Monitoring transfection efficiency by green fluorescent protein (GFP) detection with the Agilent 2100 bioanalyzer

Application

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Abstract

This Application Note describes how the Agilent 2100 bioanalyzer and the Cell Fluorescence LabChip® kit can be used to determine the efficiency of transfection of mammalian cells using green fluorescent protein (GFP) as a reporter molecule. Transfection of CHO-K1 cells with expression vectors encoding GFP from two different sources, and optimization of transfection conditions for both plasmids were performed. Histogram quality and the percent transfected cells determined, based on the low number of fluorescent cells counted with the microfluidic system, is in good agreement with data obtained with a conventional flow cytometer. Detailed protocols and reagent recommendations for analyzing transfection reactions are given. High reproducibility of chip results, low cell consumption and ease-of-use are advantages the compact Agilent 2100 bioanalyzer offers for monitoring transfection efficiency using GFP.

Agilent Technologies

Introduction

The Agilent 2100 bioanalyzer was introduced by Agilent Technologies as the first commercially available lab-on-a-chip analysis system for the life science laboratory using LabChip®products, developed by Caliper Technologies Corp. Chip-based approaches for a variety of separation-based techniques have been introduced, addressing DNA, RNA, and protein separations. The Agilent 2100 bioanalyzer is capable of two-color fluorescence detection and runs disposable microfluidic glass chips. The application presented here is based on the controlled movement of cells through these channels by pressure-driven flow. Cells are hydrodynamically focused in the channels before passing the fluorescence detector in single file. Each chip accommodates several samples and data acquisition of all samples is fully automated while analysis allows for user-specific settings. Specific advantages of the instrument are the low number of cells required for analysis and the ease-of-use. Among the first applications investigated is the monitoring of cell transfection efficiency.

Transfection

Transfection, the introduction of foreign DNA into a eukaryotic cell, is an important tool for studying the regulation of gene expression as well as protein expression and function. In stable transfection, the foreign DNA becomes integrated into the genomic DNA of the cell so that it is passed on in the cell lineage and continues to express the encoded gene of interest. More commonly used is transient transfection, in which higher copy numbers of the foreign DNA and hence higher levels of gene expression are present in the cell for a brief period of time. There are several methods available for cell transfection such as formation of complexes of the DNA with either DEAE dextran or calcium phosphate, to facilitate entry into the cell by endocytosis, or electroporation, which uses high voltage pulses to form transient pores in the cell membrane through which the DNA can enter. Currently, the most widely used method for transfecting cells is with cationic lipids that result in very high transfection efficiencies with low cytotoxicity.

In order to determine the percentage of cells in a transfection experiment that have received and are expressing the foreign DNA sequence, a reporter gene can be used. The reporter gene can be present on the same vector as the gene of interest or can be on a separate plasmid. The reporter gene can also be used to create a fusion protein with the gene of interest for protein localization studies. A convenient reporter for monitoring transfection efficiency is the green fluorescent protein (GFP). When excited by blue or UV light, the protein emits bright green fluorescence light through cyclization of a tripeptide chromophore embedded within the complete amino acid sequence. Genes encoding green fluorescent proteins have been cloned from various coelenterates such as the jellyfish *Aequorea victoria* and the sea pansy *Renilla reniformis*. To facilitate their use as reporters, several GFP variants have been developed by introducing amino acid substitutions into the chromophore, which result in a shift in the emission wavelength as well as an increase in fluorescence intensity. Additional mutations have been introduced to create preferred human codons in order to increase expression efficiency in mammalian cells. Many expression vectors containing these humanized GFP variants are commercially available. Expression of GFP is typically detected by fluorescence microscopy or flow cytometry.

In this Application Note, we describe the use of the Agilent 2100 bioanalyzer to monitor transfection efficiency using GFP as a reporter. CHO-K1 cells were transfected with GFP expression vectors using a cationic lipid reagent, and the percentage of GFP expressing cells within the live cell population was determined.

