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Jean-Philippe Carralot, Tae-Kyu Kim, Boris Lenseigne, Annette S Boese, Peter Sommer, et al.. Automated high-throughput siRNA transfection in raw 264.7 macrophages: a case study for optimization procedure.. Journal of Biomolecular Screening, 2009, 14 (2), pp.151-60. 10.1177/1087057108328762 . pasteur-00683854

HAL Id: pasteur-00683854
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J Biomol Screen OnlineFirst, published on February 4, 2009 as doi:10.1177/1087057108328762

Automated High-Throughput siRNA Transfection in Raw 264.7 macrophages: A Case Study for Optimization Procedure

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RNAi using siRNA is a very powerful tool for functional genomics to identify new drug targets and biological pathways. Although their use in epithelial cells is relatively easy and straightforward, transfection in other cell types is still challenging. The authors report the optimization of transfection conditions for Raw 267.4 macrophage cells. The herein described procedure makes use of automated confocal microscopy, enhanced green fluorescent protein (EGFP)–expressing macrophages, and fluorescently labeled siRNAs to simultaneously quantify both siRNA uptake and silencing efficiency. A comparison of 10 commercial transfectants was performed, leading to the selection of the transfectant giving the highest reproducible knock-down effect without inducing cell toxicity or cell activation. Several buffers used for siRNA/lipid complex assembly were tested, and such a study revealed the crucial importance of this parameter. In addition, a kinetics study led to the determination of the optimal siRNA concentration and the best time window for the assay. In an original approach aimed at simultaneously optimizing both the high-throughput screening process and biological factors, optimal reagent volumes and a process flowchart were defined to ensure robust silencing efficiencies during screening. Such an account should pave the way for future genome-wide RNAi research in macrophages and present an optimization procedure for other "hard-to-transfect" cell lines. (*Journal of Biomolecular Screening* XXXX:xx-xx)

Key words: siRNA, transfection, macrophages, automation, optimization procedure

INTRODUCTION

OVER THE PAST YEARS, RNA INTERFERENCE (RNAi) using short interfering RNA (siRNA) has been adopted as the method of choice for loss-of-function studies in a variety of mammalian cells. Indeed, as the introduction of long double-stranded RNA induces innate immune responses,[1] RNAi in mammalian cells is achieved using siRNA.[2] The emergence of commercially available genome-wide libraries of chemically synthesized siRNAs has enabled the rapid development of high-throughput screening (HTS) of siRNA. However, one major drawback for successful siRNA screening resides in an efficient delivery of siRNA into cells. Cell transfection through cationic lipid-based reagents has become one of the most popular methods as it enables protocol automation and yields efficient knock-down in many cell types.[2-4]

For various cell lines such as HeLa, a direct adaptation of the manufacturer's single-tube protocol to the HTS format is sufficient to achieve efficient siRNA delivery. Therefore, reports on the optimization of lipid-mediated transfection are typically limited to transfectant screening and the optimization of siRNA and lipofectant concentrations.[5] However, for other cell types, transfection still remains a challenge,[6] and such classical optimization approaches do not lead to levels of knock-down sufficient for loss-of-function studies. For "hard-to-transfect" cell lines, viral vector-mediated expression of short hairpin RNAs (shRNAs) offers a possible alternative to lipid-mediated siRNA delivery.[7,8] Nevertheless, several studies have reported higher variability in silencing efficacy exhibited by shRNAs compared with siRNAs.[9] In addition, viral-mediated transduction might increase off-target effect risk due to an excessive presence of siRNA.[10,11]

Macrophages are key players of the immune system as they constitute the first cellular mediators of innate immune defense together with neutrophils and dendritic cells (DCs). They are involved in many inflammatory diseases[12] and are the target for many intracellular pathogens such as *Listeria monocytogenes*, *Mycobacterium tuberculosis*, or *Leishmania* spp.[13] Despite such a central role in many diseases, the only genome-wide siRNA screenings in macrophage-like cells, to our knowledge, were

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Received Jul 4, 2008 and in revised form Oct 12, 2008. Accepted for publication Oct 16, 2008.

Journal of Biomolecular Screening XX(X); XXXX
DOI: 10.1177/1087057108328762

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performed in *Drosophila* S2 cells,[14,15] and to date, no report exists on a siRNA screen using mammalian macrophages. The lack of such report might be explained by the fact that accepted models of macrophage-like cell lines, such as J774.1, Raw 264.7, and THP-1, are particularly difficult to transfect.

In the present study, several parameters were tested to increase silencing efficacy in Raw 264.7 cells. In addition, method adaptation to the HTS format was embedded in biological parameter optimization to ensure consistent knockdown levels during HTS. This original procedure led to the delivery of an automated high-throughput siRNA transfection protocol in murine macrophage-like Raw 264.7 cells.

MATERIALS AND METHODS

Generation of EGFP-expressing Raw 264.7 cell using the lentiviral system

Vector particles were produced by transient cotransfection of HEK 293T/17 cells (ATCC CRL-11268) with a pTRIP-LTR-EGFP plasmid, an encapsidation plasmid (p8.71), and a VSV-g envelope expression plasmid (pVSV-G) using calcium phosphate coprecipitation as described elsewhere.[16,17] Cell culture supernatants were harvested 48 h after transfection. Retroviral particles were recovered from the cell supernatant and concentrated by ultracentrifugation (48,000 *g*/1h45/4 °C) on a Sorvall RC-6 Plus Superspeed Centrifuge (Thermo Fisher Scientific, Waltham, MA). After resuspension of the pellet in pure Dulbecco's modified Eagle's medium (DMEM) media (Gibco, Carlsbad, CA), virus was stored at –80 °C.

Raw 264.7 (ATCC TIB-71) cells expressing enhanced green fluorescent protein (EGFP) were generated by Lentiviral transduction. Briefly, Raw 264.7 cells were plated on 96-well plates (Falcon) and incubated with a half dilution of thawed viral stock. Plates were centrifuged twice (300 *g*, 2 × 30 min) to increase infection efficiency and then placed at 37 °C. At 72 h posttransduction, more than 90% of the Raw 264.7 cells were expressing EGFP, as confirmed by fluorescence microscopy.

