siRNA transfection optimization with the Agilent 2100 bioanalyzer

A new method for effective gene silencing

Application Note

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Abstract

When working in the field of target validation, or when studying functional assays requiring the transfection of small interfering RNA (siRNA) into a cell, the presence of having a fast, easy-to-use method to optimize transfection conditions for given cell lines would be of great value. Three to four siRNA sequences are generally tested for each gene in order to suppress the expression of a protein with a corresponding mRNA sequence. Before any sequence comparison can be performed, the integrity and purity of siRNA, the siRNA uptake and the cell viability must be monitored and optimized. Once optimal transfection conditions have been established, different siRNA sequences can be evaluated for the lower target protein expression. There are many influencing factors for transfection efficiency, among them the cell line, siRNA concentration and its ratio to the transfection reagent, cell confluence during transfection, incubation time and media composition all need to be taken into consideration. In this Application Note we describe the use of Agilent's 2100 bioanalyzer to quickly verify the siRNA integrity and to determine the optimal transfection conditions for gene silencing experiments with the help of red-fluorescently labeled siRNA and cell fluorescence assays. Cell viability and delivery efficacy are simultaneously evaluated with fluorescent probes, therefore helping to decide on the optimal transfection conditions.

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Introduction

Over recent years the scientific community has seen an explosion in the number of research studies related to RNA interference (RNAi) induced gene silencing. Benefits, such as genome usability to generate knockout phenotypes, easy automation with the potential for high throughput, specificity, and low cost when compared to animal knockout models are seen as a huge opportunity for functional analysis, target validation and gene-specific therapeutics.

RNAi is a well-conserved cellular mechanism believed to have anti-retroviral effects. dsRNA fragments with homologous sequence to a translated mRNA fragment will effectively silence the expression of the protein by catalyzing the degradation of the complementary mRNA[1-4]. In RNAi Dicer, an RNase like enzyme, is responsible for processing dsRNA into double stranded small interfering RNA (siRNA). *In vitro* studies in Drosophila suggested that siRNAs assemble into endoribonuclease enzyme complexes known as RNA-induced silencing complexes (RISCs). After the siRNA strands are unwound, activated RISCs are guided to complementary mRNA molecules, where they cleave the associated RNA (for recent reviews, Refs. 5-6).

While in some organisms, such as C. elegans or Arabidopsis, RNAi requires an RNA amplification step[7], the direct introduction of siRNA in mammalian cells has shown to produce transient silencing of more than 90 % in protein expression. Recovery from a single treatment occurs after 4 to 6 days[8], therefore suggesting that no copying takes place.

There are currently several methods for siRNA delivery into cells. siRNA can be produced *in vitro* by chemical synthesis, *in vitro* transcription or RNase III-type digestion of dsRNA or siRNA can be directly expressed *in vivo* by use of plasmids, viral vectors or a PCR cassette. All of these methods require the optimization of the transfection, for cell viability, siRNA uptake/production and silencing effectiveness. It is known that optimal transfection conditions strongly vary between cell lines. However, transfection is not affected by the specific sequence of the nucleic acid or the presence of a fluorescence tag, which allows the use of the optimized conditions for the screening of several sequences. Also, *in vitro* preparation requires additional purification steps where the verification of purity and integrity of nucleic acids is required. Given the expense and complexity of monitoring and optimizing these types of experiments, a new tool that allows for minimal sample and reagent consumption in a fast and automated format would be of great benefit.

The Agilent 2100 bioanalyzer combines electrophoretic analysis of nucleic acids with dual-color flow cytometry capabilities. The DNA 1000 LabChip kit can be used for verifying purity and integrity of dsRNA from siRNAs (19-23 bp) up to 1000 bp dsRNAs while the Cell Fluorescence LabChip® kit is the ideal tool for monitoring transfection efficiency of fluorescently tagged siRNAs or green fluorescent protein (GFP) producing vectors. Compared to fluorescence microscopy, the Agilent 2100 bioanalyzer automates the measurements, increasing the total cell count to approximately a thousand cells per sample and provides quantitative information on the transfection degree and viability of each cell. In addition, the silencing effect of RNAi can be quickly monitored with the Agilent 2100 bioanalyzer by antibody staining of cells or analysis of endpoint RT-PCR results. A typical workflow for a siRNA transfection optimization experiment is outlined in figure 1, showing analysis time reduced to 60 minutes. We show data on electrophoretic quality assessment of siRNA and transfection efficiency measurements of red fluorescence tagged siRNA. Varying amounts of siRNA, different transfection reagents and its ratios were evaluated.