Experimental

Cell Culture

CHO-K1 cells were obtained from ATCC and cultured in F12 medium containing 10 % FBS, 10 mM HEPES, Pen/Strep, 1 mM sodium pyruvate and 2 mM L-glutamine.

Transfection

pEGFP-C2 (Clontech) and phrGFP (Stratagene) plasmid DNA was purified using the Perfectprep Plasmid XL kit (Eppendorf). Twenty hours before transfection, CHO-K1 cells were seeded in a 6-well tissue culture plate at a density of 5×10^5 in 2 ml of growth medium and incubated overnight. On the day of transfection, 1 µg of plasmid DNA was diluted into OPTI-MEM (Life Technologies) and mixed with 6 µl of Lipofectamine 2000 (Life Technologies) according to supplier's protocol. Prior to transfection, the growth medium was replaced with 2 ml of OPTI-MEM. DNA-Lipofectamine complexes were added to the cells and incubated for 6 h. The transfection medium was then replaced by growth medium and cells were incubated for an additional 18 h.

Measuring transfection efficiency with the 2100 bioanalyzer

Ordering information for the reagents is listed in table 1 on page 8.

1. Cells were harvested and resuspended at $1x10^6$ cells/ml in HBSS with 0.05 % pluronic acid (Molecular Probes).
2. Cells were stained with 0.5µM of the live cell dye carboxy-naphthofluorescein diacetate (CBNF, Molecular Probes) in the same buffer for 15 min at room temperature.
3. Afterwards, cells were washed in HBSS with 0.05 % pluronic acid.
4. Stained cells were centrifuged (500 x g, 5 minutes) and resuspended in cell buffer (supplied with the Cell Fluorescence LabChip kit) at 2×10^6 cells/ml
5. 10 µl of the cell suspension was applied to the sample wells of the cell assay chip and analyzed on the Agilent 2100 bioanalyzer.

Approximately 600-800 cell events were counted per sample. Parallel samples were prepared for conventional flow cytometer measurement. Cells were photographed on a Nikon fluorescence microscope.

Results and Discussion

For successful transfection of GFP plasmid DNA into CHO-K1 cells, the optimal DNA:lipid ratio was initially determined. As a result, 6 µl of Lipofectamine 2000 was chosen as the optimal amount for transfection of 1 µg of DNA in 6-well culture plates.

Assay of transfection efficiency on the 2100 bioanalyzer

In this experiment, pEGFP-C2, which encodes a red-shifted variant of wild-type *Aeqourea* GFP was used for transfection of CHO-K1 cells. Mock- or EGFP-transfected cells were stained with the live cell dye CBNF and analyzed using the 2100 bioanalyzer. Approximately 600 cell events were collected for each sample. Figure 1A shows the dot plots of the fluorescence data (CBNF versus EGFP) of the control and EGFP-transfected cells. The population within the rectangular region represents live (CBNF-positive) and EGFP-expressing CHO-K1 cells. The data can also be displayed as frequency histograms, as depicted in figure 1B. In this case, in order to determine the percentage of EGFP-expressing cells, live cells in the CBNF-positive population were cross-gated onto the EGFP histogram. A parallel measurement of the same cell samples on a flow cytometer yielded a comparable result (figure 1C). On average, 600 to 800 cell events were collected for each sample run on the cell assay chip, whereas 10,000 events were collected using the flow cytometer. The histogram quality on both instruments was very similar (figure 1C). Figure 2 shows a photograph of the cell sample obtained with a fluorescence microscope.

