | 12 | 49 | 0.3325 | 60 | 42 | 0.5985 | 24 | 77 | 0.09 | 24 | 76 |
| HeLa | <1 | 24 | 98[c] | nd | 48 | 40[e] | 0.1995 | 48 | 75 | 2.66 | 36 | 45[f] | 0.5985 | 48 | 73 | 0.09 | 36 | 22[g] |
| NIH/3T3 | 1 | 48 | 64 | 0.6 | nd | nd | 0.1995 | 48 | 75 | 4.655 | 48 | 77 | 0.5985 | 48 | 83 | 0.4 | 48 | 84 |

[a] KD = measured knockdown relative to positive controls at recommended conditions (exceptions noted below); nd = could not be determined. [b] At 1 $\mu$L/well. [c] At 2 $\mu$L/well. [d] At 0.45 $\mu$L/well. [e] At 0.6 $\mu$L/well. [f] At 4.655 $\mu$L/well. [g] At 48 h.

luciferase expression occurred at 72 h (46% reduction compared to positive controls), although this result was not statistically significant. The largest statistically significant reduction in luciferase expression (40%) occurred at 48 h. siPORT *Lipid* did not appear to mediate luciferase knockdown when a volume of 0.6 $\mu$L/well was used in the NIH/3T3 cell line in the knockdown kinetics study (see the Supporting Information). siPORT *Lipid* was one of the less cytotoxic reagents for the HR5-CL11 and HeLa cell lines. The NIH/3T3 cell line did not appear to be sensitive to the siPORT *Lipid*, as the cell viability never dropped substantially below that measured for untreated cells over the range of siPORT *Lipid* concentrations used in the cytotoxicity study, and no $IC_{50}$ value could be calculated.

**RNAiFect.** In our initial screening study, the knockdown in luciferase expression in a given cell line was similar regardless of the volume of RNAiFect used (Figure 1c). However, the largest knockdowns relative to the positive controls (80%, 75%, and 75% for the HR5-CL11, HeLa, and NIH/3T3 cell lines, respectively) were observed when all three cell lines were treated with 0.1995 $\mu$L/well RNAiFect and anti-luciferase siRNA. The knockdown at this volume of RNAiFect was statistically significant for all three cell lines compared both to the positive controls and to cells that were treated with the nonspecific control siRNA. The largest knockdown relative to the nonspecific siRNA controls (87%) occurred at a volume of 0.1995 $\mu$L/well for the HeLa cell line. Although the largest knockdown relative to the nonspecific siRNA controls occurred at a volume of 0.599 $\mu$L/well for the HR5-CL11 and NIH/3T3 cells (100% and 156%, respectively), this volume of reagent appeared to cause an increase in luciferase expression (solid and open bars in Figure 1c), perhaps due to a stimulatory effect of the delivery reagent.

Significant knockdown was observed in the HR5-CL11 cell line when 0.1995 $\mu$L/well RNAiFect was used in the subsequent knockdown kinetics study. The maximum reduction in luciferase expression (49%) was observed in the HR5-CL11 cell line at 12 h post-transfection (Figure 2c), and the effect of RNAiFect on luciferase expression seemed to gradually diminish over the 72 h of the study. Although the amount of knockdown in the initial screening study was similar when either the HeLa or NIH/3T3 cells received either 0.1995 or 0.399 $\mu$L/well RNAiFect, the larger volume was used in the knockdown kinetics studies with these two cell lines in the hope that it would promote more substantial knockdown. This did not occur, however. Only a very slight reduction in luciferase expression (1%) was observed at 12 h post-transfection when HeLa cells were treated with 0.399 $\mu$L/well RNAiFect and anti-luciferase siRNA (Figure 3c), and the largest reduction in luciferase expression (66%) was observed 48 h post-transfection in the NIH/3T3 cell line (see the Supporting Information). RNAiFect was of intermediate cytotoxicity to the HR5-CL11 and HeLa cell lines, and appeared to be more cytotoxic to the NIH/3T3 cells than the HeLa or HR5-CL11 cells. No $IC_{50}$ value could be calculated for the NIH/3T3 cell line due to a plateau in cell viability at reagent volumes larger than 2 $\mu$L/well (2% v/v, see the Supporting Information).

