Detection of antibody-stained cell surface and intracellular protein targets 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 detect cell surface and intracellular protein targets by antibody staining. CD3 staining on Jurkat cells as well as CD4 staining on CCRF-CEM cells and intracellular glucocorticoid receptor (GR) staining of H4 rat hepatocytes were performed. Histogram quality and the percentage of stained cells counted with the microfluidic system are in good agreement with data obtained with a conventional flow cytometer. Detailed protocols and reagent recommendations for staining cell surface and intracellular protein targets are given. The high reproducibility of the chip results, low cell consumption and ease-of-use are advantages of the compact bioanalyzer system for monitoring cell surface and intracellular protein target expression by antibody staining.

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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 applications presented here are 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 up to six samples and data acquisition of all samples is fully automated while data 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.

Antibody staining

Monitoring cellular protein expression is a critical step for quality control and characterization of cell populations or assay optimization and can be achieved by staining the protein of interest with a specific antibody. These specific reagents can either be directly labeled with a fluorescent probe or they can be detected with a secondary fluorescent reagent. An antibody is defined as "an immunoglobulin capable of specific combination with the antigen that caused its production in a susceptible animal" and usually consists of a pair of two light and two heavy amino acid chains. They are produced in response to the invasion of foreign molecules in the body. Antibodies can be divided into five subclasses based on their heavy chain: IgG, IgM, IgA, IgD and IgE. The most commonly used antibody for specific protein detection is IgG, which can be cleaved by the protease papain into three parts, two F(ab) regions and one Fc, or into two parts, one $F(ab')_2$ and one Fc by the proteolytic enzyme pepsin. The F(ab) regions comprise the "arms" of the antibody, which are critical for antigen binding. The Fc region comprises the "tail" of the antibody and plays a role in immune response.

Cells carry antigens (lipids, proteins or carbohydrates) which can be detected with specific antibodies. This can be achieved by treating cells with specific antibodies recognizing, for example, the protein of interest. The detection antibody may be directly conjugated to a fluorescent probe, for example allophycocyanine (APC), or a fluorescently-labeled secondary antibody may be used which specifically binds to the Fc-part of the primary antibody. The application of antibody staining is not restricted to the use of antigens that are expressed on the cell surface. Following fixation and permeabilization of cells, intracellular antigens can be easily detected by antibody staining.

Detection of proteins generally depends on the availability of a suitable antibody, many of which are commercially available in a fluorescently conjugated form. 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, APC, or Cy5® can be used as dyes. Counterstaining the cells with the live cell dye calcein allows the discrimination between live and dead antibody stained cells. In contrast, the measurement of intracellular proteins requires permeabilization and fixation of cells prior to antibody labeling. Therefore, staining with Calcein is not advisable. In this case cells can be counterstained with the nucleic acid-specific-dye SYTO16®.

Staining of 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 [1]. On the other hand, the extracellular part of the receptor molecule may be shed from the cell surface, as described for CD44[2, 3]. 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.

Experimental

The Agilent 2100 bioanalyzer and Cell Assay Extension were obtained from Agilent Technologies Deutschland 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. Stained cell samples were resuspended in an isobuoyant cell buffer at 2 x 10^6 cells/ml and loaded onto the chips as described in the reagent kit guide. Data acquisition was performed using the intuitive software package supplied with the Cell Assay Extension with no requirement to manually set instrument-specific parameters.