Materials and Methods

Short interfering RNA preparation

Cy5® labeled and unlabeled short interfering RNA were synthesized by QIAGEN. For the annealing of siRNA duplexes, 5 nmol single stranded sequences targeting Lamin A/C (QIAGEN, Cat. #: 1022050), were incubated in 250 µl siRNA Buffer (QIAGEN) for one min at 91 °C followed by 1 h at 37 °C. GFP siRNA (QIAGEN, GFP-22 siRNA, sense 3'Cy5 modified sense and antisense 5'-P(Phosphate)) was supplied annealed and ready to use in sterile buffer. Samples were then stored at –20 °C until needed, and then incubated at 37 °C for 20 min.

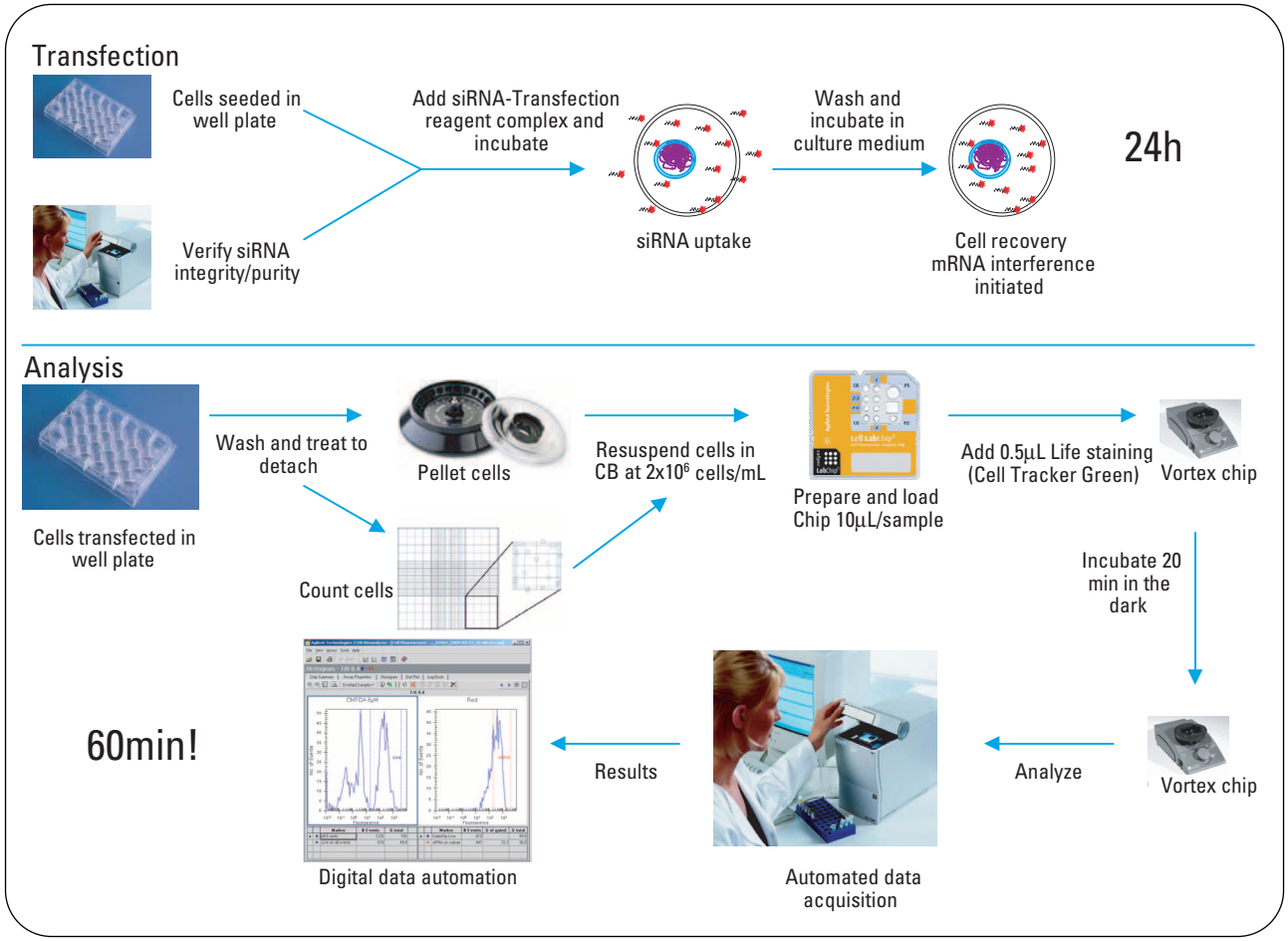

Figure 1
Transfection Optimization Workflow. The workflow consists of two steps, first the transfection of cells using different conditions (upper panel) followed by the analysis of the effect of this changes (lower panel). A streamlined protocol allows analysis of this effect in 60 minutes.