**GeneSilencer.** No knockdown relative to either the positive controls or nonspecific siRNA controls was observed when GeneSilencer was used to deliver anti-luciferase siRNA to HR5-CL11 cells in the initial screening study, and in fact the luciferase expression appeared to increase as the volume of GeneSilencer was increased (Figure 1d). Since 0.3325 $\mu$L/well GeneSilencer produced the lowest levels of luciferase expression in this cell line in the initial screening study, this volume was therefore used for the subsequent knockdown kinetics study in the HR5-CL11 cell line. Modest knockdown was observed in the HR5-CL11 cell line over the course of 72 h (Figure 2d). The largest reduction in luciferase expression, to 58% of the expression of the positive controls, occurred at 60 h post-transfection. In contrast to the HR5-CL11 cell line, a statistically significant 53% reduction in

luciferase expression compared to the positive controls was observed in HeLa cells treated with 4.655 $\mu$L/well GeneSilencer (Figure 1d). The largest knockdown relative to the nonspecific siRNA controls (68%) occurred at a volume of 0.3325 $\mu$L/well. However, neither result was statistically significant compared to cells that were treated with nonspecific control siRNA. A 47% reduction in luciferase expression relative to the positive controls was observed at a volume of 2.66 $\mu$L/well, and this volume of reagent appeared to avoid the nonspecific knockdown observed at larger reagent volumes. When 4.655 $\mu$L/well GeneSilencer was used in the knockdown kinetics study of the HeLa cell line, the maximum knockdown in luciferase expression (45%) was observed at 36 h post-transfection (Figure 3d). GeneSilencer is the least cytotoxic reagent of those tested in the present study for the HR5-CL11 and HeLa cell lines, and the calculated $IC_{50}$ values in the two cell lines are comparable (15.76 and 11.85% v/v, respectively, Table 2).

Statistically significant knockdown was observed in the NIH/3T3 cell line in the initial screening study. The maximum knockdown relative to the positive controls (77%) occurred with a volume of 4.655 $\mu$L/well GeneSilencer. Similar knockdown was observed at the other volumes of GeneSilencer that were tested. The maximum knockdown relative to the nonspecific siRNA controls (138%) occurred at a volume of 2.66 $\mu$L/well. The knockdown relative to the nonspecific siRNA controls was 103% at a volume of 4.655 $\mu$L/well, however, and this result had greater statistical significance than the result at 2.66 $\mu$L/well ($p = 0.006$ and $p = 0.039$ for 4.655 and 2.66 $\mu$L/well, respectively). The maximum knockdown (73%) in the kinetics study was observed at 48 h when 4.655 $\mu$L/well GeneSilencer was used to deliver anti-luciferase siRNA to the NIH/3T3 cells (see the Supporting Information). GeneSilencer was relatively nontoxic to the NIH/3T3 cells, as indicated by the large calculated $IC_{50}$ value (Table 2).

**X-tremeGENE.** X-tremeGENE appeared to have a stimulatory effect on luciferase expression, as indicated by the increased measured luciferase for cells receiving the nonspecific siRNA (solid bars in Figure 1e). The largest reduction in luciferase expression in the HR5-CL11 cells relative to both the positive controls and nonspecific siRNA controls (82% and 246%, respectively) occurred when 0.5985 $\mu$L/well X-tremeGENE was used in the initial screening study (Figure 1e). When this volume of reagent was subsequently used to deliver anti-luciferase siRNA to the HR5-CL11 cells in the knockdown kinetics study, the largest statistically significant ($p < 0.01$) reduction in luciferase expression (77%) was observed 24 h after transfection (Figure 2e). The 34% reduction in luciferase expression observed at 48 h was statistically significant ($p < 0.05$), but less so than the measured knockdown at 24 h.

