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

Application

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Abstract

This Application Note describes a new protocol for detecting cell surface protein targets with the Agilent 2100 bioanalyzer and the cell fluorescence LabChip® kit. The specific advantage of an on-chip staining reaction is the amount of time, cells and reagents saved. CD3 on-chip staining on Jurkat cells as well as CD80 (B7-1) and CD86 (B7-2) on-chip staining on 293 cells stably transfected with a CD80 or CD86 expression plasmid were performed. The histogram quality and the percentage of stained cells counted with the microfluidic system are in good agreement with data obtained using a conventional flow cytometer. Detailed protocols and reagent recommendations for on-chip staining of cell surface proteins are provided. The new procedure requires 17 fold less cells per sample analysis compared to the conventional staining procedure, while reducing antibody reagent costs by a factor of 80. The high reproducibility of the chip results, very low cell and reagent consumption as well as speed and ease-of-use are advantages the Agilent 2100 bioanalyzer offers for monitoring cell surface protein targets by on-chip antibody staining.

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]. The Agilent 2100 bioanalyzer system is capable of two-color fluorescence detection and runs disposable microfluidic glass chips. Most recently a set of applications has been presented based on the controlled movement of cells by pressure-driven flow inside the interconnected networks of microfluidic channels. Cells are hydrodynamically focused in these channels before passing the 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 5988-4320EN)
- "Detecting cell surface and intra cellular proteins with the Agilent 2100 bioanalyzer by antibody staining" (Agilent publication number 5988-4322EN)

This Application Note describes on-chip antibody staining of extracellular protein targets.

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.

Antibody staining

Monitoring cellular protein expression is a critical step for quality control, characterization of cell populations or optimization of functional cell assays. It can be achieved by staining the protein of interest with a specific antibody. These specific reagents can either be labeled directly with a fluorescent dye or they can be detected with a secondary fluorescent reagent that specifically binds to the Fc-region of the primary antibody.

As the Agilent 2100 bioanalyzer has the highest sensitivity in the red, with an excitation wavelength of 635 nm and a detection wavelength of 685 nm, allophycocyanine (APC) or Cy5® can be used as a dye. Counterstaining the cells with the live cell dye calcein allows the discrimination between live and dead cells. Staining cell surface proteins with antibodies may introduce artifacts that should be eliminated by special experimental procedures and by carefully and critically examining the results obtained. On the one hand, crosslinking of several receptor molecules by bivalent antibodies may lead to receptor internalization, where the receptor becomes internalized together with the bound antibodies as described for endothelial growth factor (EGF)-Receptor [4]. On the other hand, the extracellular part of the receptor molecule may be shed from the cell surface, as described for CD44 [5, 6]. As these effects require membrane alterations, they are prevented if the cells are fixed or kept continuously on ice and in a buffer containing low amounts of sodium azide.

B7-1 (CD80) and B7-2 (CD86) proteins are important players in the induction of an immune response.
In mammals T-cells are activated if they encounter antigen presenting cells (APCs) carrying an appropriate processed antigen presented by the major histocompatibility complex (MHC) molecules. Upon ligation of the T-cell receptor (TCR) with the loaded MHC a signal is transmitted via the CD3 molecules associated with the TCR. Interactions between TCRs and MHC involving the CD3 signal cascade are generally not sufficient to initiate an immune response. Effective regulation of this response requires a number of antigen-independent co-stimulatory signals[7], the most important of which are provided by the related T-cell surface molecules, CTLA-4 and CD28[8]. The respective ligands, B7-1 (CD80) and B7-2 (CD86) are expressed on certain APCs[9] and provide T-cell co-stimulatory signals through ligation with CD28. Failure to receive a co-stimulatory signal after antigen presentation induces a state of anergy in T-cells. Once activated, T-cells express an additional receptor, CTLA-4, which binds B7 molecules with high affinity. The B7-CTLA-4 pair activates the T helper 1 (Th1) and Th2 pathways of T-cell maturation. The temporal expression of B7-1 and B7-2, and where they are expressed, can greatly affect the development and progression of the immune response, shifting the relative strengths of the Th1 and Th2 pathways in healthy and autoimmune responses[10]. Studies indicate that CTLA-4 both disrupts stimulatory signaling complexes by competing with CD28 for binding the B7 isoforms, and promotes the assembly of inhibitory signaling complexes[11]. Interestingly, expression of the B7-1 and B7-2 proteins can be induced by lipopolysaccharide.

