Monitoring transfection efficiency in cells using an on-chip staining protocol

A rapid and accurate method to detect green fluorescent protein expression

Application

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Abstract

Optimization of transfection procedures is an important step to obtain optimal results in downstream experiments. 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 by on-chip staining. Transfection of CHO-K1 cells with an expression vector encoding GFP was performed and cells were stained on-chip with the live-dye CBNF (carboxynaphthofluorescein). The histogram quality and the percent transfected cells determined are in good agreement with data obtained using a conventional flow cytometer. Detailed protocols and reagent recommendations for analyzing transfection reactions are provided. Speed of the on-chip staining procedure, high reproducibility of the chip results, low cell consumption and ease-of-use are advantages the 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 commerially 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[1, 2, 3]. Most recently a set of applications has been developed for the measurement of cell fluorescence using the same platform. Cells move through the networks of microfluidic channels in the cell chip by precisely regulated pressure driven flow. Cells are hydrodynamically focused in the channels before passing the two-color fluorescence detector in single file. Each chip accommodates six samples and data acquisition of all samples is fully automated while analysis allows for user-specific settings. These applications include:

- "Apoptosis detection by Annexin V and active Caspase 3 with the Agilent 2100 bioanalyzer" (Agilent publication number 5988-4319EN)
- "Monitoring transfection efficiency by green fluorescent protein (GFP) detection with the Agilent 2100 bioanalyzer" (Agilent publication number 5988-4320EN)
- "Detecting cell surface and intracellular proteins with the Agilent 2100 bioanalyzer by antibody staining" (Agilent publication number 5988-4322EN)

This Application Note describes on-chip staining of cells for monitoring transfection efficiency.

Specific advantages of the on-chip staining procedure include the low number of cells and the low amount of reagents required for analysis, as well as speed and ease-of-use of the protocol.

Transfection, the introduction of foreign DNA into an eukaryotic cell, is an important tool to study 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 involves the use of cationic lipids. The transfection process greatly depends on cell line, plasmid and its preparation as well as type of transfection reagent and its concentration. The process must be optimized for each new experiment in order to obtain optimal transfection efficiencies. A reporter gene can be used to determine the percentage of cells in a transfection experiment that have received and are expressing the foreign DNA. 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 green fluorescent protein (GFP). When excited by blue or UV light, the protein emits bright green fluorescence through cyclization of a tripeptide chromophore embedded within the complete amino acid sequence[4]. 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[5]. Additional mutations have been introduced to create preferred human codons in order to increase expression efficiency in mammalian cells. Many expression vectors containing the GFP variants are commercially available. Expression of the GFP proteins is typically detected by fluorescence microscopy or flow cytometry.
The transfection procedure, as well as the introduction of DNA or the expression of the genes introduced may reduce the

viability of the transfected cells significantly. Especially *Aequorea* GFP is often cytotoxic when expressed in mammalian cells[6]. Therefore it is of special interest to not only know the number of cells expressing the GFP protein, but to determine the number of cells expressing the GFP protein within the live cell population. Counterstaining with the live dye Carboxynaphthofluorescein Diacetate (CBNF), which is cleaved by intracellular esterases to yield red-fluorescent carboxynaphthofluorescein can be passively loaded into live cells and allows the discrimination between live and dead cells[7].

In this Application Note, we describe a protocol for directly staining EGFP-transfected CHO-K1 cells within the wells of a cell chip with the live-cell stain CBNF. The cells were subsequently analyzed on the Agilent 2100 bioanalyzer to determine the transfection efficiency. Optimization of a lipofection method was demonstrated along with a comparison of results from fluorescence microscopy and conventional flow cytometry.

Experimental

Cell Culture

Adherent growing chinese hamster ovary (CHO-K1) cells were obtained from ATCC (Manassas, VA, USA) and cultured in F12 medium containing 10%FBS, 10 mM HEPES, Pen/Strep, 1 mM sodium pyruvate and 2 mM L-glutamine (Life Technologies).