Electrophoretic measurement of purity and integrity

dsRNA electrophoresis analysis was performed using the DNA 1000 LabChip kit with the Agilent 2100 bioanalyzer. The kit includes dsDNA internal standards at 15 and 1500 bp for sample alignment (sizing) and quantitation. Lamin A/C siRNA was analyzed after 1:10 dilution in PBS.

Transfection and culture conditions

Lipid based TransMessenger™ and RNAiFect™ transfection reagents where kindly provided by QIAGEN GmbH. Human cervix carcinoma Hela S3 cells (DSMZ, ACC 161) were kept in culture with 10 % FBS in Ham's F12 medium at 5 % CO_2 atmosphere and 37 °C. Twenty-four hours before transfection, $5x10^4$ cells where seeded in 24 well plates. Transfection protocol was followed as described in QIAGEN's manual for each transfection reagent, being the specific siRNA incubations times of 3 hours in serum free medium for TransMessenger and 17 hours in complete medium when RNAiFect was used. After transfection, cells were kept in culture medium until time of analysis, 24 hours after transfection was initiated.

Transfection efficiency measurements
Flow cytometry measurements were performed using the Cell Fluorescence LabChip kit with the Agilent 2100 bioanalyzer. After enzymatic cell detachment with Accutase (PAA laboratories, Cat. # L11-007) cells where resuspended in Cell Buffer at a concentration of $2x10^6$ cells/ml. For on-chip staining procedure, cells were loaded onto a cell chip (10µl/sample), vortexed for 1 min (1000 rpm) and incubated for 20 min in the dark with 0.5µl of the life staining dye CellTracker Green CMFDA (5-chloromethyl-fluorescein diacetate, Molecular Probes, Cat. # C-7025) at a final concentration of 6 µM. After 1 min vortexing (1000 rpm) the chip was loaded into the instrument and the six samples were analyzed automatically.

Results and Discussion

The first step of an RNAi experiment is the assessment of purity and integrity of the interfering RNA. Figure 2 shows the electropherogram and gel-like image of a chemically synthesized siRNA. The size range and resolution of the DNA 1000 assay match the requirements for siRNA quality control, verifying that a single sharp peak for siRNA is obtained and undercovering any impurities ranging 15 to 1000 bp coming from digestion, synthesis, purification or degradation products. In case of RNAse III or Dicer digestion of dsRNA the appearance of fragments above 30 bp would indicate incomplete digestion and potentially induce the interferon mediated unspecific suppression of gene expression in mammalian cells[10].