The Toll Like Receptors (TLRs) are the mammalian homologues of the Drosophila Toll transmembrane receptor and represent an ancient well conserved host defense mechanism[12]. TLRs have been shown to orchestrate the recognition of pathogens by the innate immune system. Bacterial components cause an intracellular signaling cascade via multiple TLRs that leads to activation of NF-kappaB and c-Jun N-terminal kinase (JNK), which in turn, initiate the transcription of pro-inflammatory cytokine genes and finally regulate the expression of B7 proteins[13]. TLRs and the downstream signaling pathway play an important role in linking the innate immune recognition with the subsequent activation of adaptive immunity[14].

Experimental

The Agilent 2100 bioanalyzer and cell fluorescence LabChip kit were obtained from Agilent Technologies GmbH (Waldbronn, Germany). Detection of antibody-stained cells was performed on the Agilent 2100 bioanalyzer in combination with the cell fluorescence LabChip kit and the cell fluorescence software. The kit includes 25 chips and reagents required to perform the analysis. Cell samples were stained on-chip in an isobuoyant cell buffer and measured on the Agilent 2100 bioanalyzer with the cell assay extension installed. Data acquisition was performed by an intuitive software package that eliminates the need to manually set instrument specific parameters. Ordering information for reagents is listed in table 1 on page 10.

Results

1. On-chip extracellular CD80/ CD86 staining of 293 cells

Determination of optimal antibody concentration

Determination of the optimal antibody concentration for an antibody staining is a tedious and time-consuming step as it requires staining of several samples with different concentrations of antibodies. While antibody concentrations that are too high will increase unspecific signals, antibody concentrations that are too low will result in low staining intensities. This optimization procedure is greatly simplified by on-chip staining. 10 µl of cell suspension with a cell density adjusted to 3×10^6 cells/ml in cell buffer were incubated together with 2 µl Calcein-AM (1:50 in CB) and 2 µl prediluted antibody in CB (1:10-1:80 final dilution) directly in the wells of a cell chip. Mixing was achieved by vortexing the loaded chip for 1 minute on an IKA vortexer (as supplied with the Agilent 2100 bioanalyzer) with speed adjusted to 12 o'clock position as described in the kit guide. After an incubation time of 25 minutes at room temperature in the dark the chip was vortexed again and measured in the Agilent 2100 bioanalyzer. During incubation chips can either be stored in a humidified chamber or stacked with a