X-tremeGENE appeared to have a stimulatory effect on luciferase expression in the HeLa cell line at a reagent volume of 0.5985 $\mu$L/well. The knockdown in the HeLa cell line was similar to that observed in the HR5-CL11 cell line in the initial screening study. The luciferase expression of HeLa cells was reduced by 79% and 73% when they received 0.2394 and 0.5985 $\mu$L/well, respectively, of X-tremeGENE. The largest reduction in luciferase expression relative to the nonspecific siRNA controls (163%) occurred at a volume of 0.5985 $\mu$L/well. When 0.5985 $\mu$L/well X-tremeGENE was subsequently used in the knockdown kinetics study, the largest statistically significant reduction in luciferase expression (62%) was observed at 48 h in the HeLa cell line. The cytotoxicity of the X-tremeGENE was similar in the HR5-CL11 and HeLa cell lines. The $IC_{50}$ values (Table 2) and cell survival trends were similar, and X-tremeGENE had intermediate cytotoxicity for both cell lines.

X-tremeGENE was also an effective delivery reagent in the NIH/3T3 cell line. Luciferase expression was reduced by a maximum of 83% and 159% relative to the positive controls and nonspecific siRNA controls, respectively, when 0.5985 $\mu$L/well X-tremeGENE was used in the initial screening study. A 74% maximum reduction in luciferase expression was measured in the subsequent knockdown kinetics study 60 h after transfection with this same volume of X-tremeGENE (Supporting Information). X-tremeGENE appeared to have a moderately stimulatory effect on luciferase expression at the smaller reagent volumes that were used (see Figure 1e).

***Trans*IT-siQUEST.** *Trans*IT-siQUEST was extremely effective at reducing luciferase expression in the HR5-CL11 and NIH/3T3 cell lines. The best knockdown in the initial screening study occurred in the HR5-CL11 cell line (78% and 229% relative to the positive controls and nonspecific siRNA controls, respectively) when 0.09 $\mu$L/well *Trans*IT-siQUEST was used to deliver anti-luciferase siRNA (Figure 1f). In the subsequent knockdown kinetics study, the maximum reduction in luciferase expression (76%) was measured in this cell line 24 h after transfection with 0.09 $\mu$L/well *Trans*IT-siQUEST (Figure 2f).

Although similar knockdown (79% relative to the positive controls) to that observed in the HR5-CL11 cell line was achieved in the initial screening study of *Trans*IT-siQUEST in the HeLa cell line, a volume of 0.6 $\mu$L/well *Trans*IT-siQUEST was required to achieve this result. However, no statistically significant reduction in luciferase expression compared to the cells that received nonspecific control siRNA was observed in the HeLa cell lines at any of the reagent volumes used (see Figure 1f). The largest reduction in luciferase expression relative to the nonspecific siRNA controls (30%) occurred at a reagent volume of 0.09 $\mu$L/well. The luciferase knockdown relative to the positive controls at this reagent volume was 22%. Although the largest knockdown was observed at a volume of 0.6 $\mu$L/well *Trans*IT-siQUEST, a volume of 0.2 $\mu$L/well *Trans*IT-siQUEST was used in the subsequent knockdown kinetics study to avoid cytotoxicity since the calculated $IC_{50}$ of *Trans*IT-siQUEST in the HeLa cells was 0.35% v/v (0.21 $\mu$L/well, see Table 2). In the knockdown kinetics study of the HeLa cell line the largest reduction in luciferase expression (67% relative to positive controls) was measured

36 h after transfection with 0.2 μL/well *Trans*IT-siQUEST (Figure 3f). The measured knockdowns at 12 and 24 h (63% and 61%, respectively) were comparable to the measured knockdown at 36 h.

The magnitude of knockdown achieved in the NIH/3T3 cell line in the initial screening study was similar to that observed in the HR5-CL11 cell line. The maximum luciferase knockdown relative to both the positive controls and the nonspecific siRNA controls (84% and 150%, respectively) occurred at a volume of 0.4 μL/well *Trans*IT-siQUEST. Although the $IC_{50}$ of *Trans*IT-siQUEST in the NIH/3T3 cell line was larger than in the other two cell lines (1.06% v/v, or approximately 0.636 μL/well, see Table 3), a conservative volume of 0.2 μL/well *Trans*IT-siQUEST was used in the subsequent knockdown kinetics study to avoid any potential cytotoxicity. The largest reduction in luciferase expression (only 30%) was observed 48 h after transfection in the knockdown kinetics study of the NIH/3T3 cells (see the Supporting Information). This knockdown is comparable to the 40% knockdown observed when this volume of *Trans*IT-siQUEST was used in the initial screening study. The observed knockdown in the kinetics study, however, was not statistically significant compared to the positive controls at any of the time points.

