Confirming gene silencing mechanism by pGFP/GFP22 – siRNA co-transfection

Application

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Abstract

In addition to the specificity and efficiency of an siRNA sequence for a target gene, which are of high importance in post-transcriptional gene silencing (PTGS), the delivery of siRNA into cells is crucial. An efficient method to optimize transfection and gene silencing is critical for successful RNAi experiments. In this Application Note HeLaS3 cells were transfected with GFP expressing plasmid and Cy5®-labeled siRNA against GFP, both individually and in combination, using QIAGEN transfection reagents. Uptake of labeled siRNA and the gene silencing effect were monitored for up to 24 hours after transfection. Efficient gene silencing was achieved which confirms the operation of RNAi mechanism in the selected cell line.

Introduction

The technique of RNAi has the potential to revolutionize functional genomics. The pathways and mechanisms of RNAi are currently under intense investigation, and a good understanding of these is essential to allow scientists to exploit the applications of RNAi. Simple and efficient methods for monitoring gene silencing are important tools for RNAi experiments. In this Application Note we describe the evaluation of a method using fluorescent labels to determine transfection efficiency and the gene silencing effect.

HeLaS3 cells were transfected with siRNA using RNAiFect™ Reagent. TransMessenger™ Reagent was used for the transfection of GFP expressing plasmid (pGFP). Cells were also co-transfected with both plasmid DNA and siRNA using TransMessenger Reagent. The uptake of siRNA into mammalian cells was monitored using fluorescent labeled siRNA and the gene silencing effect and level of cellular protein expression were quantified at 4, 8, and 24 hours after transfection.

Materials and methods

HeLa S3 cells(ATCC, CCL-2.2) were plated at 5 x 10^4 cells /well in 24-well plates and incubated for 24 hours before transfection. Cells were transfected with 0.9 µg of GFP22-siRNA (QIAGEN, Cat. # 1022064) per well using RNAiFect Reagent (QIAGEN, Cat. #301605). GFP22-siRNA has a homologous sequence to GFP mRNA. It was transfected unlabeled or labeled

with Cy5. For GFP expression, cells were transfected with 0.2 µg of pGFP in each well using TransMessenger Reagent (QIAGEN, Cat. # 301525). For co-transfection of GFP22-siRNA and pGFP, TransMessenger Reagent was also used. The recommended protocols, described in the QIAGEN RNAiFect handbook and the supplementary protocol for co-transfection of adherent cells with siRNA and plasmid DNA using TansMessenger Reagent was followed, using the optimized ratio of RNAiFect Reagent : siRNA and TransMessenger Reagent : siRNA of 1:5 as determined previously.

Analysis of GFP expression and Cy5 fluorescence was performed using the Agilent 2100 bioanalyzer with pressure cartridge for flow cytometric studies.

For the analysis with the Agilent 2100 bioanalyzer, cells were trypsinized and washed twice in phosphate buffered saline (PBS), and then resuspended in Cell Buffer (CB) at a dilution of 1 x 10^6 -1.5 x 10^6 per milliliter. Cells were stained for 30 minutes at RT with the live dyes calcein-AM (Molecular Probes, Cat. # C-3099) or carboxynaphthofluorescein diacetate(CBNF) (Molecular Probes, Cat. # C-13196). Dye concentrations of 2.5 µM were used for staining. Ten microliters of each sample were loaded on a microfluidic chip for analysis. Microfluidic chips and CB buffer were supplied with the Agilent Cell Fluorescence LabChip kit. Cy5 fluorescence indicated the efficiency of siRNA uptake and calcein-AM staining monitored the cell viability. In