Transfection

pEGFP-C2 (Clontech, Palo Alto, CA, USA) plasmid DNA was purified using the Perfectprep XL kit (Eppendorf, Hamburg, Germany). Twenty hours before transfection, CHO-K1 cells were seeded in a 6-well tissue culture plate at a density of 5 x 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, Carlsbad, CA, USA) and mixed with different volumes of Lipofectamine 2000 (Life Technologies) as described below. Prior to transfection, the growth medium was replaced with 2 ml of OPTI-MEM (Life Technologies). 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 additional 18 h.

Measuring transfection efficiency with the 2100 bioanalyzer

Ordering information for the reagents is listed on page 8.
Chips, CB, PS and FD buffers are provided in the Cell Fluorescence LabChip® kit. All chip vortexing steps were performed on the IKA vortexer supplied together with the Agilent 2100 bioanalyzer.

1. Cells were trypsinized, harvested and carefully resuspended at a density of 3x10^6 cells/ml in cell buffer provided in the Cell Fluorescence LabChip® kit.
2. A cell chip was primed with 10 µl PS.
3. After 1 min 10 µl FD were added to the dye well and 30 µl of CB were added to the buffer wells.
4. 10 µl of the cell suspension were placed into each sample well.
5. 4 µl of a 1:15 dilution of CBNF in CB (a stock solution of 5 mM in DMSO was prepared, final CBNF-concentration in the sample well was 10 µM) were added to each sample well.
6. The chip was vortexed for 1 min on an IKA vortexer (see kit guide for use of vortexer and vortexer setting) and then incubated in the dark for 15 min at room temperature in a humidified chamber. Alternatively, chips can be stacked using a used or unused chip on top to prevent excessive evaporation.
7. The chip was vortexed for 1 min again, loaded into the instrument and run.

Approximately 600-800 cell events were counted per sample. Parallel samples were stained according to the conventional protocol as described in another Application Note[8] and measured by conventional flow cytometry.

Results and Discussion

Using the Agilent 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. In fluorescence microscopy the process is manual, tedious and no electronic data is generated. Conventional flow cytometry requires expensive hardware, a high degree of expertise and more sample is needed for the analysis.

Here we demonstrate that the Agilent 2100 bioanalyzer Lab-on-a-Chip system can be used to quantitatively monitor the expression of GFP in an exercise to optimize a transfection protocol for CHO-K1 cells. Typically, transfection parameters have to be varied in order to find the optimal conditions for achieving a maximum transfection efficiency. For successful transfection of GFP plasmid DNA into CHO-K1 cells, the optimal DNA:lipid ratio was initially determined. pEGFP-C2, which encodes a red-shifted variant of wild-type *Aequorea* GFP was used for transfection of CHO-K1 cells. Control- or EGFP-transfected cells were stained with the live cell dye CBNF on-chip and analyzed using the 2100 bioanalyzer. In the experiment, titration of Lipofectamine 2000 reagent was performed to determine the optimal DNA:lipid ratios that yielded the highest transfection efficiency in 6-well culture plates. While keeping the amount of EGFP plasmid DNA constant at 1 µg (1 µl), the amount of lipid was varied from 2 to 10 µl (DNA:lipid ratios of 1:2, 1:4, 1:6, 1:8 and 1:10).