## Discussion

siPORT *Amine* produced relatively good knockdown in all three of the investigated cell lines. The transfection efficacy and cytotoxicity results of siPORT *Amine* in the HR5-CL11 and HeLa cell lines were similar, and the siPORT *Amine* was relatively less cytotoxic to the NIH/3T3 cell line. Although the largest knockdown occurred in HeLa cells treated with 2 μL/well siPORT *Amine*, cytotoxicity was observed even at 1 μL/well. Results reported by Ambion, the manufacturer of both siPORT *Amine* and siPORT *Lipid*, suggest that the optimal volume of siPORT *Amine* is similar to the volume at which cytotoxicity effects appear in some cell lines; the best knockdown of GAPDH in COS-7 cells was observed at 4 μL per well in a 24-well plate (approximately 0.68 μL/well of a 96-well plate, scaled by area, see ref 20 for tissue culture plate area conversion factors), but less than 50% of COS-7 cells survived 48 h after transfection with 5 μL per well in a 24-well plate (approximately 0.85 μL/well of a 96-well plate).[21] In fact, Ambion does not recommend the use of siPORT *Amine* in the HeLa cell line.[22] siPORT *Amine* volumes less than 1 μL are therefore recommended for both the HeLa and HeLa-derived HR5-CL11 cell lines (see Table 3). The optimal assay time for maximum knockdown appears to be 12 h for the HR5-CL11 cell line and 24 h post-transfection for the HeLa cell line (Table 3). Our observation that the efficacy and cytotoxicity of the siPORT *Amine* are different in HeLa-derived and NIH/3T3 cell lines is consistent with the differential recommendations by Ambion. While Ambion does not recommend siPORT *Amine* in the HeLa cell line, siPORT *Amine* is recommended for use with NIH/3T3 cells.[23] Although the largest knockdown relative to the positive controls occurred at a volume of 2 μL/well siPORT *Amine* in the NIH/3T3 cell line, this result was not statistically significant when compared to the nonspecific siRNA controls, possibly due to nonspecific knockdown effects of the siPORT *Amine* (see Figure 1f). While the $IC_{50}$ of siPORT *Amine* was nearly 30% v/v (30 μL/well) for the NIH/3T3 cells, a volume of 1 μL of siPORT *Amine* per well is conservatively recommended for the NIH/3T3 cells to avoid the nonspecific knockdown observed in Figure 1f, with the optimal assay time at 48 h post-transfection (Table 3).

The substantial knockdown observed in all three cell lines with siPORT *Amine* is in stark contrast to the observed poor transfection efficacy of siPORT *Lipid* (Figures 1b, 2b, and 3b). The generally poor knockdown results we observed with siPORT *Lipid* are in contrast to the 95% knockdown of GAPDH at 48 h in HeLa cells reported by Ambion.[22] However, Ambion's results are not reported in a peer-reviewed publication and do not include the transfection conditions used, making direct comparisons difficult. The poor transfection efficacy we observed is unlikely to be caused by cytotoxicity of the siPORT *Lipid* since the calculated $IC_{50}$ values of siPORT *Lipid* (11.27% and 4.04% v/v in the HR5-CL11 and HeLa cell lines, respectively) were larger than the volumes used in the initial screening and knockdown kinetics experiments, and the viability of the NIH/3T3 cell line never dropped substantially below that measured for untreated cells over the range of siPORT *Lipid* concentrations used in the cytotoxicity study. We recommend an optimal reagent volume of 0.6 μL/well siPORT *Lipid* for both the HR5-CL11 and NIH/3T3 cell lines based on the knockdown relative to the nonspecific siRNA controls in the initial screening study, and assay times of 36 h for HR5-CL11 cells and 48 h for HeLa cells based on the results of the knockdown kinetics studies in Figures 2b and 3b (see Table 3). We cannot recommend an optimal volume of siPORT *Lipid* for the HeLa cell line due to the poor knockdown results observed in the initial screening study.