EGFP-expressing Raw 264.7 cells were cultured at 37 °C/5% CO_2 in RPMI 1640 medium (Gibco) supplemented with 10% of fetal bovine serum (FBS; Gibco).

siRNA stocks

All siRNAs were labeled with DY647 fluorochrome and were purchased from Dharmacon Thermofisher (Boulder, CO). Four labeled siRNAs targeting EGFP (target sequences: 5′-GGC TAC GTC CAG GAG CGC ACC-3′, 5′-GCA AGC TGA CCC TGA AGT TC-3′, 5′-GGC AAG CTG ACC CTG AAG TTC-3′, 5′- GCG ACG TAA ACG GCC ACA AGT TC-3′) were resuspended at 20 μM in resuspension buffer (Dharmacon Thermofisher) and pooled together. DY647-labeled nontargeting siRNA (target sequence: 5′-U AAG GCU AUG AAG AGA UAC-3′) was resuspended at 20 μM and used as a control.

Transfectants

To determine the best transfectant for Raw 264.7 cells, 10 different lipofectants were tested. Dharmafect 1 (cat. T2001-02), Dharmafect 2 (cat. T2002-02), Dharmafect 3 (cat. T2003-02), and Dharmafect 4 (cat. T2004-02) were purchased from Dharmacon. HiPerFect (cat. 301704) and Effectene (cat. 301425) were purchased from QIAGEN GmbH (Hilden, Germany). INTERFERin™ (cat. 409-10) was purchased from Polyplus transfection SA (Illkirch, France). Lipofectamine 2000 (cat. 11668-027), Lipofectamine RNAiMax (cat. 13778-075), and Lipofectamine LTX (cat. 15338-100) were purchased from Invitrogen (Carlsbad, CA). All transfectants were stored at 4 °C.

siRNA transfection

DY647-labeled siRNAs were diluted in OPTIMEM medium (Gibco), and 5 μL of the diluted siRNA was distributed into each well of 384-well EVOTEC plates (cat. 781058) from Greiner Bio-One (Frickenhausen, Germany). Transfectant was gently mixed and diluted in OPTIMEM medium, and 5 μL of the diluted lipofectant was distributed onto the siRNA in each well. Lipid/siRNA complexes were allowed to form during 30 min at room temperature.

EGFP-expressing Raw 264.7 cells at 60% to 80% confluence were detached from culture vessels, washed with RPMI 1640 medium, and counted. Cells were resuspended at 8.3×10^4 cells/mL, and 30 μL (2500 cells) of the resulting cell suspension was dispensed onto the siRNA/lipid complexes. For siRNA transfection, cells were always maintained at less than 5 passages.

Cell viability assay

On indicated day posttransfection, cell viability was evaluated with 100 μg/mL of Resazurin (cat. R7017-5G) purchased from Sigma (St. Louis, MO). After 2 h of incubation at 37 °C/5% CO_2, resofurin fluorescence was measured at ex 544/em 595 nm on a spectraMax M5 microplate reader from Molecular Devices (Sunnyvale, CA). Relative cell viability was expressed as a percentage, taking nontransfected cells as a reference.

Automated fluorescence microscopy

Fluorescence images of transfected EGFP-expressing Raw 264.7 cells in 384-well plates were acquired using an OPERA automated fluorescence microscope from EVOTEC AG (Hamburg, Germany) equipped with a 20×/0.7 NA water immersion lens. Of the 4 available light sources, 488- and

635-nm confocal excitation lines were used to excite EGFP and DY647, respectively. Emission was detected simultaneously using 2 CCD cameras in spectral bands at 535 and 690 nm. Five fields within each well were collected.

Image analysis

An appropriate threshold value set by the user led to an implicit cell/background separation and produced images that could be used for binary morphologic operations. Under a given value, all pixels were set to 0, whereas all pixels with a higher intensity were set to 1. In the same way, a second threshold on the red channel allowed for the identification of DY647-labeled siRNAs. EGFP fluorescence of the cells was measured by considering the global intensity of the green signal above threshold. The global fluorescence within the well was therefore considered rather than the fluorescence of the transfected cell subpopulation only. The percentage of EGFP expression was calculated taking EGFP fluorescence in nontransfected wells as a reference.

A morphological reconstruction was then performed using the masked red channel to reconstruct the threshold green channel. This operation selected in the reconstructed channel all objects whose intersection with the mask was not empty and enabled the selection of transfected cells only. The percentage of transfected cells was determined as the ratio of transfected cells to total cells [(red signal-containing cells/(cells without red signal + red signal-containing cells))*100].

Finally, the average fluorescence of objects in the red channel was used to quantify the amount of siRNA inside the transfected cells.

RESULTS

Transfectant screening

To optimize the transfection of Raw 264.7 macrophages in 384-well format, we first compared a series of transfectants. The optimal lipofectant should trigger the highest level of siRNA entry into the maximum number of cells and should produce the highest silencing effect. In addition, the selected product should limit the toxicity toward the cells. Ten transfectants were chosen among the products available on the market. EGFP-expressing Raw 264.7 cells were transfected with fluorescently labeled siRNAs using 3 different amounts of each lipofectant. On day 4 posttransfection, the percentage of transfected cells (cells containing siRNA compared to the total number of cells), average EGFP fluorescence intensity, and average DY647 fluorescence were determined from confocal pictures using dedicated image analysis (see **Fig. 1**).[18] Cell viability was assessed with resazurin and expressed as the percentage of viability compared to untransfected cells. As a negative control, cells that had been transfected with nontargeting siRNA (scramble) or with lipofectant only (mock transfection) did not show any reduction of EGFP expression (data not shown).

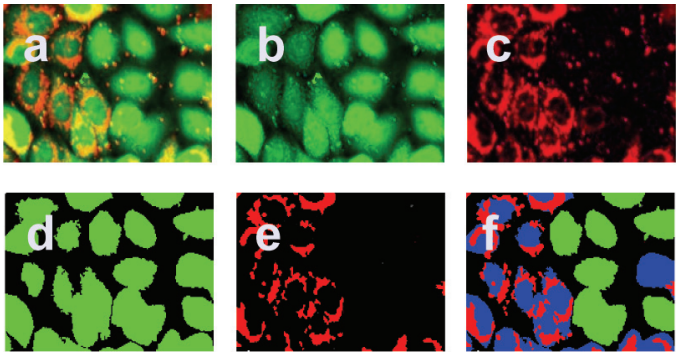


FIG. 1. Image analysis flowchart. (**a**) Original image. (**b, c**) Green enhanced green fluorescent protein (EGFP) and red DY647-labeled siRNAs. (**d, e**) Threshold green and red channels leading to object/background separation. (**f**) Segmented image presenting in red DY647-labeled siRNAs, in green nontransfected cells, and in blue transfected cells.