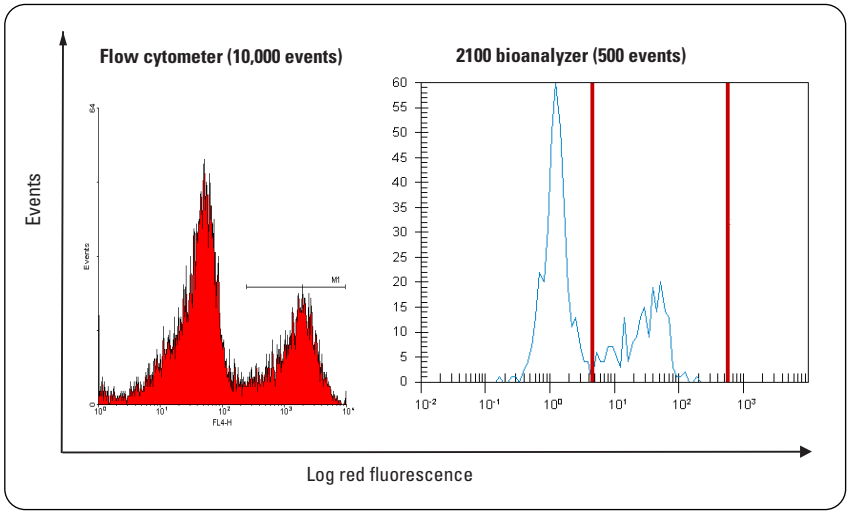

Figure 1
Comparison of histogram quality from the 2100 bioanalyzer and a conventional flow cytometer. Using the 2100 bioanalyzer, a gate was set on the calcein-positive cells to determine the percentage of antibody-stained cells within the live population. Jurkat cells were stained with 1 µM calcein alone or with 1 µM calcein and APC-labeled anti hCD3-antibody. After washing a mixture of both populations was prepared and measured with both instruments.

Results

a) Extracellular CD3 staining of Jurkat cells

Jurkat cells, a cell line derived from an acute human T-cell leukemia, were harvested and washed. After staining with calcein, the cells were incubated with APC-labeled anti hCD3-antibody. After washing and resuspending the cells in cell buffer the samples were loaded onto the chip and measured in the Agilent 2100 bioanalyzer. Figure 1 shows representative histograms of a mixture of calcein-only and calcein and antibody treated cells. The data obtained with the 2100 bioanalyzer are of comparable quality as data generated with the same samples on a conventional flow cytometer. Figure 2 shows the reproducibility of the measurement over several chips and compares the data obtained from the Agilent 2100 bioanalyzer with flow cytometer measurements.

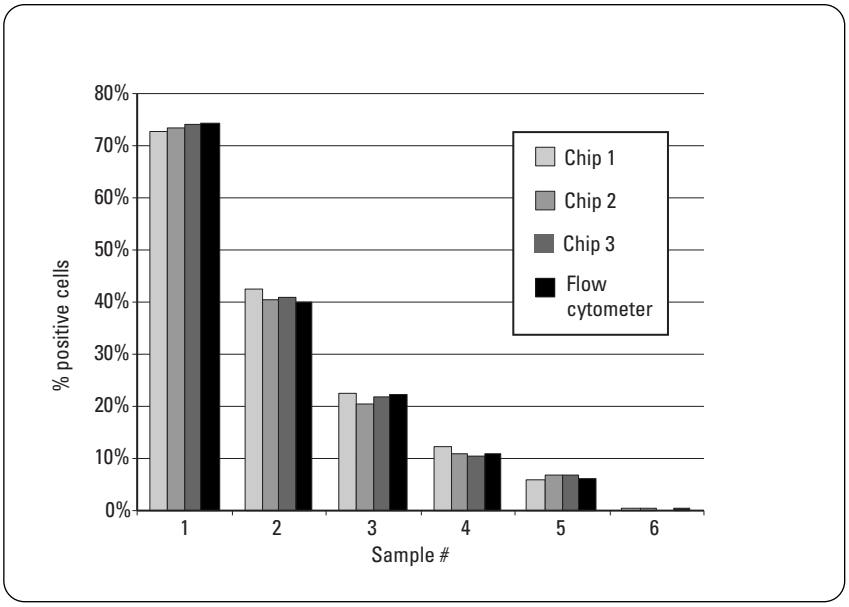

Figure 2
Reproducibility of data from the 2100 bioanalyzer and a conventional flow cytometer. A gate was set on the calcein-positive cells to determine the percentage of antibody-stained cells within the live population. Jurkat cells were stained with 1 µM calcein alone or with 1 µM calcein and APC-labeled anti hCD3-antibody. After washing mixtures of both populations were prepared at various ratios of antibody/calcein and calcein only-stained cells to simulate different percentages of CD3 expressing cells.

b) Extracellular CD4 staining of CCRF-CEM cells

CCRF-CEM cells, a cell line derived from an acute human lymphoblastic leukemia, were harvested and washed. After staining with calcein, the cells were incubated with APC-labeled anti hCD4-antibody. After washing and resuspending the cells in cell buffer the samples were loaded onto the chip and measured in the Agilent 2100 bioanalyzer. Figure 3 shows representative histograms of a mixture of calcein-only and calcein and antibody treated cells. These data are of comparable quality as data generated with the same samples on a conventional flow cytometer. Figure 4 shows the reproducibility of the measurement over several chips and compares the 2100 bioanalyzer data with that obtained from the conventional flow cytometer.