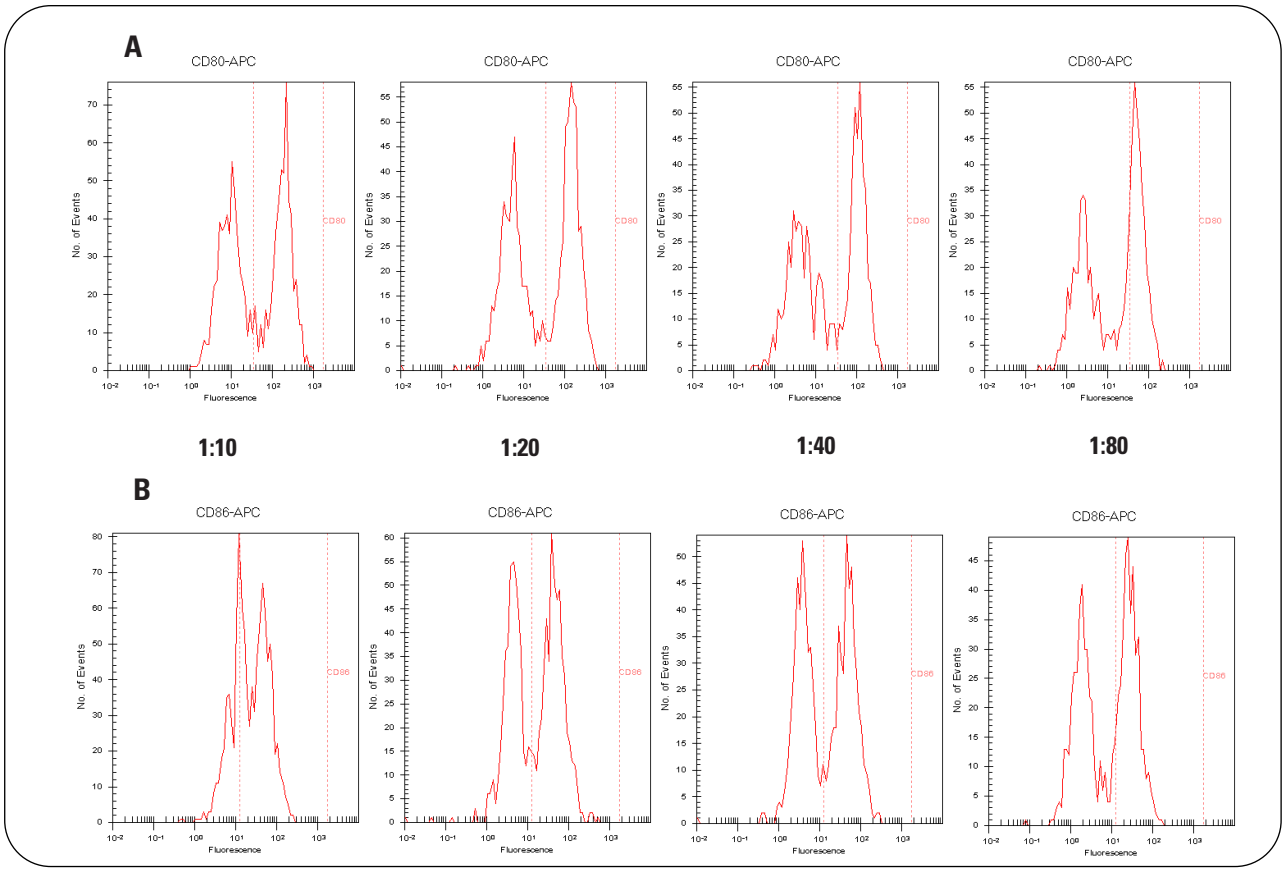


Figure 1

293 clones stably transfected with B7-1 (CD80) or B7-2 (CD86), designated CD80-D2 or CD86-V6 respectively were washed and harvested. Cell density was adjusted to 3×10^6 cells/ml in cell buffer. Cells were stained on-chip with antibodies diluted 1:10 to 1:80 (final dilution). After an incubation time of 25 min at room temperature in a humified chamber the chip was measured in the Agilent 2100 bioanalyzer. Only histograms of the red channel are displayed. All samples were gated on Calcein positive live population.

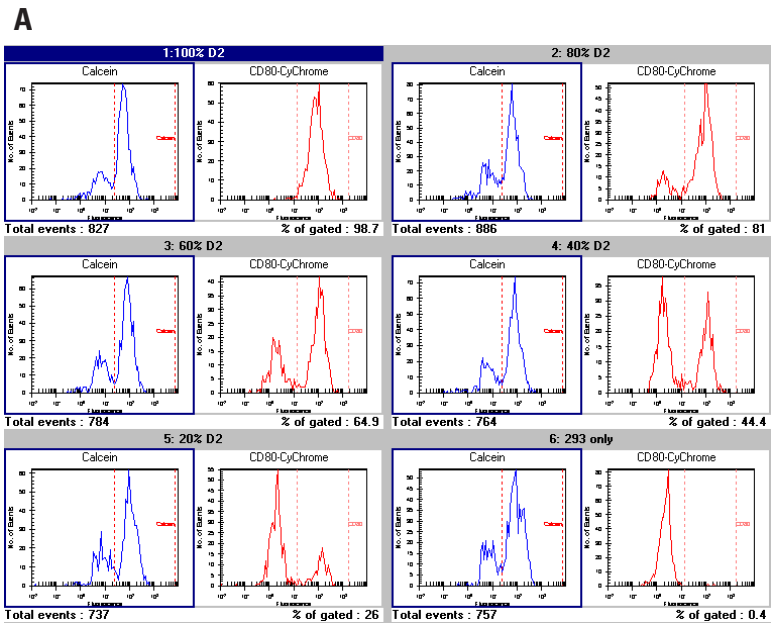
A) On-chip staining optimization using CD80-D2 cells expressing B7-1 and hCD80-APC antibody.