Figure 1 shows the transfected samples as viewed through a fluorescence microscope after 18 hours of transfection. Usually a quick view gives the answer if the transfection was successful, but getting quantitative results with this method is quite tedious. In addition live and dead cells are indistinguishable. Control cells and the 5 transfected cell samples were harvested, loaded into the wells of a cell assay chip, stained on-chip with CBNF and analyzed on the 2100 bioanalyzer (figure 2). At a DNA:lipid ratio of 1:2, expression of the GFP reporter gene was barely detectable (5.3% transfected cells). There was an 8-fold enhancement in activity when the ratio was increased to

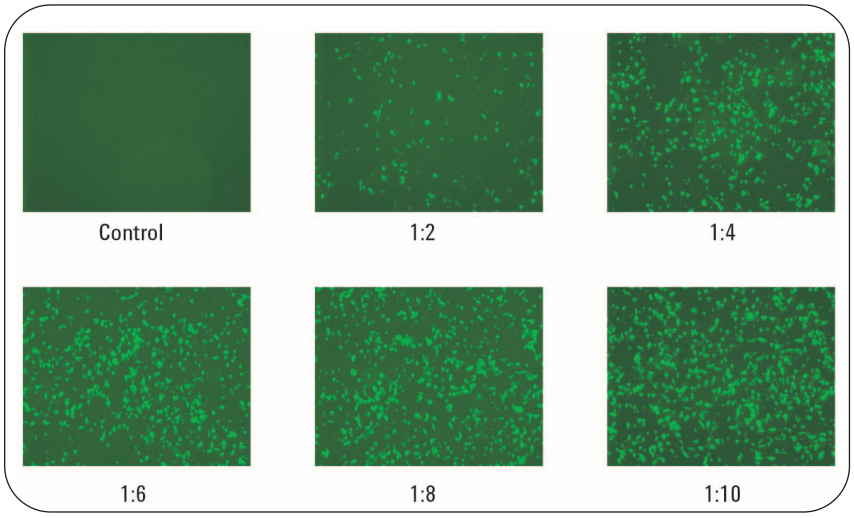

Figure 1
Optimization of transfection conditions for EGFP. CHO-K1 cells were transfected with EGFP using Lipofectamine 2000 and EGFP DNA at various DNA:lipid ratios (1:2 to 1:10) or only lipofectamine (control). Photographs of transfected cells were obtained 18h post transfection by fluorescence microscopy.

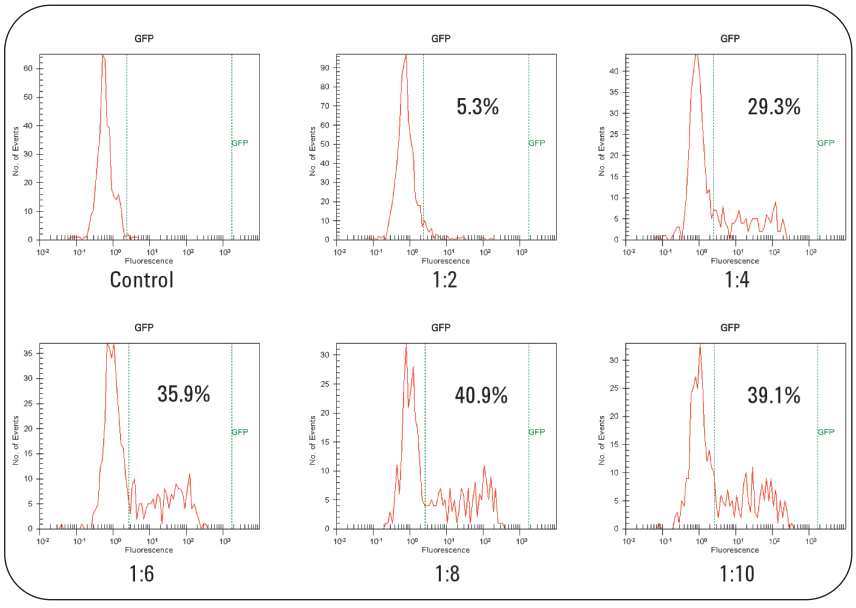

Figure 2
Optimization of transfection conditions for EGFP. CHO-K1 cells were transfected with EGFP using Lipofectamine 2000 and EGFP DNA at various DNA:lipid ratios (1:2 to 1:10) or only with lipofectamine (control). Cells were then stained on-chip with the live-cell stain CBNF and analyzed on the 2100 bioanalyzer. Blue fluorescence histograms of CBNF positive cells and percentages of transfection are shown. 600–800 events were measured per sample.