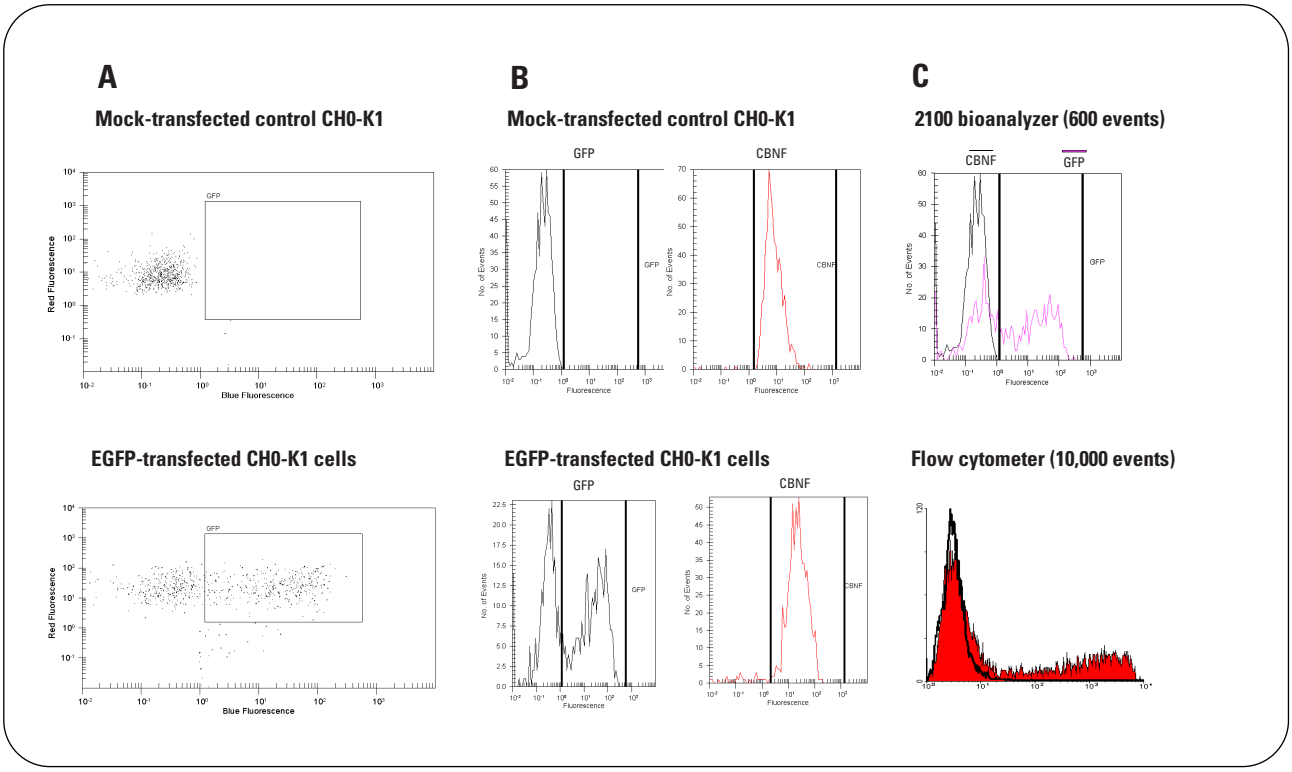


Figure 1
Determination of transfection efficiency. CHO-K1 cells were transfected with pEGFP-C2. Cells were stained with the live cell dye CBNF and analyzed on the 2100 bioanalyzer and on a conventional flow cytometer.
A. Dot plots of mock- and EGFP-transfected cells.
B. GFP and CBNF histograms of mock- and GFP-transfected cells.
C. Comparison of histograms of the GFP-transfected cells obtained from 600 events measured on the 2100 bioanalyzer and from 10,000 events measured on a flow cytometer.