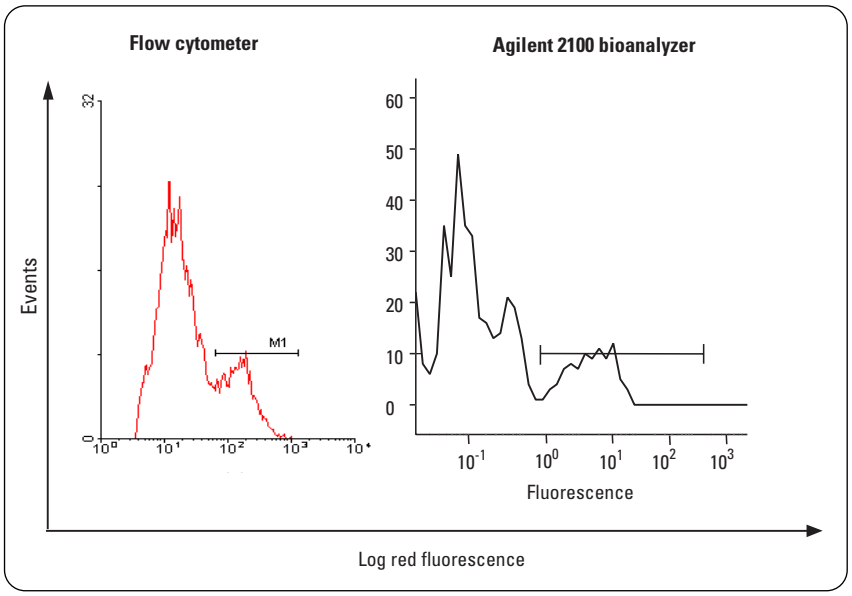

Figure 3
Comparison of histogram quality from the 2100 bioanalyzer and a conventional flow cytometer. A gate was set on the calcein-positive cells to determine the percentage of antibody-stained cells within the live population. CCRF-CEM cells were stained with 1 µM calcein alone or with 1 µM calcein and APC-labeled anti hCD4-antibody. After washing, mixtures of both populations were prepared at various ratios of antibody/calcein and calcein only-stained cells to simulate different percentages of CD4 expressing cells, and measured on both instruments.

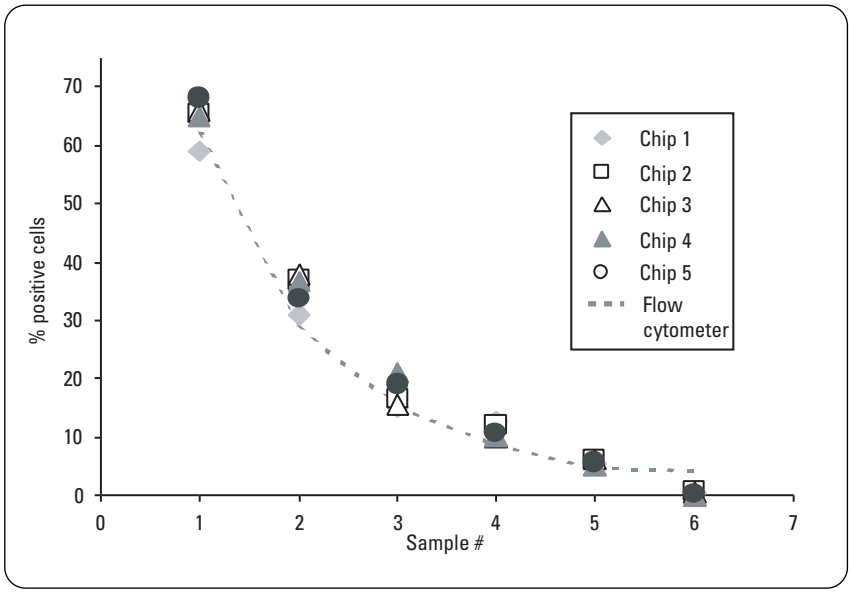

Figure 4
Comparative data from the 2100 bioanalyzer and a conventional flow cytometer. CCRF-CEM cells were stained with 1 µM calcein alone or with 1 µM calcein and APC-labeled anti hCD4-antibody. After washing, mixtures of both populations were prepared at various ratios of antibody/calcein and calcein only-stained cells to simulate different percentages of CD4 expressing cells.