B) On-chip staining optimization using CD86-V6 cells expressing B7-2 and hCD86-APC antibody.

used or unused chip on top to avoid excessive evaporation. As shown in figure 1 concentrations were varied between 1:10 and 1:80 in an on-chip staining experiment. As expected, upon reducing antibody concentrations the unspecific signal (left histogram peak) decreases, however, the specific signal (right histogram peak) only decreases slightly down to the optimal antibody concentration. Upon reducing the antibody concentration further, the specific signal also begins to decrease. From these experiments an antibody dilution of 1:40 was selected as the optimal dilution for further experiments. Optimization was performed in one chip and the time to result was approximately 60 minutes.

Performance of the assay

To test the performance of the assay, mixtures of non-transfected parental 293 cells and 293 cell lines stably transfected with expression plasmids coding for the B7-1 (clone D2) and B7-2 (clone V6) proteins were prepared at different ratios. Cell density was adjusted to 3×10^6 cells/ml in the isoboyant cell buffer. Cell mixtures were stained on-chip as described above. Figures 2 and 3 show representative histograms of calcein- and antibody-stained cells. The data compare well to data generated with the same cell samples stained by a classic staining approach and analyzed on a conventional flow cytometer[15].



Sample	% of Gated	Sample	% of Gated	Sample	% of Gated
1:100% D2	98.7	3: 60% D2	64.9	5: 20% D2	26
2: 80% D2	81.0	4: 40% D2	44.4	6: 293 only	0.4