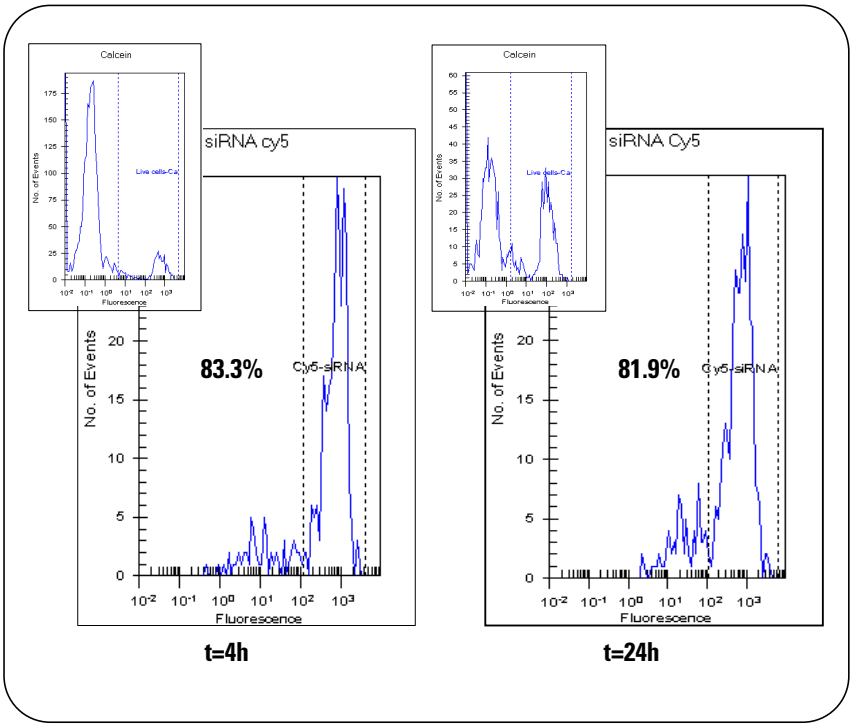

Figure 1
Fast uptake of siRNA. Histogram view of the Agilent 2100 bioanalyzer shows high transfection efficiencies on the RNAiFect transfected cells already 4 hours after transfection, with 83.3 % of live cell population showing a strong Cy5-siRNA signal. The signal remains constant after 24 hours.

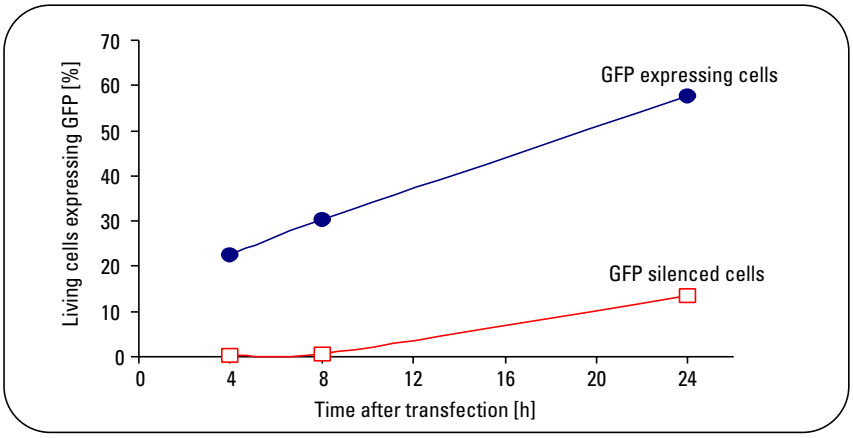

Figure 2
Strong GFP silencing was observed until 24 hours after transfection. pGFP transfected cells show increasing expression from 22.4 to 57.6 % after 24 hours. Co-transfection of pGFP and GFP22-siRNA with TransMessenger resulted in efficient gene silencing, with <1 % of cells expressing GFP after 4 and 8 hours, and only 13.5 % of live cells expressing it after 24 hours. The measured down-regulation of expression of GFP at 24 hours is up to 77 %.

cells transfected with pGFP, GFP fluorescence indicated the transfection efficiency and the proportion of living cells was monitored by CBNF staining. The efficiency of gene silencing in cells co-transfected with Cy5-siRNA and pGFP was monitored by GFP fluorescence, and the viability of the cells was monitored by CBNF staining. Fluorescence microscopy was used to compare the 2100 bioanalyzer results 24 hours after transfection.

Results and discussion

Cells were analyzed 4, 8, and 24 hours after transfection using the 2100 bioanalyzer. As controls, untransfected cells were used, either unstained, stained with calcein-AM or CBNF alone. Controls were analyzed in parallel with the transfected cells (data not shown). In cells transfected with Cy5-labeled siRNA, Cy5 positive cells were detectable after 4 hours (figure 1). Transfection efficiency measurements after calcein staining showed that over 80 % of the live cells were Cy5-positive. This picture remained constant after 24 hours. In cells transfected with pGFP, the proportions of live cells expressing GFP increased from 22.4 % after 4 hours to 57.6 % after 24 hours. Co-transfection of pGFP and siRNA resulted in efficient gene silencing and results after each time point were compared and plotted (figure 2). A strong gene silencing effect was measured and by 24 hours the measured down-regulation of GFP expression was up to 77 %.