8 (40.9% transfected cells). Transfection efficiency did not increase further by increasing the DNA:lipid ratio to 1:10, whereas the number of live cells decreased (data not shown). This indicated that optimal transfection and expression of EGFP were achieved when a DNA:lipid ratio of 1:8 was used.
The optimal DNA:lipid ratio of 1:8 was then used for further experiments. A comparable result was obtained when samples were measured in parallel on a conventional flow cytometer (table 1).

A comparison of figure 2 and 3 shows that the histogram quality is good and comparable to that of a conventional flow cytometer. On average, 600 to 800 cell events were collected per sample on the bioanalyzer for each sample run on the cell assay chip, whereas 10,000 events were collected using the flow cytometer.
As a result of the transfection optimization, 8 µl of Lipofectamine 2000 was chosen as the optimal amount for transfection of 1 µg of DNA in 6-well culture plates. Figure 4A shows the dot plots of the control and EGFP-transfected cells as generated by the instrument with a DNA:lipid ratio of 1:8. 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 4B (EGFP) and figure 4C (CBNF). To determine the percentage of transfected cells in the histogram view a gate was first set on the CBNF-positive, live cells. This subpopulation was then analyzed in the blue channel by

DNA:lipid ratio	Agilent 2100 bioanalyzer 600-800 cell events	Conventional flow cytometer 10,000 cell events
1:2	5.3%	4.5%
1:4	29.3%	26.4%
1:6	35.9%	35.1%
1:8	40.9%	39.3%
1:10	39.1%	39.5%

Table 1
Instrument comparison of parallel samples

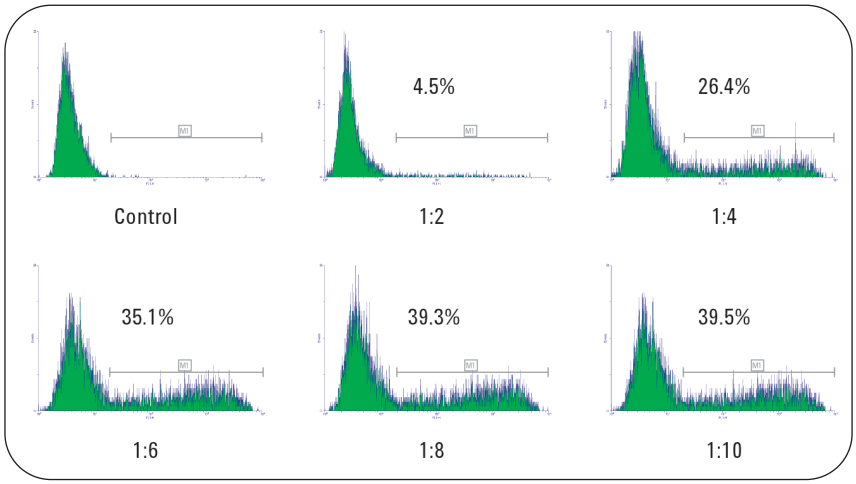

Figure 3
Optimization of transfection conditions for EGFP. CHO-K1 cells were transfected with EGFP using Lipofectamine 2000 and EGFP DNA at various DNA:lipid ratios (1:2 to 1:10) or only with lipofectamine (control). Cells were then stained according to the original protocol and analyzed on a flow cytometer. 10,000 cells were measured per sample.