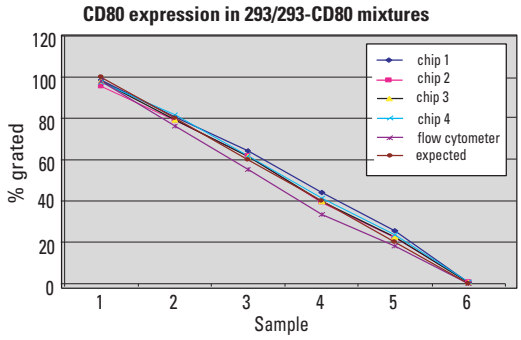


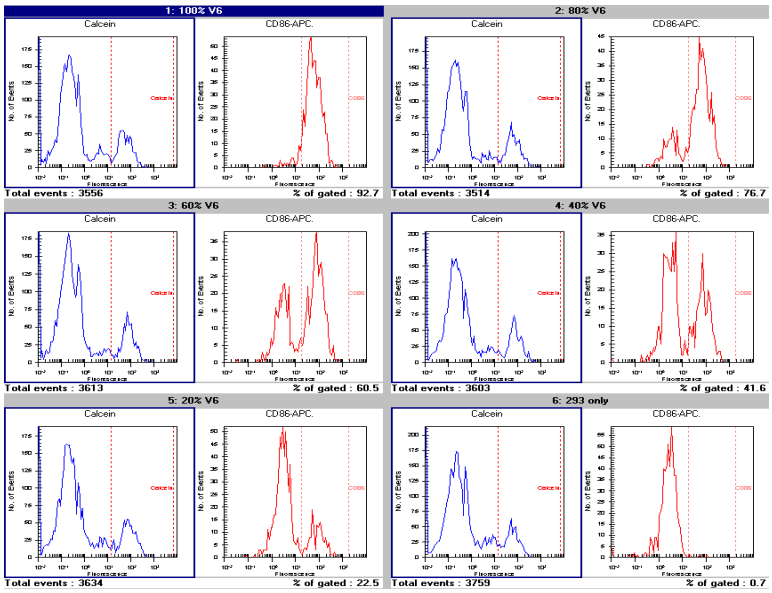
Figure 2

293 cells and the clone CD80-D2 expressing B7-1 were harvested, washed and cell density was adjusted to 3x10^6 cells/ml in an isobuoyant cell buffer. Mixtures of the parental 293 cell line and the transfectants were prepared at different ratios. 10 µl of cell suspension were incubated together with Calcein-AM and anti hCD80-CY Chrome antibody (prediluted 1:8 in CB) directly in the wells of a cell chip as described.

A) Representative histograms of calcein and antibody stained cells.

B) Comparison of on-chip staining results in 4 different experiments with the data obtained with conventionally stained samples[15] measured on a flow cytometer. Data are in very good agreement with the data obtained by standard techniques and the theoretical prediction.

A



Sample	% of Gated	Sample	% of Gated	Sample	% of Gated
1:100% V6	92.7	3: 60% V6	60.5	5: 20% V6	22.5
2: 80% V6	76.7	4: 40% V6	41.6	6: 293 only	0.7

B

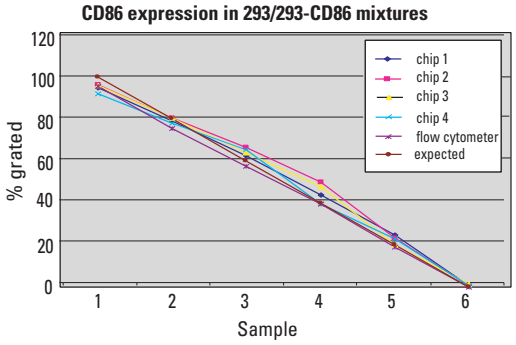
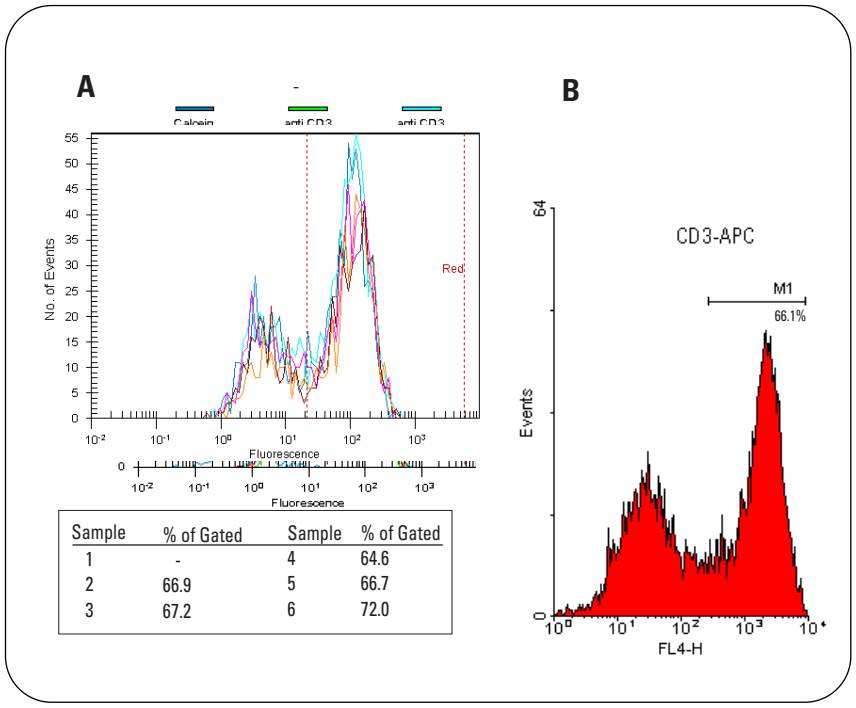