As shown in **Table 1**, only Lipofectamine LTX, Lipofectamine RNAiMax, and HiPerfect were able to trigger significant siRNA uptake in Raw 264.7 cells and significant EGFP silencing without toxicity. Over 20% reduction of EGFP expression was observed, and HiPerfect even triggered up to 41% of reporter protein knock-down. Moreover, these 3 products displayed the highest transfection efficiency with at least 70% of the cells containing siRNA and up to 90% for Lipofectamine LTX. Intracellular siRNA fluorescence intensity for these 3 lipofectants was also higher compared to the other products. In particular, a significant increased siRNA signal was obtained using HiPerfect. Lipofectamine LTX, Lipofectamine RNAiMax, and HiPerfect were chosen for the next selection round.

To determine the most efficient transfectant among the 3 selected ones, we transfected EGFP-expressing cells using Lipofectamine LTX, Lipofectamine RNAiMax, and HiPerfect at 5 different concentrations. As shown in **Table 2**, HiPerfect was the best transfectant, resulting in the highest intracellular siRNA signal and an EGFP knock-down of 60% when used at 1.5 µL/well. However, at this concentration, HiPerfect also displayed over 30% cytotoxicity to the cells. Despite a much lower intracellular siRNA signal, Lipofectamine LTX was able to trigger comparable EGFP silencing (55%) associated with reduced cell toxicity (16%). In addition, the required amount of Lipofectamine LTX was over 10-fold lower in comparison to the amount of HiPerfect needed to achieve comparable EGFP silencing, resulting in a 9-fold cost reduction for each screening. Therefore, Lipofectamine LTX was chosen for all subsequent experiments as it represented the best compromise between high transfection efficacy, low toxicity, and reduced cost.

Table 1. Lipid Reagents Primary Screening

	Reagent Volume, µL/Well	*% of Transfected Cells*	*% of Silencing*	*siRNA (DY647) Fluorescence*	*% of Viability*
Dharmafect 1	0.1	57 ± 3	—	217 ± 6	114 ± 1
	0.075	61 ± 2	—	223 ± 8	107 ± 1
	0.05	67 ± 3	6 ± 2	264 ± 21	100 ± 2
Dharmafect 2	0.1	67 ± 4	—	264 ± 27	111 ± 2
	0.075	85 ± 4	—	277 ± 10	102 ± 1
	0.05	71 ± 9	1 ± 7	261 ± 19	97 ± 2
Dharmafect 3	0.1	81 ± 4	2 ± 7	284 ± 13	109 ± 0
	0.075	78 ± 2	8 ± 3	270 ± 12	102 ± 4
	0.05	69 ± 3	—	262 ± 12	97 ± 5
Dharmafect 4	0.075	71 ± 4	—	266 ± 15	108 ± 3
	0.05	86 ± 4	—	275 ± 3	102 ± 1
	0.025	76 ± 12	—	270 ± 15	97 ± 53
Lipofectamine RNAiMax	0.075	80 ± 9	32 ± 12	264 ± 5	106 ± 2
	0.05	91 ± 4	33 ± 5	276 ± 5	100 ± 2
	0.025	86 ± 4	10 ± 8	267 ± 8	95 ± 1
Lipofectamine LTX	0.075	94 ± 2	26 ± 4	285 ± 1	108 ± 1
	0.05	93 ± 8	23 ± 1	297 ± 1	102 ± 2
	0.025	92 ± 7	13 ± 7	285 ± 9	98 ± 2
Lipofectamine 2000	0.075	78 ± 4	—	285 ± 5	111 ± 5
	0.05	75 ± 7	—	269 ± 13	105 ± 3
	0.025	75 ± 3	3 ± 9	267 ± 13	98 ± 1
InterferIN	0.25	24 ± 10	5 ± 7	173 ± 21	94 ± 5
	0.1	37 ± 12	5 ± 6	208 ± 14	88 ± 6
	0.075	57 ± 6	—	251 ± 9	92 ± 3
HiPerfect	1	68 ± 13	39 ± 7	314 ± 23	97 ± 1
	0.75	78 ± 21	41 ± 6	332 ± 18	94 ± 1
	0.5	67 ± 12	30 ± 5	281 ± 12	96 ± 0
Effectene	1	35 ± 12	—	226 ± 4	106 ± 1
	0.75	67 ± 23	—	256 ± 34	99 ± 2
	0.5	82 ± 9	1 ± 13	265 ± 16	96 ± 1

Raw 264.7 cells expressing enhanced green fluorescent protein (EGFP) were transfected with 75 nM of DY647-labeled siRNA targeting EGFP using different lipofectants at the indicated concentrations. On day 4 posttransfection, pictures were taken at 488 and 635 nm on an OPERA automated confocal microscope. Cells and siRNA fluorescence were analyzed using in-house developed IM software. Cells were stained with 100 µg/mL of resazurin, and after 8 h at 37 °C, resofurin fluorescence was measured at em 544/ex 595 nm. Percentage of silencing and viability were calculated using nontransfected cells as a reference. For each condition, average and standard deviation (± SD) values were determined for 3 replicate wells.

Table 2. Lipid Reagents Secondary Screening

	Lipofectamine LTX					*Lipofectamine RNAiMax*						*HiPerfect*			
µL/Well	*0.15*	*0.125*	*0.1*	*0.075*	*0.05*	*0.15*	*0.125*	*0.1*	*0.075*	*0.05*	*2*	*1.5*	*1*	*0.75*	*0.5*
% of silencing	70 ± 5	52 ± 18	55 ± 0	24 ± 0	35 ± 8	59 ± 3	31 ± 0	33 ± 6	—	10 ± 18	58 ± 12	60 ± 5	52 ± 17	50 ± 24	51 ± 6
% of viability	73 ± 5	86 ± 30	84 ± 1	100 ± 22	91 ± 1	77 ± 1	88 ± 34	88 ± 3	99 ± 26	69 ± 27	48 ± 33	69 ± 7	92 ± 17	70 ± 7	75 ± 3
siRNA	206 ± 28	197 ± 13	217 ± 2	210 ± 19	210 ± 27	223 ± 1	241 ± 12	241 ± 2	245 ± 11	225 ± 1	624 ± 37	840 ± 51	426 ± 23	203 ± 21	147 ± 13

Raw 264.7 cells expressing enhanced green fluorescent protein (EGFP) were transfected with 50 nM of DY647-labeled siRNA targeting EGFP using Lipofectamine LTX, Lipofectamine RNAiMax, and HiPerfect at the indicated concentrations. On day 4 after transfection, pictures were taken at 488 and 635 nm on an OPERA automated confocal microscope. Cells and siRNA fluorescence were analyzed using in-house developed IM software. On day 5 posttransfection, cells were stained with 100 µg/mL of resazurin, and after 8 h at 37 °C, resofurin fluorescence was measured at em 544/ex 595 nm. Percentages of silencing and cell viability were calculated using nontransfected cells as a reference. For each condition, average and standard deviation (± SD) values were determined for 2 replicate wells.