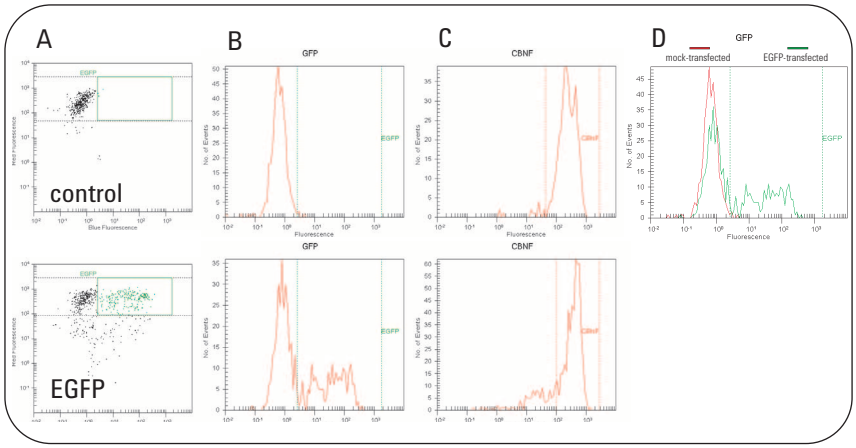

Figure 4
Determination of transfection efficiency. CHO-K1 cells were transfected with EGFP using Lipofectamine 2000 and EGFP DNA at a DNA:lipid ratio of 1:8 (EGFP) or only lipofectamine (control). Cells were then stained on-chip with the live-cell stain CBNF and analyzed on the 2100 bioanalyzer. A) Dot plots of control and EGFP transfected cells. B) GFP histograms of control and EGFP transfected cells. C) CBNF histograms of control and EGFP transfected cells. D) GFP histogram overlays of control (red line) and EGFP transfected sample (green line).

setting a marker on the GFP-positive cells. Figure 4D shows the overlay of the GFP histograms of the control and the transfected sample.

Reproducibility of GFP Transfection Efficiency Assay

To determine the reproducibility of the assay control- or EGFP-transfected CHO-K1 cells of 3 transfection experiments were run on multiple cell assay chips. On each chip, control-transfected cells were loaded in cell well 1 and EGFP-transfected cells in wells 2 to 6. Twenty transfected samples were measured per experiment (4 chips; 5 transfected samples and 1 control each). Figure 5A shows the individual data points of each transfected sample. Chip to chip reproducibility is shown in figure 5B. The transfection efficiency as determined on the 2100 bioanalyzer is practically identical to the result obtained using a conventional flow cytometer.