RNAiFect was a modestly effective delivery reagent in all three cell lines. Interestingly, the optimal reagent volume based on the results of the present studies is the same in all three cell lines (0.1995 μL/well; see Table 3). Although the knockdown in luciferase expression of the HR5-CL11 and NIH/3T3 cell lines was largest compared to the nonspecific

(20) *Retroviral Gene Transfer and Expression User Manual*; Protocol No. PT3132-1, Version No. PR37004; BD Biosciences Clontech; July 2003.

(21) Jarvis, R. Optimize Transfection of siRNAs for RNAi. *Ambion TechNotes* **2002**, *9* (6). http://www.ambion.com/techlib/tn/96/9612.html.

(22) Choose the Right Transfection Reagent for Your RNAi Experiment. *Ambion TechNotes* **2003**, *10* (3). http://www.ambion.com/techlib/tn/103/5.html (accessed July 25, 2006).

(23) Recommended siPORT™ Transfection Reagent for Various Cell Types. http://www.ambion.co.jp/techlib/resources/delivery/efficiency_chart.html (accessed July 25, 2006).

controls when a volume of 0.599 $\mu$L/well was used in the initial screening study, both this volume and 0.399 $\mu$L/well RNAiFect seemed to cause a stimulatory effect in luciferase expression. The time at which the optimal knockdown occurred was different in the HR5-CL11 cell line than in the HeLa and NIH/3T3 cell lines. The effect of RNAiFect was maximal at 12 h post-transfection in the HR5-CL11 cell line and seemed to gradually diminish over the 72 h of the study. We therefore recommend an assay time of 12 h post-transfection with a volume of 0.1995 $\mu$L/well RNAiFect for the HR5-CL11 cell line (Table 3). In contrast, only a very slight reduction in luciferase expression (1%) was observed at 12 h post-transfection when HeLa cells were treated with 0.399 $\mu$L/well RNAiFect and anti-luciferase siRNA (Figure 3c). Maximal knockdown in the kinetics study was observed at 48 h post-transfection in the NIH/3T3 cell line, but the amount of knockdown was smaller with 0.399 $\mu$L/well than with the 0.1995 $\mu$L/well used in the initial screening study. These are surprising results, given the 75% knockdown observed in the initial screening study when 0.1995 $\mu$L/well RNAiFect was used with both cell lines. The initial conditions of 0.1995 $\mu$L/well RNAiFect with assay 48 h post-transfection therefore appear to be optimal for both the HeLa and NIH/3T3 cell lines (Table 3). Although the manufacturer (Qiagen) reports approximately 90% knockdown of lamin A/C mRNA in HeLa-S3 cells 48 h after transfection, the conditions that were used are unspecified. Qiagen also reported less than a 10% increase in LDH (lactate dehydrogenase) activity, a measure of cytotoxicity, at 48 h in HeLa-S3 cells.[24] However, the volume of reagent used is not specified, and therefore our results cannot be directly compared. In a separate study Qiagen reported optimal transfection viability at 24 h when HeLa-S3 cells were treated with 0.9 $\mu$g of siRNA and 5.4 $\mu$L of RNAiFect per well of a 24-well plate[25] (corresponding to approximately 0.225 $\mu$g of siRNA and 1.35 $\mu$L of RNAiFect per well of a 96-well plate[26]). For comparison, the present studies used 50 nM, or approximately 66.5 ng, of siRNA per well of a 96-well plate. In experiments using human mammary epithelial cells (HMEC) in 24-well plates, Qiagen reports that approximately 80−85% knockdown of lamin A/C was achieved 48 h after transfection with 200 nM siRNA and a 1:6 or 1:9 ratio of $\mu$g of siRNA:$\mu$L of RNAiFect.[27] Although these studies reported slightly larger knockdown than what we observed in either the HR5-CL11 or HeLa cell lines, they used larger amounts of siRNA and RNAiFect than what we tested, since the siRNA concentration was kept constant at 50 nM for all of our studies.