Transfectant buffer selection

Using Lipofectamine LTX, silencing typically ranged from 40% to 60%. To increase the knock-down efficacy, we then investigated the effect of the buffer used for the preparation of siRNA/lipofectant complexes. EGFP-expressing Raw 264.7 cells were transfected with DY647-labeled siRNA using 0.1 µL of Lipofectamine LTX diluted in different media or buffers. Percent EGFP silencing, viability, and intracellular siRNA signal obtained with the different media/buffers are summarized in **Figure 2**. Dulbecco's phosphate-buffered saline (DPBS) was the only buffer that showed increased EGFP knock-down (73% vs. 59%) compared to OPTIMEM, which was used in the first

	OPTIMEM	RPMI	MEM	DMEM	DMEM Glutamax	Neurobasal	DPBS
% of silencing	**59** (±3)	46 (±17)	32 (±6)	11 (±17)	6 (±5)	32 (±18)	**73** (±6)
% of viability	**86** (±7)	76 (±2)	83 (±9)	94 (±0)	115 (±18)	121 (±30)	**86** (±1)
siRNA fluorescence	**81** (±1)	52 (±2)	61 (±8)	86 (±6)	67 (±1)	35 (±2)	**261** (±11)

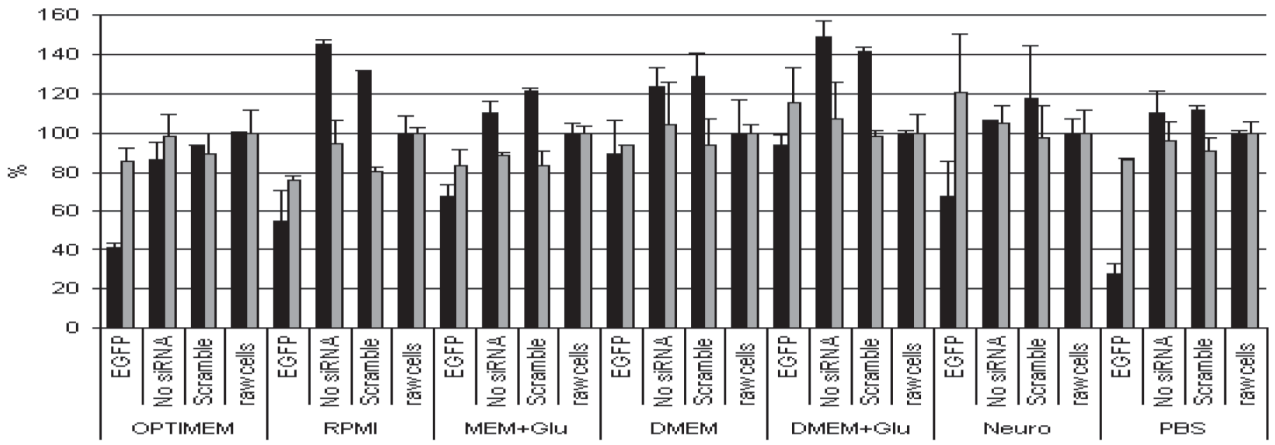


FIG. 2. Screening of transfection mix buffer. Raw 264.7 cells expressing enhanced green fluorescent protein (EGFP) were transfected with 75 nM of DY647-labeled siRNA targeting EGFP using Lipofectamine LTX at 0.1 μL/well. On day 4 posttransfection, pictures were taken at 488 and 635 nm on an OPERA automated confocal microscope. Cells and siRNA fluorescence were analyzed using in-house developed IM software. On day 5 posttransfection, cells were stained with 100 μg/mL of resazurin, and after 8 h at 37 °C, resofurin fluorescence was measured at em 544/ex 595 nm. Percentage of silencing and viability were calculated using nontransfected cells as a reference. For each condition, average and standard deviation (± SD) values were determined for 2 replicate wells. MEM, minimal essential medium; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; DPBS, Dulbecco's phosphate-buffered saline.

set of experiments. This difference was likely due to a much more efficient siRNA uptake triggered by DPBS as an over 3-fold increase in DY647 fluorescence was observed.

siRNA optimal concentration and silencing kinetics

To determine the optimal siRNA concentration, we transfected EGFP-expressing cells with different amounts of DY647-labeled siRNA (**Fig. 3**). On day 4 posttransfection, no significant difference in EGFP silencing was observed between samples (**Fig. 3B**). Although knock-down efficiency reached ~75% at all siRNA concentrations, intracellular DY647-labeled siRNA signals varied significantly. Entry of siRNA increased proportionally to the concentration used until 75 nM, when the intracellular siRNA signal reached a plateau (**Fig. 3C**, small box). Differential ribonucleic acid uptake influenced the kinetics of silencing as siRNA clearance from cells correlated with the end of gene expression repression. As shown in **Figure 3B**, maximal silencing reached at day 4 was comparable for all concentrations. However, when lower siRNA concentrations were applied (25 and 50 nM), EGFP expression began again from day 5 while knock-down was still maintained with higher siRNA concentrations (75 and 100 nM).

Optimal reagent volumes

The effect of reagent volumes on the silencing efficacy was then investigated with the aim of adapting the method to HTS genome-wide siRNA screening. Optimal buffer volume for the assembly of siRNA/lipofectant complexes was first studied and did not have any noticeable effect on siRNA transfection efficiency (data not shown).