Figure 3
293 cells and the clone CD86-V6 expressing B7-2 were harvested, washed and cell density was adjusted to 3 × 10^6 cells/ml in an isobuoyant cell buffer. Mixtures of the parental 293 cell line and the transfectants were prepared at different ratios. 10 µl of cell suspension were incubated together with Calcein-AM and anti hCD86-APC antibody (prediluted 1:8 in CB) directly in the wells of a cell chip.
A) Representative histograms of calcein and antibody stained cells.
B) Comparison between on-chip staining results in 4 different experiments with the data obtained with classically stained samples[15] measured on a conventional flow cytometer. Data are in very good agreement with the data obtained by standard techniques and the theoretical prediction.

2. On-chip extracellular CD3 staining of Jurkat Cells

To verify the performance of the on-chip staining procedure with a different cell line growing in suspension, an on-chip CD3 staining of Jurkat cells was performed. Jurkat cells were harvested and washed and cell density was adjusted to 3×10^6 cells/ml in an isobuoyant cell buffer. 10 µl of cell suspension were incubated together with 2 µl Calcein-AM (1:50 in CB) and 2 µl anti hCD3-APC antibody (prediluted 1:5.5 in CB) directly in the wells of a cell chip as described before (optimization of antibody concentration not shown). Figure 4 shows representative histograms of Calcein and antibody-stained cells. The data closely match data generated with the same samples on a conventional flow cytometer by conventional staining, as described previously[15].



Sample	% of Gated	Sample	% of Gated
1	-	4	64.6
2	66.9	5	66.7
3	67.2	6	72.0

Figure 4
Performance and reproducibility of on-chip antibody staining. Jurkat cells were harvested and washed. Cell density was adjusted to 3×10^6 cells/ml in cell buffer. Cells were stained on-chip with anti hCD3-APC prediluted 1:5.5 in CB and Calcein (1:50 in CB). After an incubation time of 25 minutes the chip was measured in the Agilent 2100 bioanalyzer.
A) Overlay of representative histograms from one chip of calcein and antibody stained cells.
B) Comparison between on-chip staining data and data obtained by measuring cells stained by conventional staining on a flow cytometer.

Working with few cells

Since the on-chip staining procedure might prove especially useful when working with limited amounts of cells and reagents we developed the following steps to facilitate working with few cells. 293 cells and cells of the clone CD80-D2 were trypsinized, harvested and washed, and a 1:1 mixture of the cells was prepared. 1,000,000 down to 15,000 cells were placed into vials and resuspended in 2 × CB according to the steps for handling few cells as described on page 9 (procedure B). On-chip staining was performed as described on page 9. Briefly, 10 µl of cell suspension were incubated together with 2 µl Calcein-AM (diluted 1:50 in CB) and 2 µl antibody (diluted 1:8 in CB) directly in the wells of a cell chip. Figure 5 shows representative histograms of Calcein-positive and antibody-stained cells. The results compare very well with the expected values. The low amount of expensive antibody needed for on chip staining significantly reduces reagent costs. A typical fluorescently labeled antibody may cost about $400 for 2 ml (100 tests, 20 µl each). This translates to $4/sample. With on-chip staining the same volume is good for 8000 tests (2 µl of a 1:8 dilution each), which reduces the reagent cost by a factor of 80 to 5 cent per sample.