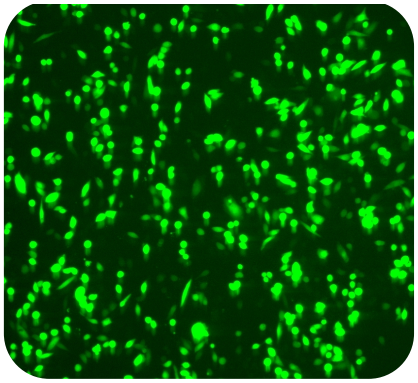


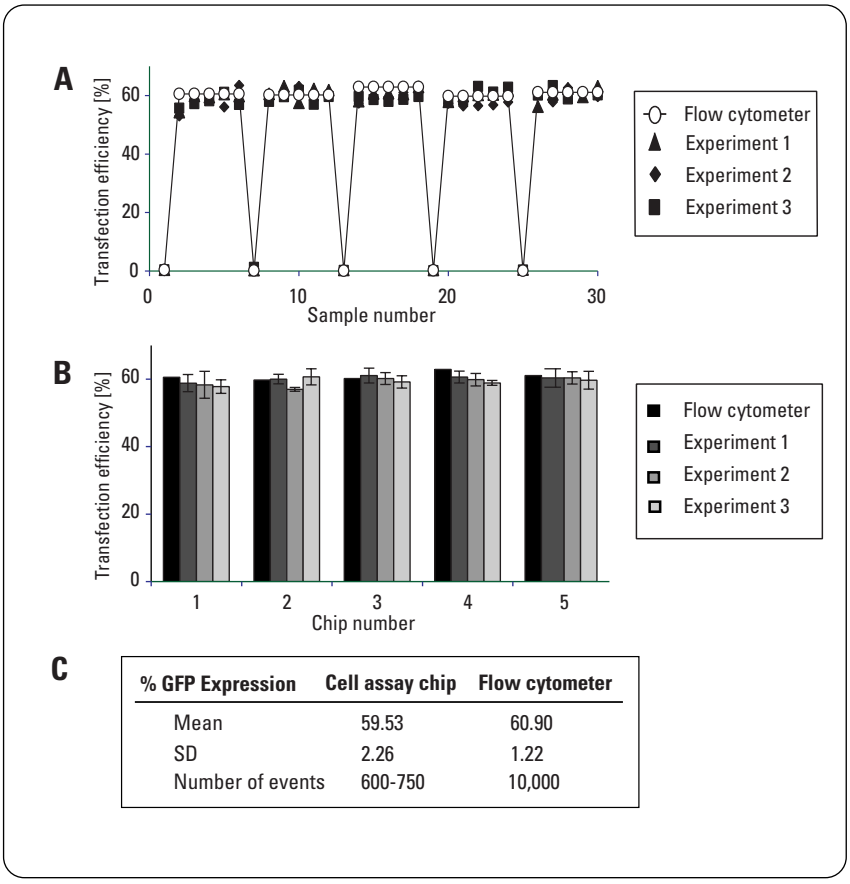
Figure 2
Photograph of the EGFP-transfected cell sample obtained with a fluorescence microscope

Reproducibility of GFP transfection efficiency assay

To determine the reproducibility of the assay method using the 2100 bioanalyzer, the same mock- or EGFP-transfected CHO-K1 cells were run on multiple cell assay chips. On each chip, mock-transfected cells were loaded in cell well 1 and EGFP-transfected cells in cell wells 2 to 6. Figure 3A shows the individual data points from 15 chips run on three different instruments (5 chips per instrument). Chip to chip reproducibility of the assay is shown in figure 3B. Figure 3C is a summary of the transfection data from 75 measurements of the same cell preparation assayed on the 2100 bioanalyzer. The transfection efficiency as determined on the 2100 bioanalyzer is identical to the result obtained using a conventional flow cytometer.

Using the 2100 bioanalyzer to monitor optimization of transfection conditions

Measurement of the transfection efficiency and expression of GFP in cells are typically done by fluorescence microscopy or flow cytometry. Each of these methods has its drawbacks. Here we demonstrate that the 2100 bioanalyzer can be used to monitor quantitatively the expression of GFP in an exercise to develop an optimized transfection protocol for CHO-K1 cells. In the experiment, titration of Lipofectamine 2000 reagent was performed to determine the optimal DNA:lipid ratios that gave the best transfection efficiency in 6-well culture plates. In order to see if there are differences in transfection and expression levels of GFP from different species, reporter plasmids encoding green fluorescent proteins from *Aequorea* (EGFP) and *Renilla* (hrGFP) were used to transfect CHO-K1 cells. While keeping the amount of GFP (EGFP or hrGFP) plasmid DNA at 1 µg, the DNA:lipid ratio was varied from 1 to 8 (ratios of 1, 2, 4, 6, and 8). Twenty hours after transfection, control cells and the 5 transfected cell samples were stained with