To determine the optimal volume to resuspend the genome-wide siRNA library, we studied the influence of siRNA stock concentration. Indeed, resuspension at low concentration could facilitate siRNA transfer through the handling of larger volumes. On the other hand, a larger volume of siRNA (resuspended in siRNA resuspension buffer) could dilute the buffer used for the assembly of siRNA/Lipofectant, a parameter that was shown to be essential (**Fig. 2**). To determine the optimal volume for siRNA, we transfected cells with 0.2, 1, 2, and 4 μL of siRNA solution at 20, 4, 2, and 1 μM, respectively. After cell addition (resuspended in 20 μL), the siRNA final concentration was 100 nM in all wells. As shown in **Figure 4A**, no significant difference was observed in EGFP knock-down efficiency for any of the volumes tested.

Finally, in terms of automation and with the objective of determining the best compromise between handling comfort

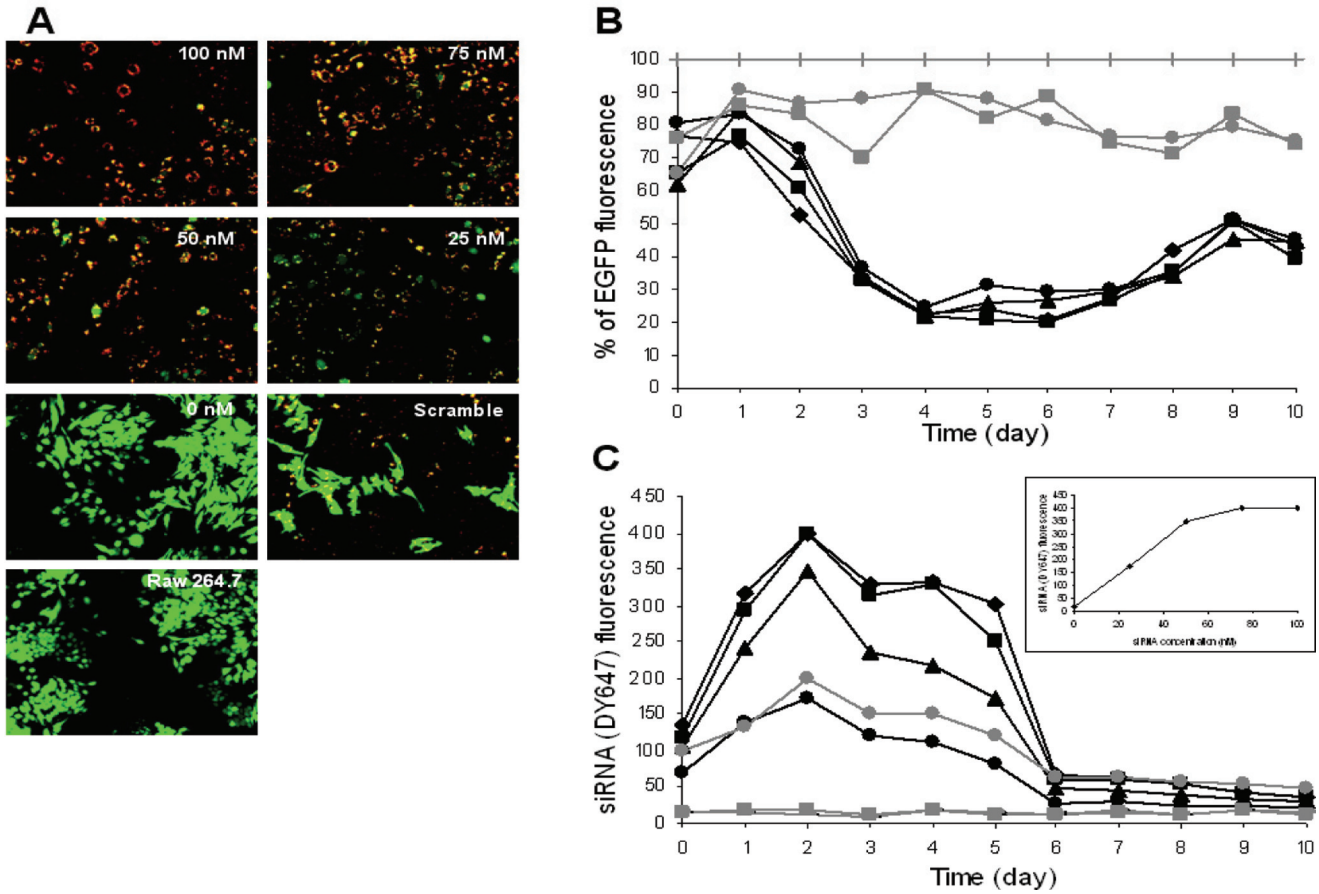


FIG. 3. siRNA optimal concentration. Raw 264.7 cells expressing enhanced green fluorescent protein (EGFP) were transfected with 100 (♦), 75(■), 50 (▲), and 25 (●) nM of DY647-labeled siRNA targeting EGFP using Lipofectamine LTX at 0.1 µL/well. As controls, cells were also transfected with 100 nM of DY647-labeled nontargeting (Scramble) siRNA (●) or without siRNA (■). Over a 10-day period, pictures (**A**) were taken at 488 and 635 nm on an OPERA automated confocal microscope. Both (**B**) cells and (**C**) siRNA fluorescence were analyzed using in-house developed IM software. Percentage of silencing and viability were calculated using nontransfected cells (+) as a reference.

and silencing efficacy, the optimal total reaction volume was determined. It was hypothesized that minimizing reaction volume would increase transfection efficiency. As evidenced with **Figure 4B**, minimizing the total reaction volume correlated with a more sustained silencing of EGFP expression. At day 3 after transfection, optimal knock-down was similar between samples. However, from day 4, clear differences could be observed in EGFP expression. With total reaction volumes of 40, 50, and 60 µL, silencing remained below 50% during 4 days, whereas at 70 and 80 µL, 60% of the EGFP expression was already restored after 48 h. Surprisingly, even if RPMI medium was diluted up to 2-fold (40 µL of total reaction volume), no increased cell toxicity was observed compared with control wells where Raw 264.7 cells were incubated in pure RPMI (data not shown).