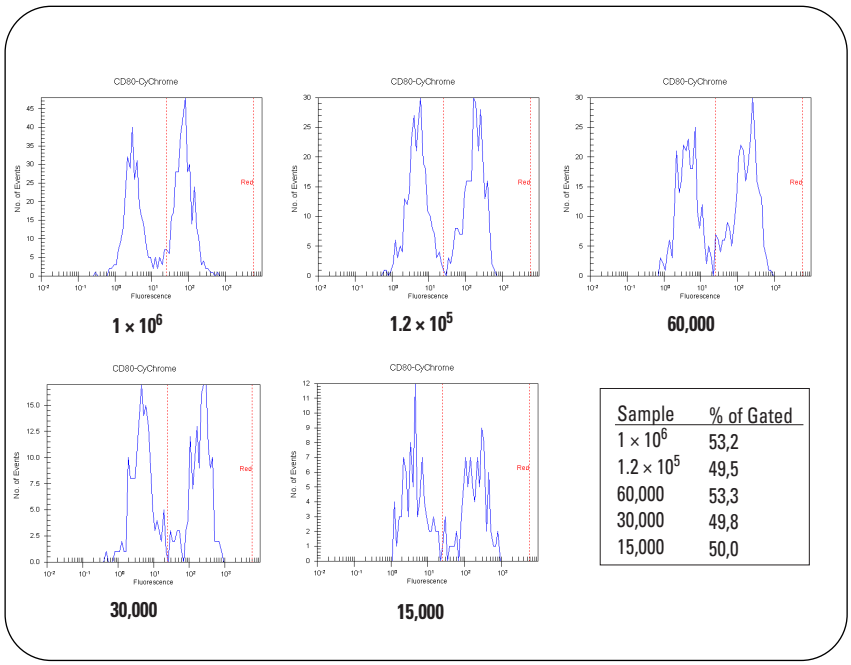


Figure 5
Assay performance when working with few cells. Parental 293 cells and 293 cells stably transfected with expression plasmids coding for the B7-1 (clone D2) protein were prepared. Cells were trypsinized, washed and a 1:1 mixture of the cells was prepared. 1,000,000 down to 15,000 cells were placed into vials and resuspended in 2×CB according to the steps for handling few cells as described on page 9 (procedure B). On-chip staining was performed as described on page 9. Even when using 15,000 cells, the results are not distinguishable from the results obtained with 1,000,000 cells.

Material & Methods

On-chip staining of 293 cells transfected with B7-1 (CD80) or B7-2 (CD86)

Reagents

- anti hCD80 CyChrome antibody
- anti hCD86 APC antibody
- anti hCD3-APC
- Calcein-AM (diluting the original stock with DMSO to yield a 500 µM solution)
- 293 cells and clones CD80-D2 and CD86-V6, expressing B7-1 and B7-2, respectively (Cells were kindly provided by Dr. M. Sester, University of Homburg, Germany.)
- Jurkat cells were purchased from ATCC (Manassas, USA)

On-chip staining protocol

1. Harvest cells and adjust cell density to 3 × 10^6 cells/ml in CB. Adherent cells should be trypsinized properly.
2. Prime chip with 10 µl PS and wait for 1 minute.
3. Add 10 µl FD to the focussing dye well.
4. Place 30 µl CB in the buffer wells.

5. Place 10 µl cell suspension into each sample well.
6. Add 2 µl of a 1:50 dilution of calcein in CB (final calcein concentration 1.4 µM).
7. Add 2 µl of the diluted antibody (prediluted 1:8 for CD80/CD86 or 1:5.5 for CD3 in CB) to the sample wells**.
8. Vortex chip for 1 min on an IKA vortexer (as supplied with the 2100 Agilent bioanalyzer) with speed adjusted to 12 o'clock position as described in the kit guide.
9. Incubate for 25 min at RT* in a humidified chamber. Alternatively chips may be stacked using an old or unused chip on top to prevent excessive evaporation.
10. Vortex chip again for 1 min as described in step 8
11. Load and run chip.

*At 4°C if antigen is shed or internalized upon antibody binding.

** If several samples are to be stained with the same antibody, calcein (Step 6) and antibody (Step 7) may be pre-mixed before adding reagents to sample wells.

Steps for handling few cells

Any of the three procedures below may be used according to user preferences. For all procedures cells were counted and centrifugations were performed at 400 × g for 2 min in 0.5 ml Eppendorf vials.