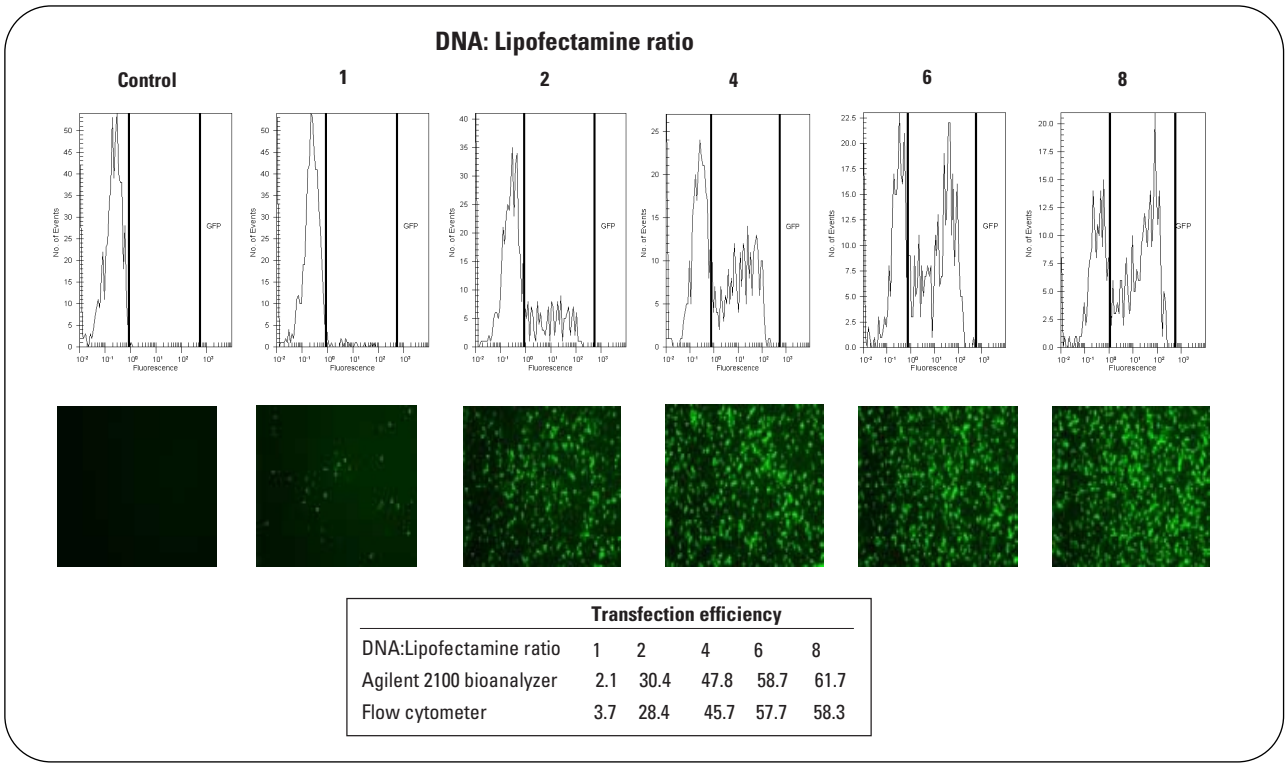
C

% GFP Expression	Cell assay chip	Flow cytometer
Mean	59.53	60.90
SD	2.26	1.22
Number of events	600-750	10,000

Figure 3
Reproducibility of transfection results obtained with the 2100 bioanalyzer. The mock- and GFP-transfected cell samples described in figure 1 were used.
A. Plot of individual data points from 15 chips where mock-transfected cells were loaded in well 1 and EGFP-transfected cells were loaded in wells 2-6.
B. Graph of the average percent EGFP-transfected cells obtained per chip, where the transfected cells were loaded in wells 2-6. The samples were run on a total of 15 chips (3 different instruments with 5 chips each).
C. The table lists the average percent transfected cells for the 75 measurements shown in B compared to the result obtained with a flow cytometer. The standard deviations for the measurements are also shown.