Transfection reagents stability

In terms of HTS procedure, transfer of siRNAs from source to assay plates might require several hours. Compared to compound handling, this issue can represent a major drawback for unstable and labile molecules such as ribonucleic acids. To evaluate the stability of each reagent involved in transfection, we transferred siRNA, Lipofectamine LTX (resuspended in DPBS) and siRNA/complexes to the assay plate and incubated them for several hours before addition of the next reagent or cells. On day 4 after transfection, EGFP expression of Raw 264.7 cells was evaluated (**Fig. 5**). Strikingly, siRNA, the component that was expected to be the most labile, appeared to be the most stable. Indeed, when siRNAs were transferred up to 5 h before addition of Lipofectamine LTX, no reduction of EGFP

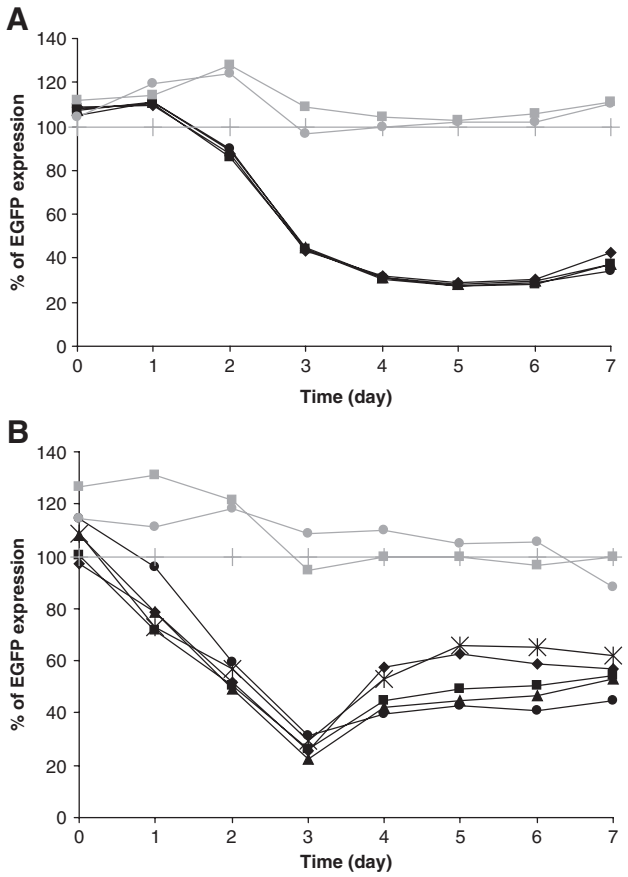


FIG. 4. Influence of siRNA stock concentration and total reaction volume on siRNA transfection in Raw 264.7 macrophages. (**A**) Enhanced green fluorescent protein (EGFP)–expressing Raw 264.7 cells were transfected with 100 nM of siRNA using stocks at 20 (♦), 4 (■), 2 (▲), and 1(●) μM of DY647-labeled siRNA targeting EGFP and using Lipofectamine LTX at 0.1 μL/well. (**B**) Raw 264.7 cells expressing EGFP were transfected with 100 nM in a total reaction volume of 80 μL (*), 70 μL (♦), 60 μL (■), 50 μL (▲), and 40 μL (●). As controls, cells were also transfected with 100 nM of DY647-labeled nontargeting (Scramble) siRNA (●) or without siRNA (■). Over a 7-day period posttransfection, pictures were taken at 488 and 635 nm on an OPERA automated confocal microscope. Cells and siRNA fluorescence were analyzed using in-house developed IM software. Percentage of EGFP expression was calculated using nontransfected cells (Raw cells) as a reference.

silencing was observed. In contrast, Lipofectamine LTX diluted in DPBS proved to be dramatically unstable as increased incubation time correlated with a clear decrease in EGFP knockdown. Finally, this present experiment confirmed that siRNA/lipofectant complexes are only stable for about an hour before losing their transfection activity. In parallel to the above experiment, a similar assay plate was performed but was kept at 4 °C instead of room temperature for the test described above. Refrigeration did not result in enhancement of the stability of any ingredient (data not shown).

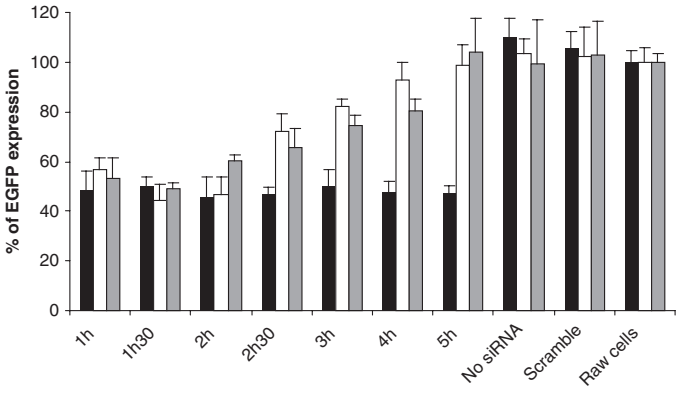


FIG. 5. Stability of siRNA transfection ingredients. siRNA (■), Lipofectamine LTX (□), or the siRNA/lipofectant complexes (■) were incubated at room temperature for 1 to 5 h before the addition of the next transfection component. Assembly of siRNA/Lipofectamine LTX complexes were authorized to perform for 30 min at room temperature before transfer of EGFP-expressing Raw 264.7 cells. On day 4 posttransfection, pictures were taken at 488 and 635 nm on an OPERA automated confocal microscope. Cells fluorescence was determined using in-house developed IM software. Average percentage of enhanced green fluorescent protein (EGFP) fluorescence and viability were calculated for 4 replicate wells using nontransfected cells (+) as a reference.

To verify the robustness of the developed protocol, we compared intraday and interday silencing efficacies. As shown in **Figure 6**, EGFP expression repression was reproducible from one well to the other with a mean standard deviation of 2%. Mean coefficient of variation (CV) ranged from 4.5% to 12.6% (mean 7.7%), a very good score for cell-based assays. Interday performance was also satisfactory as mean silencing efficacy was of 74% with a standard deviation of 5%.

Finally, the issue of macrophage activation using the above described transfection conditions was addressed. Cells were transfected with nontargeting siRNA using the optimized protocol, and samples were taken at 10, 24, 48, and 72 h after transfection. Enzyme-linked immunosorbent assay (ELISA) and fluorescence-activated cell sorting (FACS) analysis did not show any evidence of interferon-gamma production or changes in surface markers (CD14, CD11b, F4/80, I-A/I-E) expression in any of the samples compared to untransfected cells (data not shown).