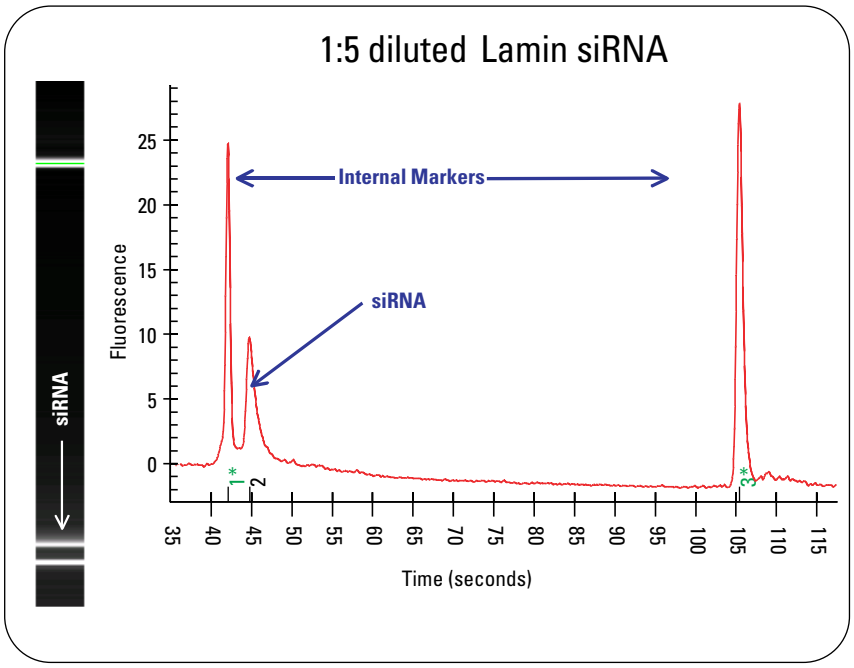

Figure 2
Integrity and purity verification of siRNA. Electrophoretic analysis of siRNA was done with the DNA1000 Labchip kit to ensure integrity and purity of siRNA. Internal marker of 15 bp is used for alignment and sizing. siRNA fragment of 21 bp is detected and correctly sized. The left panel shows gel-like image of the same run. It is known that electrophoretic mobility of nucleic acids below 70 bp may be sequence dependent, reducing sizing accuracy.

$$TV = TE \times ViT = \frac{t_v}{v} \times \frac{t_v}{t}$$

Equation 1
[TV]=Transfection Viability; [TE]=Transfection Efficiency; [ViT]=Viability in Transfected cells; [t_v]=Cy5-siRNA and CellTracker Green positive cells; [v]=CellTracker Green positive cells; [t]=Cy5-siRNA positive cells;

It is also possible to evaluate RNAse activity of culture medium with serum as the use of serum can increase transfection efficiency in some cell lines, this does however add to the risk of RNA degradation. siRNA is much more resistant to nuclease degradation, although long term incubation in serum containing medium can effectively degrade it. Following siRNA integrity validation, cells were transfected as described in the methods section. Two different lipid based transfection reagents were evaluated, cell confluence at time of transfection was kept constant at about 75 % for TransMessenger and 50 % for RNAiFect. Optimal conditions were determined by reaching the maximum of Transfection Viability (TV)(equation 1). This factor, as defined, combines two important parameters for determining the success of any transfection experiment: transfection efficiency (TE) and cell viability in transfected cells (ViT). Transfection Viability is calculated as the product of the percentage of transfected cells in the live cell population (transfection efficiency) and the ratio of live cells in the transfected population (figure 3). When evaluating the Transfection Viability with Trans-Messenger transfection reagent, we measured the effect of increasing the siRNA to TransMessenger ratio while keeping the siRNA amount fixed (0.4 µg/sample).

In this experiment, increasing amounts of TransMessenger steadily increased the transfection efficiency while gradually decreasing the viability of the transfected cells (figure 4A). The relatively low amount of siRNA suggests that the absolute amount of transfection reagent was also low, allowing for higher ratios of TransMessenger without a direct effect on viability.

The effect of changing the siRNA amount while keeping a fix ratio (1:4) was more pronounced (figure 4B). A maximum for Transfection Viability is observed at 0.4 µg siRNA/sample; it quickly drops with decreasing or increasing amounts due to too low siRNA concentration or toxicity when in excess.