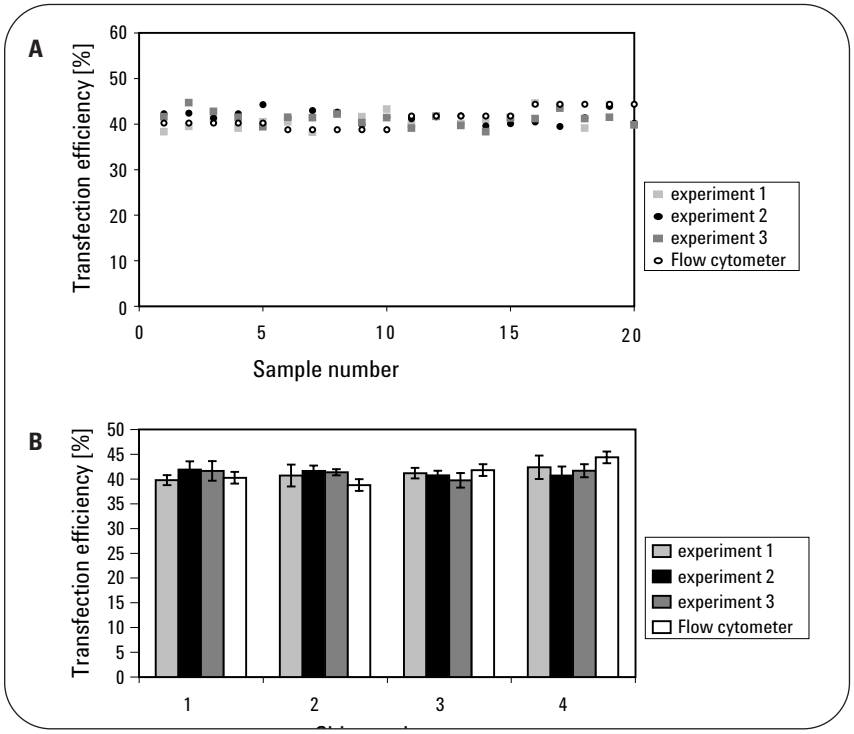

Figure 5
Reproducibility of transfection results obtained with the 2100 bioanalyzer. Comparison of results to data obtained with a standard flow cytometer. A) Plot of individual data points from 3 different transfection experiments with conditions as described for fig. 4. B) Graph of the average percent EGFP-transfected cells obtained per chip, where the transfected cells were loaded in wells 2-6. Error bars indicate standard deviation for each 5 samples. A control sample was loaded in well 1. The samples were run on a total of 12 chips (3 different experiments with 4 chips each).

Steps for handling few cells

For samples with limited cell numbers procedures were developed to guarantee accurate results and to reduce cell loss during sample preparation. For procedure B described below a special concentrated cell buffer (2x CB) is supplied with the cell fluorescence Labchip kit. Any of the three procedures described below may be used according to user preferences when working with limited cell numbers. For all procedures cells were counted and centrifugations were performed at 400xg for 2 min in 0.5 ml Eppendorf vials.

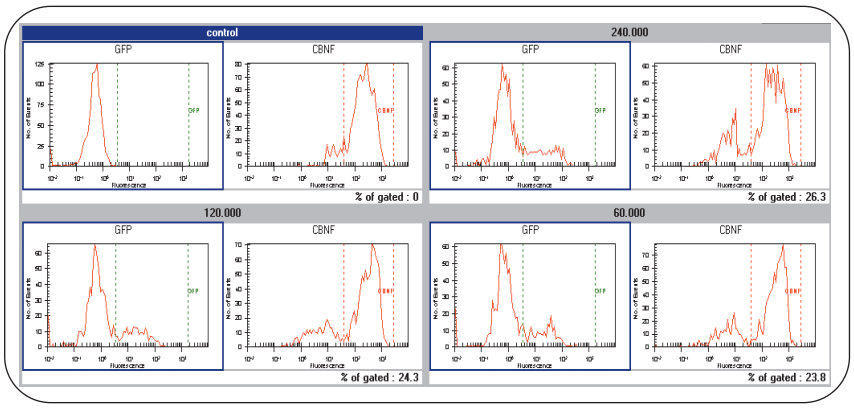

Figure 6
Assay performance when working with few cells. CHO-K1 cells were transfected with EGFP using Lipofectamine 2000 and EGFP DNA at a DNA:lipid ratio of 1:8 or only lipofectamine (control). Cells were then stained on-chip with the live-cell stain CBNF and analyzed on the 2100 bioanalyzer. Between 240,000 and 60,000 cells were used per sample and stained on-chip with CBNF. The data obtained show that 60,000 cells are sufficient to obtain accurate results.

Procedure A
1. Remove medium completely.
2. Carefully resuspend cells at 3×10^6 cells/ml in at least 20 µl CB. Strong vortexing or vigorous pipetting may damage cells.

Procedure B
1. Remove an aliquot of medium to yield 6×10^6 cells/ml.
2. Add remaining volume size of 2x CB to yield 3×10^6 cells/ml.
3. Carefully mix the cells and buffer well. Strong vortexing or vigorous pipetting may damage cells.

Procedure C
1. Remove medium by decanting.
2. Carefully resuspend cells in remaining liquid and measure the volume with a micropipette.
3. Add an equal volume of 2x CB.
4. Adjust cell density to 3×10^6 cells/ml by adding CB.
5. Carefully mix the cells and buffer well. However, strong vortexing or vigorous pipetting may damage cells.