DISCUSSION

Transfection of the "hard-to-transfect" Raw 264.7 macrophage cell line was optimized for automated HTS. The use of EGFP as a reporter gene enabled direct monitoring of silencing at the protein level by confocal microscopy. Evaluation of knockdown efficacy through exclusive follow-up of mRNA levels using techniques such as real-time PCR or Northern blotting might not reveal the actual repression of the corresponding

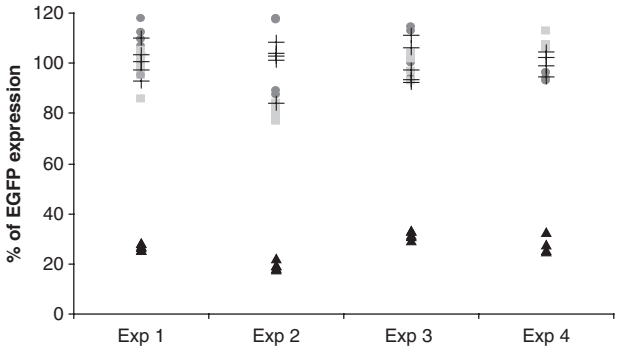


FIG. 6. Intraday and interday performance. Enhanced green fluorescent protein (EGFP)–expressing Raw 264.7 cells were transfected with 100 nM of DY647-labeled siRNA targeting EGFP using Lipofectamine LTX at 0.1 μL/well (▲). As controls, cells were also transfected with 100 nM of DY647-labeled nontargeting (Scramble) siRNA (■) or without siRNA (●). On day 4 posttransfection, pictures were taken at 488 and 635 nm on an OPERA automated confocal microscope. Cells and siRNA fluorescences were analyzed using in-house developed IM software. Percentage of EGFP expression was calculated using nontransfected cells (+) as a reference.

protein. Several recent reports have indeed indicated that protein expression levels only weakly correlate with corresponding mRNA levels,[19-21] and consequently, mRNA levels are an inappropriate surrogate for protein expression evaluation.[22] In addition, the model used for the optimization procedure was particularly challenging as transduced Raw 264.7 cells expressed relatively high levels of EGFP, a protein known for its long half-life.[23,24] Therefore, optimization of silencing of this reporter protein should probably guarantee an efficient knock-down of endogenous proteins in follow-up phenotypic assays.

DY647-labeled siRNA enabled the visualization of the internalization of siRNA into the cells and the discrimination of transfected from nontransfected cells. Toxicity to the cells could be determined through cell number monitoring and through resazurin assay. The latter was the method of choice as it measures the actual metabolic activity of the cells compared to cell number, which does not provide any information on cell viability.

In a first step, 10 commercially available transfectants were screened based on their ability to transfect the highest number of cells, produce the maximum amount of siRNA entry into the cells, induce the greatest knock-down of EGFP expression, and minimize cell toxicity (**Table 1**). Of 3 compounds able to fulfill these criteria, Lipofectamine LTX was selected as the best candidate for its capacity to induce 40% to 60% silencing with less than 20% cell mortality (**Table 2**). The optimal amount of Lipofectamine LTX amount was subsequently determined to be 0.1 μL/well; this was the maximum amount in which this reagent was triggering gene expression knock-down without major cytotoxicity. Although high levels of intracellular siRNA were obtained using Lipofectamine 2000 and Dharmafect 2, 3, and 4, no significant EGFP silencing was observed. This confirmed that an efficient transfectant should not only shuttle the siRNA into the cells but also ensure its delivery into the right subcellular compartment.[25] Such a consideration is of particular importance when dealing with macrophages that display an enhanced phagocytic activity. Indeed, siRNA/lipid complexes that uptake through the phagocytosis pathway rather than through endocytosis might dramatically change the siRNA intracellular fate.[26]

To further increase transfection efficacy, we tested different media or buffers for their effect on the assembly of siRNA/Lipofectamine LTX complexes (**Fig. 2**). Of the 8 solutions compared to the gold-standard OPTIMEM, DPBS was the only buffer that improved transfection efficacy. DPBS enhanced by 3-fold the siRNA uptake, which resulted in an increased EGFP silencing above 70%. This experiment confirmed that the physicochemical properties of buffer can greatly influence the formation of active siRNA/transfectant complexes. Similarly, several reports have demonstrated that helper molecules such as serum[27] or DOPE[28] can increase transfection activity of lipofectants. Such helper molecules can directly act on the siRNA/lipid complex formation, on its size, or in the intracellular delivery of the nucleic acid.[29,30] We introduce here a novel critical parameter that should be tested for transfection optimization in any cell line.

Direct visualization of reporter protein and siRNA allowed for the simultaneous monitoring of silencing and siRNA entry (**Fig. 3A**). These 2 parameters were of major importance in assessing siRNA optimal concentration and phenotypic assay schedule. At the different siRNA concentrations tested, EGFP knock-down was similar. However, silencing kinetics and intracellular siRNA follow-up revealed that only high siRNA concentrations guaranteed sustained gene expression inhibition (**Fig. 3B,C**). Therefore, the siRNA concentration should be adjusted based on the planned phenotypic assay. For a "short-term" assay involving a readout within the same day, a low siRNA concentration of 25 or 50 nM can be employed to reduce the risk of an off-target effect.[31] On the contrary, for "long-term" phenotypic assays, such as intracellular bacterial growth, the siRNA concentration should be raised to 75 or 100 nM to ensure gene silencing throughout the entire experiment.

To apply this transfection method to automated HTS, we optimized several parameters to match biological activity with robotized pippetting handling requirements. Handling of large volumes generally results in reliable and accurate transfers from robotic systems. However, maximizing the different reagent volumes might also reduce the biological activity. The volume used for siRNA/lipofectant complex formation was found to have no effect on transfection efficiency (data not shown) and was therefore set to 20 μL to facilitate component transfer. Similarly, several volume ratios of DPBS and siRNA resuspension buffer were tested to verify that DPBS buffer dilution would not impair

transfection efficiency. Raw 264.7 cell transfection with different siRNA volumes showed that DPBS could be diluted up to 1.25-fold without any loss of function (**Fig. 4A**). Finally, the optimal cell resuspension volume was determined. When total volume was above 60 µL, EGFP knock-down was reduced (**Fig. 4B**). Therefore, the cell resuspension volume was set to 30 µL as no increased cytotoxicity was observed under this condition.