CBNF and loaded into the wells of a cell assay chip and analyzed on the 2100 bioanalyzer. Figure 4 shows the results obtained when EGFP was used for transfection. At a DNA:lipid ratio of 1, expression of the reporter gene was barely detectable. There was a 20 to 25-fold enhancement in activity when the ratio was increased to 8. It indicated that optimal transfection and expression of EGFP were achieved when a DNA:lipid ratio of 6 or 8 was used. A comparable result was obtained when parallel cell samples were measured on a flow cytometer. The 2100 bioanalzyer results also correlated with the those obtained by fluorescence microscopy.

When CHO-K1 cells were transfected with phrGFP, which encodes a humanized *Renilla* GFP, there was a similar enhancement in transfection and expression of the reporter gene as the DNA:lipid ratio was increased from 1 to 8 (figure 5A). However, when compared to EGFP, the transfection efficiency of hrGFP was reduced (figure 5B) and the hrGFP-expressing population as a whole also showed a substantially diminished fluorescence intensity (figure 5C).



	Transfection efficiency				
DNA:Lipofectamine ratio	1	2	4	6	8
Agilent 2100 bioanalyzer	2.1	30.4	47.8	58.7	61.7
Flow cytometer	3.7	28.4	45.7	57.7	58.3

Figure 4
Optimization of transfection conditions for EGFP. CHO-K1 cells were transfected with pEGFP-C2 using Lipofectamine 2000 at various DNA:lipid ratios. Cells were stained with CBNF prior to analysis on the 2100 bioanalyzer. For each condition tested, a representative GFP histogram is shown as well as a photograph of the cells obtained with a fluorescence microscope.