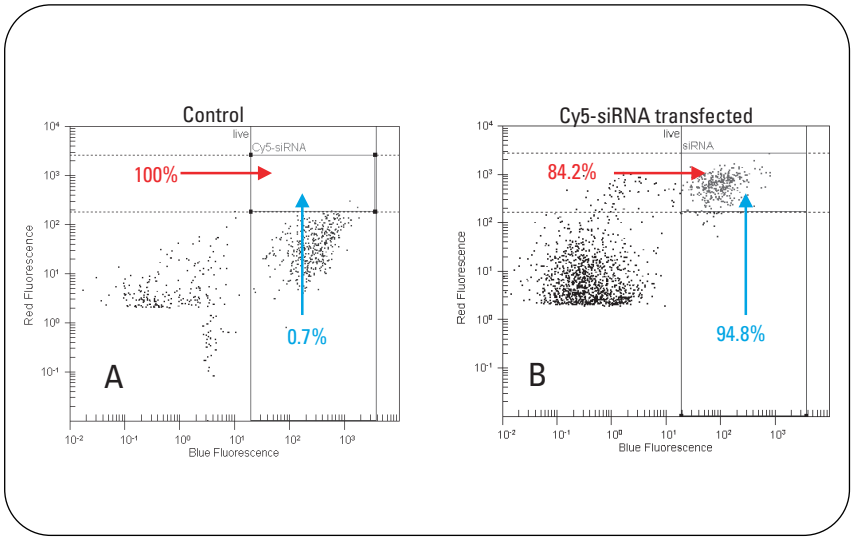

Figure 3

Transfection Viability determination. High Transfection Viability in Hela S3 is achieved with 0.9µg of Cy5–siRNA and 1:6 ratio of siRNA to RNAiFect (µg/µL). Panel B shows a typical dot plot of labeled siRNA sample. CellTracker Green staining causes life cells to fluoresce in the blue channel while Cy5-siRNA transfected cells show high red fluorescence intensity. For assessing Transfection Viability, both viability of transfected cells (red gate) and transfection in viable cells (blue gate) are measured. Transfection Viability is then defined as the product of both measurements. Panel A shows a dot plot view of negative control sample, treated only with transfection reagent, used to set the regions.