Figure 6 shows results obtained with few cells using protocol B working with the 2x CB buffer. Between 240,000 and 60,000 cells were used per sample and stained according to the on-chip protocol. The data obtained show that the performance of the on-chip protocol is comparable with the performance of the conventional protocol, and that 60,000 cells are sufficient to obtain accurate results. It is important to note, that when working with less than 200,000 cells per sample the pellets after the centrifugation steps are quite small.

Ordering Information
Ordering details are listed in table 2.

Conclusion

We demonstrated that the Agilent 2100 bioanalyzer together with an on-chip staining protocol is a versatile tool to quantitatively monitor the transfection efficiency and expression of GFP in mammalian cells. The complete staining procedure, as well as the analysis of the stained cells is carried out in disposable glass chips, preventing crossover sample contamination and requiring minimal amounts of cells and reagents. Data from the Agilent 2100 bioanalyzer compares well with that of a conventional flow cytometer in spite of a 5-fold lower cell consumption and significantly less sample preparation and hands-on time. Figure 7 compares the workflow of the conventional CBNF-staining approach of GFP transfected cells with the new on-chip staining. Importantly, the on-chip protocol saves 20 minutes hands-on-time per staining experiment, excluding two wash steps with the inherent cell loss. In addition, when working with the suggested protocols and steps for handling few cells with the new 2xCB buffer, 60,000 cells per sample are sufficient to perform on-chip staining.

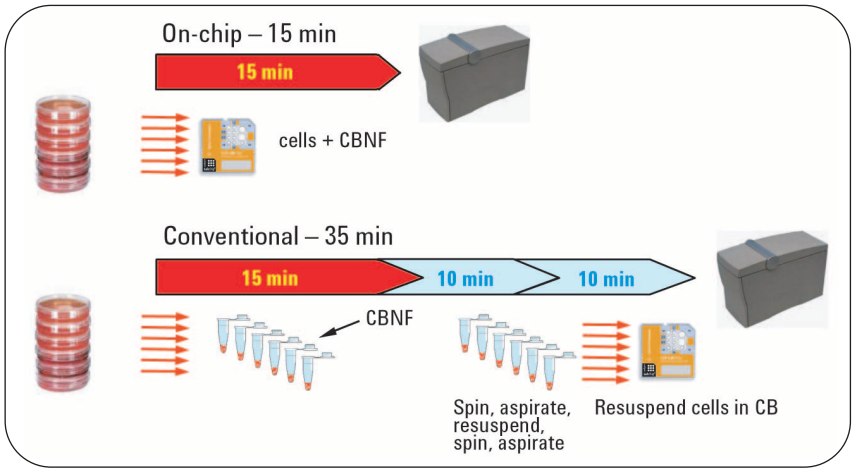

Figure 7
Comparison of workflow between conventional CBNF staining and new on-chip staining procedure.

Description	Order	
On-chip staining		
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
Agilent 2100 bioanalyzer & cell fluorescence labchip kit		
Agilent website	www.agilent.com/chem/labonachip	

Table 2
Ordering details

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Related Application Notes

- "Apoptosis detection by annexin V and active caspase 3 with the Agilent 2100 bioanalyzer" (Agilent publication number 5988-4319EN)
- Monitoring transfection efficiency by green fluorescent protein (GFP) detection with the Agilent 2100 bioanalyzer (Agilent publication number 5988-4320EN)
- Detecting cell surface and intracellular proteins with the Agilent 2100 bioanalyzer by antibody staining (Agilent publication number 5988-4322EN)
- Detecting cell surface proteins with the Agilent 2100 bioanalyzer by on-chip antibody staining. A rapid and accurate method to detect protein expression of B7-1 and B7-2 by on-chip antibody staining (Agilent publication number 5988-7111EN)
- A fast protocol for Apoptosis detection by annexin V with the Agilent 2100 bioanalyzer (Agilent publication number 5988-7297EN)

All application notes for the Agilent 2100 bioanalyzer are available from our web library at: www.agilent.com/chem/labonachip.

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