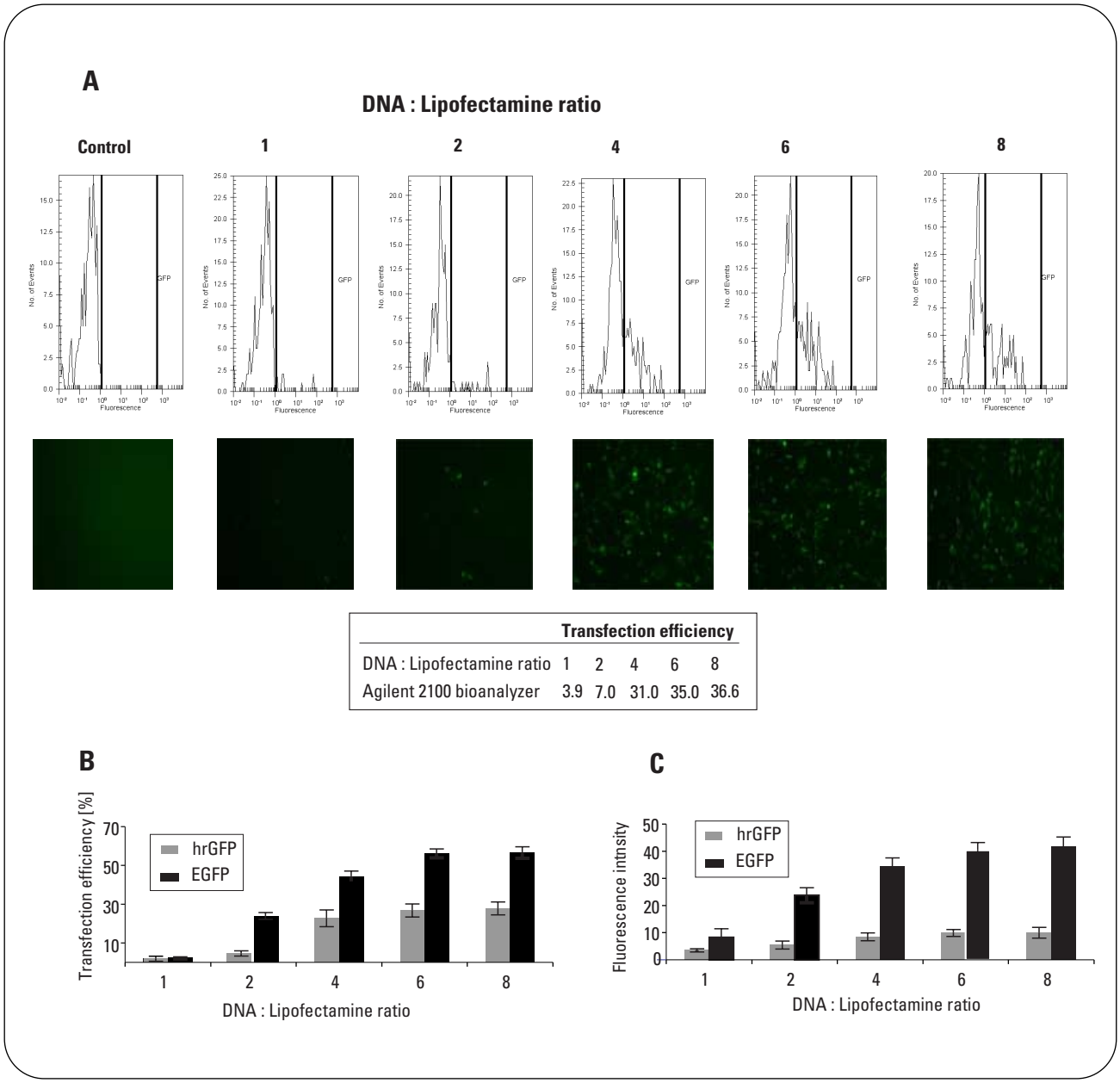


Figure 5

Optimization of transfection conditions for hrGFP. CHO-K1 cells were transfected with phrGFP using Lipofectamine 2000 at various DNA:lipid ratios. Cells were stained with CBNF prior to analysis on the 2100 bioanalyzer.

A. For each condition tested a representative GFP histogram is shown as well as a photograph of the cells obtained with a fluorescence microscope.

B. Comparison of transfection efficiencies obtained with EGFP versus hrGFP.

C. Comparison of GFP fluorescence intensities obtained for cells transfected with EGFP versus hrGFP.

Reagent	Supplier	Order No.
LIPOFECTAMINE 2000 Transfection reagent	Life Technologies http://www.lifetech.com	11668-019
OPTI-MEM I	Life Technologies	51985-018
Carboxynaphthofluorescein Diacetate, CBNF	Molecular Probes http://www.probes.com	C-13196
Pluronic Acid, 10% solution	Molecular Probes	P-6866, Pluronic F-127
HBSS, 1X	Irvine Scientific http://www.irvinesci.com	9232

Table 1
Ordering information for reagents used in the experiments described in this Application Note

Conclusion

The Agilent 2100 bioanalyzer microfluidic lab-on-a-chip system can be used to monitor the transfection efficiency and expression of the GFP reporter gene in mammalian cells. It is particularly useful as a tool for optimization of transfection conditions for different transfection methods or new cell lines. In contrast to fluorescence microscope techniques, the 2100 bioanalyzer system provides accurate, quantitative data for optimization experiments, without tedious manual cell counting. The Agilent 2100 bioanalyzer is easy to use and requires a short setup time, while results for GFP expression are comparable to data obtained using conventional flow cytometry. The cell assay chip is disposable, can accommodate up to 6 samples, and requires as little as 20,000 cells per sample.

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