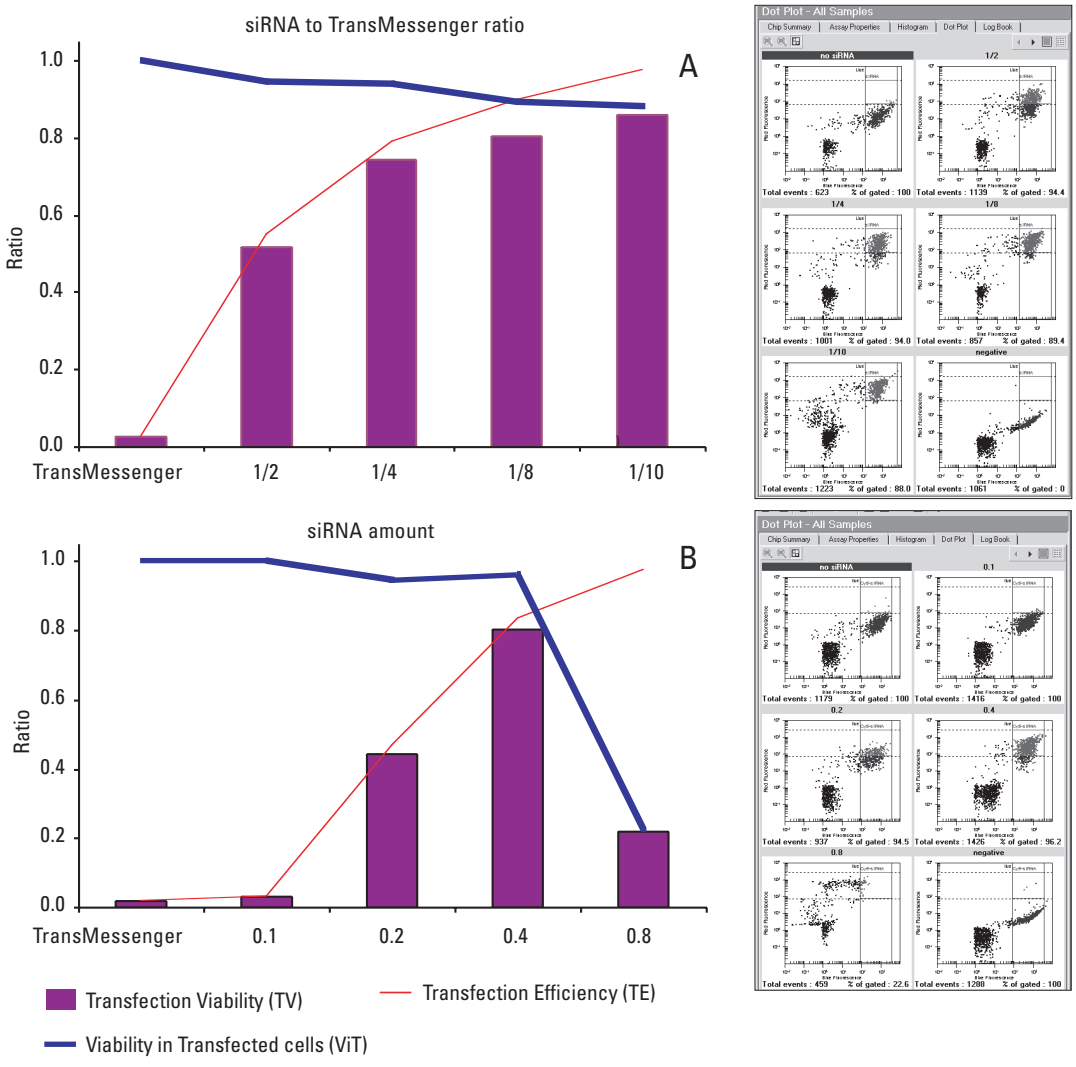

Figure 4

Optimization of Transfection Viability. Transfection with TransMessenger for 3h. Analysis 1h after transfection in two chip runs (right panel dot plots). Panel A shows the effect of increasing siRNA to TansMessenger ratio (µg/µL) in Transfection Viability (solid bars), while Cy5- siRNA amount was kept at 0.4 µg. Panel B shows a similar plot for total siRNA amount by fixing the ratio to 1:4. Transfection Viability shows a maximum at 0.4 µg while quickly decreasing under different conditions.

The Transfection Viability with RNAiFect was evaluated as suggested by QIAGEN protocols for Hela cells, in the range from 0.5 to 1.5 µg siRNA/sample in a 24 well plate and siRNA to RNAiFect ratios from 1:3 to 1:9. It shows very high Transfection Viability in Hela cells for most conditions. The effect of increasing amounts of siRNA when compared to different siRNA to RNAiFect ratios (figure 5) show that with RNAiFect lower amounts of siRNA could be successfully used with higher ratios to produce a good transfection, allowing cost per analysis to be included in the optimization criteria. The overall TV maximum appears at 0.9 µg siRNA with 5.4 µl RNAiFect (1:6), showing the highest efficiency with very high viability of the transfected cells.

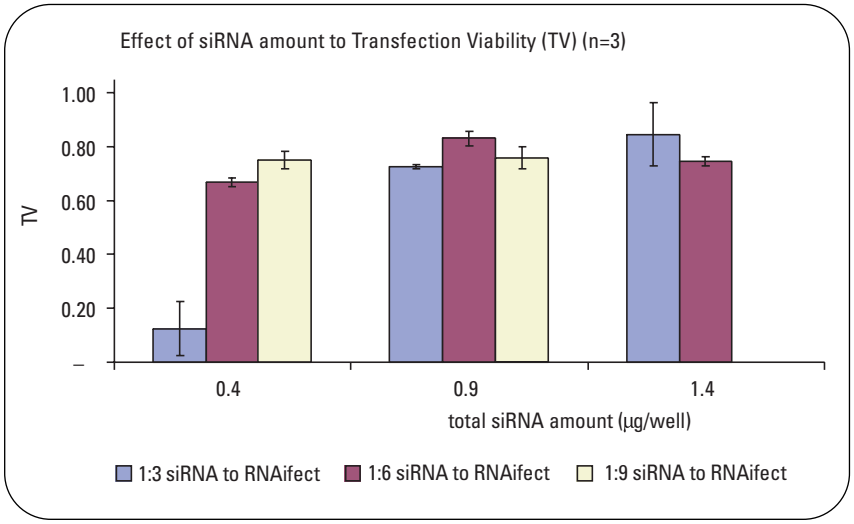

Figure 5
Effect of varying concentrations of siRNA with constant siRNA to RNAiFect ratios. Hela S3 cells were incubated for 17h with transfection complexes. Analysis was performed 24h after transfection. The plot demonstrates that increasing the amount of RNAiFect ratio to Cy5-siRNA allows very high Transfection Viability with little siRNA consumption. Maximum Transfection Viability is obtained with 0.9 µg siRNA with 1:6 siRNA to RNAiFect ratio (5.4 µL).

Conclusion

The Agilent 2100 bioanalyzer is the ideal tool for fast and easy optimization of transfection in RNAi experiments. For successful gene silencing it is essential to optimize transfection conditions for each cell line used, as siRNA delivery into cells is one of the most important steps. Transfection success is improved by the inclusion of viability of the transfected cells in its definition. The microfluidic system combines assays for transfection viability measurement, cellular protein expression analysis and siRNA integrity as well as purity on a single platform. The additional advantage that only a few cells and mimimum reagents are needed for optimization experiments with the 2100 bioanalyzer means that the remaining cells can be used for further analysis. QIAGEN's ability to deliver an integrated solution for RNAi reagents complements the Agilent 2100 bioanalyzer and provides a new analysis tool for effective gene silencing.

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A complete list of all Application Notes for the Agilent 2100 bioanalyzer and more information about RNAi can be found at:
www.qiagen.com/sirna
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