c) Intracellular glucocorticoid receptor (GR) staining of H4 hepatocytes

For intracellular detection of GR proteins H4 rat hepatocytes were harvested and washed. The cells were first stained with SYTO16®, washed again and subsequently treated with a permeabilizing/fixation solution. They were then stained with anti GR antibodies and APC-conjugated secondary antibody or APC-conjugated secondary antibody alone. Mixtures of both populations were prepared at various ratios and measured on the 2100 bioanalyzer (figure 5) and on a conventional flow cytometer. The 2100 bioanalyzer displayed good reproducibility and yielded data comparable to the flow cytometer data (figure 6).

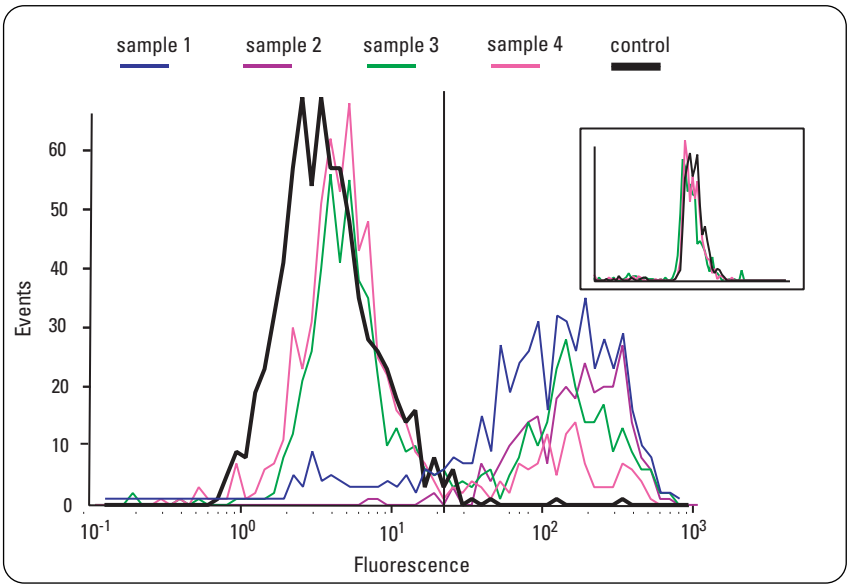

Figure 5
Histogram overlay of samples with differing percentages of GR expression cells. A gate was set on the SYTO16-positive cells to determine the percentage of antibody-stained cells within the DNA-stained population (insert). H4 hepatocytes were harvested and stained with 1.5 µM SYTO16 alone or with 1.5 µM SYTO16 and anti GR antibodies and APC-conjugated secondary antibodies. After extensive washing, mixtures of both populations were prepared at various ratios to simulate different percentages of expression of the GR receptor.