In a separate experiment, cells were transfected with pGFP alone, pGFP with unlabeled siRNA, and

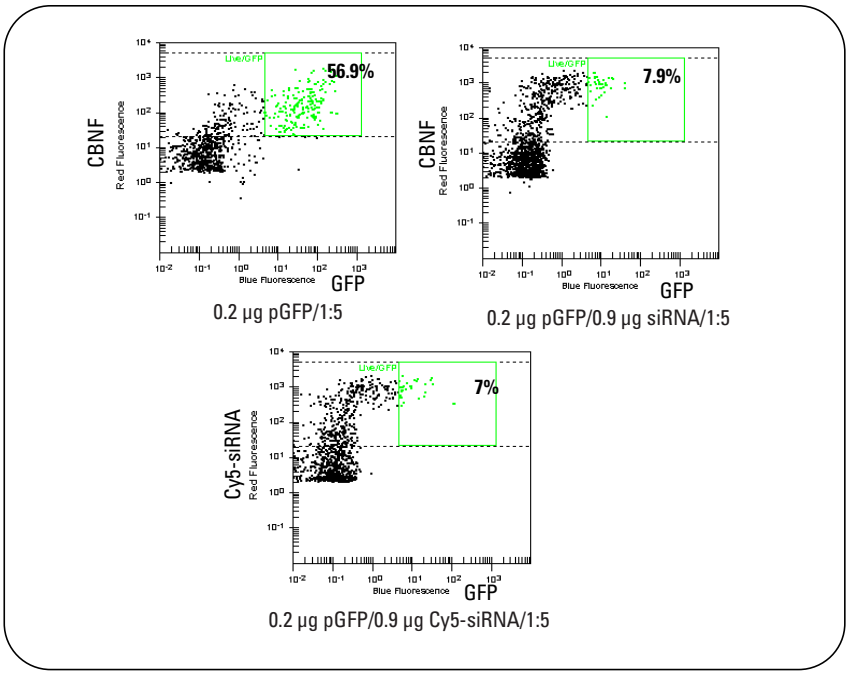

Figure 3
Fluorescent labeling does not interfere with the silencing mechanism. 56.9 % of live cells express GFP when transfected with 0.2 µg pGFP and 1:5 ratio with TranMessenger Reagent. This expression is down-regulated to below 10 % when co-transfecting the plasmid with both labeled or non-labeled GFP22-siRNA.

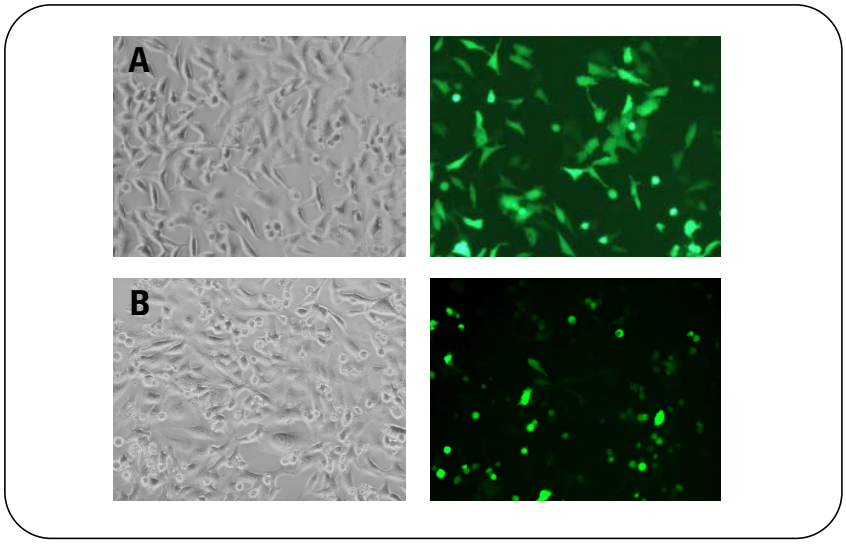

Figure 4
Fluorescence microscopy shows efficient gene silencing.
High levels of GFP fluorescence in cells transfected with pGFP are observed after 24h (A). Low levels of GFP fluorescence in silenced cells (B) are in agreement with the quantitative measurements of the 2100 bioanalyzer

pGFP with Cy5-labeled siRNA to determine whether the Cy5 label affected gene silencing. Results, 24 hours after transfection, showed similar down regulation of the GFP expression, meaning that the Cy5 label did not interfere significantly with the gene silencing effect (figure 3). Cells were also examined by fluorescence microscopy confirming the results from the 2100 bioanalyzer for GFP existence in cells (figure 4).

Conclusions

Uptake of labeled siRNA and its gene silencing effect were quantitatively monitored using the flow cytometry capability of the Agilent 2100 bioanalyzer. Successful co-transfection of pGFP and Cy5-labeled siRNA using TransMessenger reagent was achieved. GFP expression of up to 56.9 %, and silencing of GFP expression to levels below 10 % confirmed the efficient transfection and silencing mechanism in the selected cell line.

The measurement of protein expression in viable cells with the Agilent 2100 bioanalyzer is fast, accurate and automated, providing efficient methods to monitor and optimize any gene silencing experiment. QIAGEN reagents and chemically synthesized siRNA probes are highly efficient and reliable.

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