For the objective of developing a method suitable for automated HTS, the stability of the different products involved in siRNA transfection was tested because siRNA transfer from source to assay plates (~30 plates/day) might require several hours. Surprisingly, siRNA was found to be the most stable component, with no reduction in silencing efficacy for up to 5 h, whereas Lipofectamine LTX (resuspended in DPBS) and siRNA/ Lipofectamine LTX complexes were only stable for a couple of hours (**Fig. 5**). This biological result was crucial for HTS assay design, and consequently, the transfection protocol had to be adapted to match siRNA stability and Lipofectamine LTX lability.

In summary, to improve siRNA transfection, we tested several parameters close to both biological activity and the HTS process. New factors such as the buffer used for siRNA/lipofectant assembly or the stability of the different reagents were analyzed. This new, optimized procedure was successfully applied for the optimization of lipid-mediated siRNA transfection of the macrophage-like Raw 264.7 cell line. This study led to the establishment of a robust method (**Fig. 6**) for the transfection of these "hard-to-transfect" cells in an HTS format.

ACKNOWLEDGMENTS

Reagents were kindly provided by Pierre Charneau (Institut Pasteur Paris). We thank Lisa Cechetto, and Fanny Ewann for critical reading of the manuscript.

REFERENCES

1. Gil J, Esteban M: Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. *Apoptosis* 2000;5:107-114.
2. Cullen LM, Arndt GM: Genome-wide screening for gene function using RNAi in mammalian cells. *Immunol Cell Biol* 2005;83:217-223.
3. Echeverri CJ, Perrimon N: High-throughput RNAi screening in cultured cells: a user's guide. *Nat Rev Genet* 2006;7:373-384.
4. Zhang S, Zhao B, Jiang H, Wang B, Ma B: Cationic lipids and polymers mediated vectors for delivery of siRNA. *J Control Release* 2007;123:1-10.
5. Borawski J, Lindeman A, Buxton F, Labow M, Gaither LA: Optimization procedure for small interfering RNA transfection in a 384-well format. *J Biomol Screen* 2007;12:546-559.
6. Rozema DB, Lewis DL: siRNA delivery technologies for mammalian systems. *Targets* 2003;2:253-260.
7. Arts G-J, Langemeijer E, Tissingh R, Ma L, Pavliska H, Dokic K, et al: Adenoviral vectors expressing siRNAs for discovery and validation of gene function. *Genome Res* 2003;13:2325-2332.
8. Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, et al: A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* 2003;33:401-406.
9. Sachse C, Echeverri CJ: Oncology studies using siRNA libraries: the dawn of RNAi-based genomics. *Oncogene* 2004;23:8384-8391.
10. Persengiev SP, Zhu X, Green MR: Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *RNA* 2004;10:12-18.
11. Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BRG: Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 2003;5:834-839.
12. Sasaguri Y, Tanimoto A: Role of macrophage-derived histamine in atherosclerosis-chronic participation in the inflammatory response. *J Atheroscler Thromb* 2004;11:122-130.
13. Christian B: Mechanisms and consequences of persistence of intracellular pathogens: leishmaniasis as an example. *Cell Microbiol* 2008;10: 1221-1234.
14. Agaisse H, Burrack LS, Philips JA, Rubin EJ, Perrimon N, Higgins DE: Genome-wide RNAi screen for host factors required for intracellular bacterial infection. *Science* 2005;309:1248-1251.
15. Philips JA, Rubin EJ, Perrimon N: Drosophila RNAi screen reveals CD36 family member required for mycobacterial infection. *Science* 2005;309: 1251-1253.
16. Boese A, Sommer P, Gaussin A, Reimann A, Nehrbass U: Ini1/hSNF5 is dispensable for retrovirus-induced cytoplasmic accumulation of PML and does not interfere with integration. *FEBS Lett* 2004;578:291-296.
17. Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, et al: In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996;272:263-267.
18. Moon H, Genovesio A: IM, a computing approach for image mining of high throughput-high content screening. Presented at the 9th IEEE/ACM International Conference on Grid Computing 2008; September 29 to October 1, 2008; Tsukuba, Japan.
19. Tian Q, Stepaniants SB, Mao M, Weng L, Feetham MC, Doyle MJ, et al: Integrated genomic and proteomic analyses of gene expression in mammalian cells. *Mol Cell Proteomics* 2004;3:960-969.
20. Nie L, Wu G, Zhang W: Correlation between mRNA and protein abundance in *Desulfovibrio vulgaris:* a multiple regression to identify sources of variations. *Biochem Biophys Res Commun* 2006;339:603-610.
21. Chen G, Gharib TG, Huang CC, Taylor JM, Misek DE, Kardia SL, et al: Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics* 2002;1:304-313.
22. Pascal L, True L, Campbell D, Deutsch E, Risk M, Coleman I, et al: Correlation of mRNA and protein levels: cell type-specific gene expression of cluster designation antigens in the prostate. *BMC Genomics* 2008;9:246.
23. Li X, Zhao X, Fang Y, Jiang X, Duong T, Fan C, et al: Generation of destabilized green fluorescent protein as a transcription reporter. *J Biol Chem* 1998;273:34970-34975.
24. Choi I, Cho B-R, Kim D, Miyagawa S, Kubo T, Kim JY, et al: Choice of the adequate detection time for the accurate evaluation of the efficiency of siRNA-induced gene silencing. *J Biotechnol* 2005;120:251-261.
25. Zuhorn I, Engberts J, Hoekstra D: Gene delivery by cationic lipid vectors: overcoming cellular barriers. *Eur Biophys J* 2007;36:349-362.
26. Khalil IA, Kogure K, Akita H, Harashima H: Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. *Pharmacol Rev* 2006;58:32-45.

27. Zuhorn IS, Visser WH, Bakowsky U, Engberts JBFN, Hoekstra D: Interference of serum with lipoplex-cell interaction: modulation of intracellular processing. *Biochimica et Biophysica Acta (BBA)—Biomembranes* 2002;1560:25-36.
28. Ma BC, Zhang SB, Jiang HM, Zhao BD, Lv HT: Lipoplex morphologies and their influences on transfection efficiency in gene delivery. *J Control Release* 2007;123:184-194.
29. Wasungu L, Hoekstra D: Cationic lipids, lipoplexes and intracellular delivery of genes. *J Control Release* 2006;116:255-264.
30. Elouahabi A, Ruysschaert J-M: Formation and intracellular trafficking of lipoplexes and polyplexes. *Mol Ther* 2005;11:336-347.
31. Svoboda P: Off-targeting and other non-specific effects of RNAi experiments in mammalian cells. *Curr Opin Mol Ther* 2007;9:248-257.

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