GeneSilencer produced disparate knockdown results, depending on the cell line in which it was used. The largest reductions in luciferase expression in the knockdown kinetics studies were observed at different times and with different reagent volumes in the HR5-CL11 and HeLa cell lines (see Table 3). The optimal conditions for the HR5-CL11 cell line based on the knockdown kinetics study (Figure 2d) appear to be 0.3325 $\mu$L/well and assay at 60 h post-transfection. Although a volume of 4.655 $\mu$L/well GeneSilencer produced the largest knockdown relative to the positive controls in the initial screening study of the HeLa cell line, the decrease in luciferase expression for the cells treated with nonspecific siRNA at this volume of GeneSilencer suggests that nonspecific knockdown may be an issue. At the other three volumes we investigated, the luciferase expression of the nonspecific siRNA controls was statistically similar to that of the positive controls, suggesting that nonspecific knockdown is not a concern under these conditions. The 47% knockdown relative to the positive controls observed at a volume of 2.66 $\mu$L/well in the initial screening study was the next best result, and we therefore recommend this volume of GeneSilencer for use with the HeLa cell line. We recommend an assay time of 36 h based on the best result of approximately 45% knockdown relative to the positive controls that was observed in the HeLa cell line at 36 h post-transfection with 4.655 $\mu$L/well in the knockdown kinetics study. The magnitude of knockdown is similar to results reported by others. A study in which GeneSilencer was used to investigate functional genomics reported approximately 50% knockdown of a gene of interest in HeLa cells, as determined by functional assay, when cells were treated with GeneSilencer and 1000 ng of siRNA per well of a 6-well plate.[28] Although the amount of knockdown reported was similar to what we achieved, a direct comparison cannot be made due to the fact that the exact concentrations of siRNA and GeneSilencer that were used in the study were not reported. GeneSilencer was more effective at reducing luciferase expression in the NIH/3T3 cell line than in the HR5-CL11 and HeLa cell lines (see Table 3), although the nonspecific siRNA controls (open bars in Figure 1d) seem to have increased luciferase expression relative to the positive controls, possibly due to a stimulatory effect of GeneSilencer. The 62% knockdown we observed at 72 h in the kinetics study is similar to the 55% reduction in gene function 72 h after treatment with unspecified amounts of GeneSilencer and siRNA reported in a study of functional genomics in

(24) http://www1.qiagen.com/products/transfection/transfectionreagents/rnaifect.aspx?ShowInfo=1 (accessed July 26, 2006).

(25) Valer, M.; Buhlmann, C.; Andreou, I.; Weber, M. Optimizing RNAi Experiments Using QIAGEN siRNA and the Agilent 2100 Bioanalyzer. *Qiagen News* **2003**, *e33*. http://www1.qiagen.com/literature/qiagennews/weeklyarticle/sep03/e33/default.aspx (accessed July 25, 2006).

(26) *RNAiFect™ Transfection Handbook, For Transfection of Eukaryotic Cells with siRNA*; October 2003, p 14. http://www1.qiagen.com/literature/handbooks/PDF/siRNAforgenesilencing/tf_rnaifect/1025712_hb_tf_rnaifect1003.pdf (accessed July 25, 2006).

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(28) Yeng, Q.-H.; Church-Hajduk, R.; Ren, J.; Newton, M. L.; Du, C. Omi/HtrA2 Catalytic Cleavage of Inhibitor of Apoptosis (IAP) Irreversibly Inactivates IAPs and Facilitates Caspase Activity in Apoptosis. *Genes Dev.* **2003**, *17*, 1487−1496.

NIH/3T3 cells.[29] Although 4.655 μL/well GeneSilencer appeared to give the best knockdown relative to the positive controls in the NIH/3T3 cell line, noncellular aggregates were observed in the tissue culture plate wells via microscopy in the transfection studies. This was surprising, as the volumes used were much smaller than the calculated $IC_{50}$ values. A smaller volume of GeneSilencer might therefore be advantageous to avoid aggregates in the tissue culture plates, given that the luciferase knockdown in NIH/3T3 cells in the initial screening study was similar at all the tested volumes of GeneSilencer. On the basis of the results of the initial screening and knockdown kinetics studies, however, a volume of 4.655 μL/well GeneSilencer is recommended for NIH/3T3 cells, with an assay time of 48 h post-transfection.