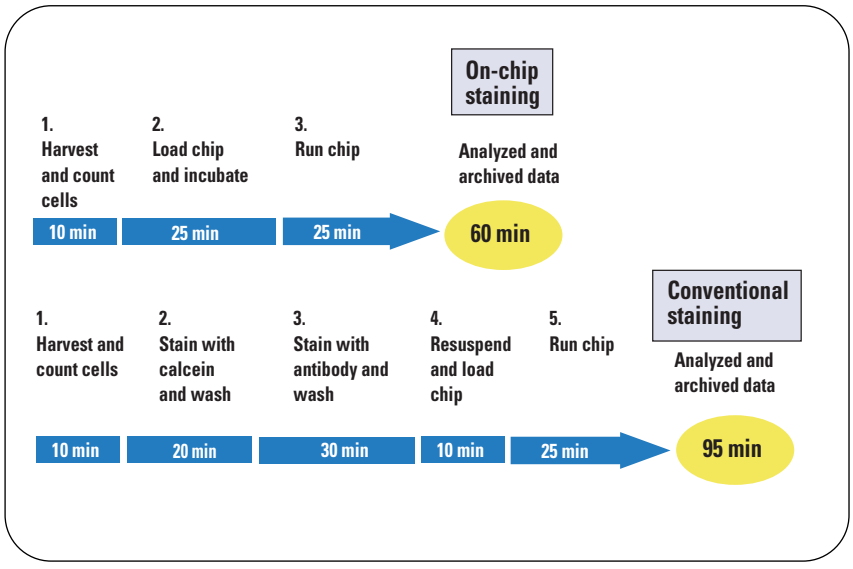


Figure 6
Comparison of the time to result for on-chip staining and the conventional staining procedure. As the on-chip staining procedure does not require washing and centrifugation steps and only small volumes, approximately 35 minutes are saved per staining experiment.

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 $2 \times$ 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 $2 \times$ 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.

Ordering Information

Ordering information for extracellular antibody staining and Agilent 2100 bioanalyzer and cell fluorescence Labchip kit is available in table 1.

Description	Order
Extracellular antibody staining	
Calcein-AM:	Molecular Probes, part number C-3099
Anti-hu-CD3-APC Antibody	Pharmingen, part number 30119X
Anti-hu-CD80-CY chrome Antibody	Pharmingen. part number 559370
Anti-hu-CD86-APC Antibody	Pharmingen, part number 555660
Agilent 2100 bioanalyzer and cell fluorescence LabChip kit	
Agilent website	www.agilent.com/chem/labonachip

Table 1
Ordering details

Conclusion

Monitoring cellular expression of protein targets is a critical step for quality control, characterization of cell populations or assay optimization. The procedure for staining of cells, however, is time-consuming and requires considerable hands-on time, which is especially due to multiple washing steps within the standard protocols. Here, we demonstrated that the Agilent 2100 bioanalyzer together with an easy to follow on-chip staining protocol is a versatile tool to detect expression levels of surface proteins. The complete staining procedure, as well as the analysis of the stained cells is carried out in disposable glass chips, preventing cross-over sample contamination and requiring minimal amounts of cells and reagents. This makes the protocols described here especially useful when time or number of cells are limited.

Protocols and a list of recommended reagents for detection of cell surface proteins are given.

Excellent reproducibility of results from different chips is demonstrated. Data from the Agilent 2100 bioanalyzer compares well with that of a conventional flow cytometer in spite of a lower cell consumption and significantly less sample preparation and hands-on time (figure 6). When working with the suggested protocols and steps, 60,000 cells are well sufficient to perform an on-chip staining. Figure 5 shows that performance with 15,000 cells is still very good. Importantly, on chip staining with antibodies greatly reduces reagent costs (80fold less antibody was needed in the experiments described). Data acquisition is done automatically and data analysis is done by an intuitive software package, which does not require manual setting of instrument related parameters.

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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)
- "Detection of antibody-stained cell surface and intracellular protein targets with the Agilent 2100 bioanalyzer" (Agilent publication number 5988-4322EN)
- "A fast protocol for apoptosis detection with the Agilent 2100 bioanalyzer" (Agilent publication number 5988-7297EN)
- "Monitoring transfection efficiency by on-chip staining of green fluorescent protein (GFP) with the Agilent 2100 bioanalyzer" (Agilent publication number 5988-7296EN)

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

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Published August 1, 2002
Publication Number 5988-7111EN

Agilent Technologies