X-tremeGENE was one of the most effective delivery reagents in all three cell lines (Figure 1e) and produced similar levels of knockdown in all three cell lines. On the basis of the results of the initial screening study and subsequent knockdown study in the HR5-CL11 cell line, a volume of 0.5985 μL/well and assay time of 24 h are recommended for this cell line (see Table 3). Although a volume of 0.2394 μL/well X-tremeGENE produced the largest knockdown relative to the positive controls in the initial screening study of the HeLa cell line, the reduction in luciferase was largest compared to the nonspecific siRNA controls at a volume of 0.5985 μL/well. Given that the largest knockdown in the kinetics study of the HeLa cell line (Figure 3e) also occurred at 48 h and with 0.5985 μL/well X-tremeGENE, an assay time of 48 h and reagent volume of 0.5985 μL/well are therefore recommended for HeLa cells transfected with X-tremeGENE (see Table 3). Although the 62% and 74% reductions in luciferase expression at 48 and 60 h in the knockdown kinetics study of the NIH/3T3 cell line were statistically significant, they had more scatter in the data than the larger reduction (83%) observed at 48 h with 0.5985 μL/well in the initial screening study. Therefore, a volume of 0.5985 μL/well X-tremeGENE and assay time of 48 h also appear to be the optimal conditions for use of this reagent in NIH/3T3 cells (see Table 3). It is worth nothing that the statistically significant 70% reduction in luciferase expression observed at 72 h in the NIH/3T3 cell line (see the Supporting Information) compares favorably with a reported 70% reduction in target protein expression in NIH/3T3 cells 72 h after treatment with 100 nM siRNA and 3 μL of X-tremeGENE per well of a 6-well plate[30] (approximately 0.10 μL/well of a 96-well plate[20]).

Although *Trans*IT-siQUEST was found to be one of the more effective delivery reagents in this study, the optimal reagent volume and assay time varied depending on the cell line. *Trans*IT-siQUEST appeared to stimulate luciferase production in the HR5-CL11 cell line, as evidenced by the greater luciferase expression of cells that received nonspecific siRNA compared to the positive controls (see solid bars in Figure 1f). Based on both the initial screening and subsequent knockdown kinetics studies, the best results were obtained in the HR5-CL11 cell line with a reagent volume of 0.09 μL/well and assay time of 24 h post-transfection. These conditions are therefore recommended for this cell line (see Table 3). In contrast with the HR5-CL11 cell line, the HeLa cells showed a reduction in luciferase expression when treated with either the anti-luciferase siRNA or the nonspecific siRNA at volumes larger than 0.09 μL/well (shaded and horizontally striped bars in Figure 1f, respectively), suggesting that the luciferase knockdown in this cell line under these conditions was not specifically mediated by siRNA. A substantial reduction in luciferase expression relative to the nonspecific siRNA controls was only observed at a *Trans*IT-siQUEST volume of 0.09 μL/well in the initial screening study, and we therefore recommend a volume of 0.09 μL/well for this cell line. An assay time of 36 h is recommended for the HeLa cell line based on the results of the knockdown kinetics study. The largest knockdown relative to both the positive controls and nonspecific siRNA controls for the NIH/3T3 cell line in the initial screening study were obtained with a *Trans*IT-siQUEST volume of 0.4 μL/well, and we therefore recommend this reagent volume for use with the NIH/3T3 cell line. Although smaller knockdown was achieved in the kinetics study of the NIH/3T3 cell line when a volume of 0.2 μL/well *Trans*IT-siQUEST was used, the optimal assay time was determined to be 48 h post-transfection. The reductions in measured luciferase expression under the optimal conditions were different for the HeLa and NIH/3T3 cell lines (22% and 84%, respectively). Although the manufacturer (Mirus Bio Corporation) reports much larger knockdown in HeLa cells than what we observed here (approximately 85% and 82% knockdown of transiently and stably expressed firefly luciferase, respectively, were reported in HeLa cells 24 h after transfection with 25 nM siRNA and *Trans*IT-siQUEST[31]), the amount of reagent used and the statistical significance of the results are not indicated. However, the knockdown we observed in the NIH/3T3 cell line is in good agreement with the approximately 79% and 89% knockdown of transiently and stably expressed firefly luciferase, respectively, reported in NIH/3T3 cells 24 h after transfection with 25 nM siRNA and *Trans*IT-siQUEST.[31] Again, however, the amount of reagent used and the statistical significance of the results are not indicated.

Unfortunately it is not possible to ascertain specific structure−function relationships for the investigated reagents since the chemical composition of each reagent is held

(29) Bertling, E.; Hotulainen, P.; Mattila, P. K.; Matilainen, T.; Salminen, M.; Lappalainen, P. Cyclase-Associated Protein 1 (CAP1) Promotes Cofilin-Induced Actin Dynamics in Mammalian Nonmuscle Cells. *Mol. Biol. Cell* **2004**, *15*, 2324−2334.

(30) Daugaard, M.; Fehrenbacher, N. Progress on siRNA delivery in mammalian cell lines. *Biochemica* **2004**, *4*, 12−14. http://www.roche-applied-science.com/PROD_INF/BIOCHEMI/no4_04/pdf/p12.pdf (accessed July 25, 2006).

(31) Brazas, R.; Millard, S.; Kawleski, T.; Hagstrom, J. *Trans*IT® siRNA Transfection Reagents for High Efficiency siRNA Delivery In Vitro, Technical Report, Mirus Bio Corporation, **2004**. http://www.mirusbio.com/pdf/siRNA-Delivery-Reagents-Technical-report.pdf (accessed July 25, 2006).

proprietary. However, several trends are apparent when examining Table 2 (calculated $IC_{50}$ values) and Table 3 (optimal transfection conditions). First, a comparison of the calculated $IC_{50}$ values shows overall remarkably similar results for the HeLa and HR5-CL11 cell lines, which is not surprising given that the HR5-CL11 cell line is derived from the HeLa parent cell line. Second, in some cases the calculated $IC_{50}$ values for the HR5-CL11 and HeLa cell lines are quite different from those calculated for the NIH/3T3 cell line, again showing the relevance of cell lineage. Third, a comparison of the optimal transfection conditions for the two transiently transfected cell lines (HeLa and NIH/3T3) shows that the time of maximal knockdown occurred in the NIH/3T3 cells at the same time as or later than in the HeLa cells. Fourth, a comparison of the transiently expressing HeLa cell line and stably expressing HeLa-derived HR5-CL11 cell line shows that the time of optimal knockdown almost always occurred in the HR5-CL11 cell line earlier than or at the same time as in the HeLa cell line. Fifth, the maximum amount of knockdown varied widely, from no knockdown with siPORT *Lipid* in the NIH/3T3 cell line to 98% knockdown with 2 $\mu$L/well siPORT *Amine* in the HeLa cell line. However, knockdown efficiencies generally ranged from approximately 70% to 85%. The knockdown efficiency of a particular reagent also varied depending on the cell line in which it was used. The siPORT *Amine*, X-tremeGENE, and *Trans*IT-siQUEST appeared to have the best overall performance, as evaluated both by the amount of knockdown achieved and by the consistency of performance between cell lines. These comparative results provide an initial basis for reagent selection and experimental design in RNA interference studies in these particular cell lines, and underscore the importance of reagent compatibility with the cell lineage(s) of interest.

In conclusion, the knockdown kinetics, transfection efficacy, and cytotoxicity of six commercial siRNA transfection reagents were correlated using cells from two different lineages, as well as two different marker protein expression profiles (i.e., stable vs transient gene expression kinetics). The results show that the efficacy and knockdown kinetics of each reagent depend on the cell lineage, and to some degree depend on the protein expression profile. Reagent cytotoxicity appears to depend on the cell lineage, but not on the protein expression profile. To our knowledge, this is the first investigation of whether protein expression profiles affect observed gene knockdown in a cell lineage, as well as the first broad and systematic comparison of siRNA delivery reagents in the peer-reviewed literature.

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**Supporting Information Available:** Summary of transfection conditions used for commercial transfection reagents, luciferase expression knockdown kinetics for the NIH/3T3 cell line, and cytotoxicity data for each commercial reagent in HR5-CL11, HeLa, and NIH/3T3 cell lines. This information is available free of charge via the Internet at http://pubs.acs.org.

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