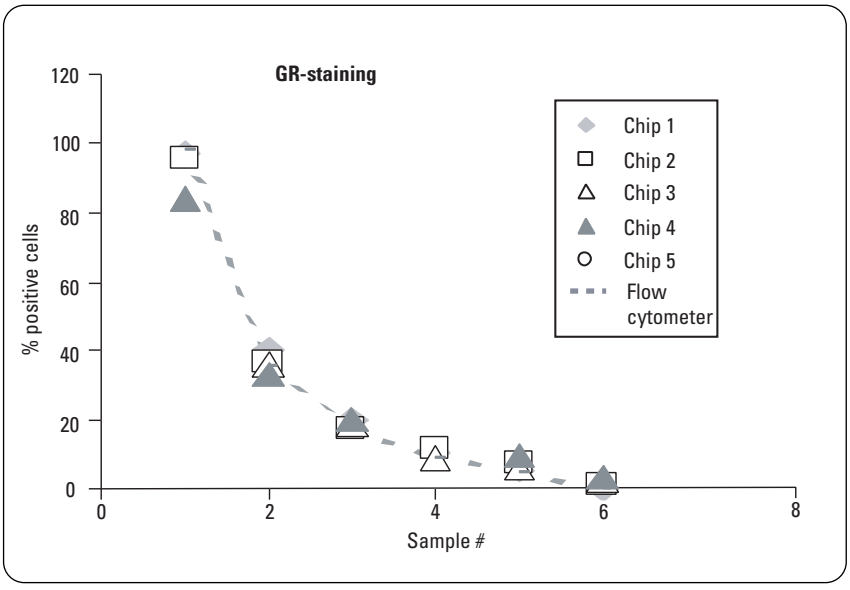

Figure 6
Comparative data measured on the 2100 bioanalyzer and a conventional flow cytometer. Four chips including 0-90% GR expressing cells were analyzed on the 2100 bioanalyzer and a conventional flow cytometer. H4 hepatocytes were harvested and stained with 1.5 µM SYTO16 and APC conjugated secondary antibodies alone or with 1.5 µM SYTO16, anti GR antibodies and APC conjugated secondary antibodies. After extensive washing, mixtures of both populations were prepared at various ratios to simulate different percentages of expression of the GR receptor.

Materials and methods

Ordering information for the reagents used is given in table 1. Cells were cultured in RPMI medium containing 10 % fetal calf serum, penicillin/streptomycine, 1 mM sodium pyruvate and 2 mM L-glutamine

a) Extracellular staining of CD3 or CD4

Reagents

- anti hCD3 APC antibody, anti hCD4 APC antibody
- Calcein-AM (diluting the original stock with DMSO to yield a 500 µM solution)
- Staining buffer (PBS, 2% BSA, 0.05 % NaN_3)
- Dye loading buffer (HBSS, 20 mM HEPES, 1 % BSA)

Protocol

1. Wash cells once in dye loading buffer after harvesting.
2. Stain cells with calcein (up to 5 x 10^6/ml, 0.5 µM calcein, 15 minutes, 37 °C) in dye loading buffer.
3. Wash once in staining buffer.
4. Stain cells (1 x 10^6) in a total volume of 100 µl (80 µl staining buffer and 20 µl antibody) for 25 minutes on ice in the dark. For more cells (up to 5 x 10^6), adjust amount of antibody but don't increase total volume of 100 µl.
5. Wash twice with 1 ml staining buffer.
6. *Optional step if cells are to be stored overnight and measured later:*
   Resuspend cells well at a cell density of 1 x 10^6 cells /ml in PBS. Make sure there are no cell clumps in the suspension.
   Add 70 µl paraformaldehyde (16 %-solution) while stirring. Incubate for 10 minutes at room temperature (RT) and store sample at 4 °C in the dark.
7. Resuspend cells in cell buffer at 2 x 10^6 cells/ml and load onto the chip.

b) Intracellular GR staining of H4 hepatocytes

Reagents

- BD FACS Permeabilizing Solution 2 (500 tests)
  Alternatively, use Triton-X100 and Paraformaldehyde 16 % for fixation and permeabilization.
- Anti rat GR antibody
- Phycoprobe AP Anti-Mouse IgG
- SYTO16
- Staining buffer (PBS, 2 % BSA, 0.05% NaN_3)

Protocol

1. Harvest cells and adjust cell density to 1 x 10^6 cells /ml in PBS.
2. Add 1.5 µl SYTO16/ml and incubate 10 minutes at 37 °C in the dark.
3. Wash cells in 2 ml staining buffer and centrifuge (500 x g, 5 minutes).
4. Resuspend cells in 500 µl 1x BD permeabilizing solution (dilute in distilled water) and incubate 10 minutes at RT.
5. *Two alternative steps, if permeabilization and fixation is done without BD permeabilizing solution 2:*
   Resuspend in 1 ml HBSS.
   Add 125 µl paraformaldehyde (16 %-solution) and incubate 10 minutes on ice.
   Add 75 µl Triton (1 %-solution in distilled water) and incubate 10 minutes on ice.
6. Wash cells in 2 ml staining buffer and centrifuge (500 x g, 5 minutes).
7. Stain cells (1 x 10^6) with GR antibody in a total volume of 100 µl. Add antibody to a final concentration of 15 µg/ml (6 µl ad 100 µl staining buffer) and incubate for 30 minutes at RT. For more cells (up to 5 x 10^6) adjust amount of antibody but do not in-crease total volume of 100 µl.
8. Wash cells in 2 ml staining buffer and centrifuge (500 x g, 5 minutes).
9. Stain cells (1 x 10^6) with secondary antibody (Phycoprobe AP anti-mouse IgG) in a total volume of 100 µl. Add secondary antibody to a final concentration of 15 µg/ml (1.5 µl and 100 µl staining buffer) and incubate for 30 minutes at RT. For more cells (up to 5 x 10^6) adjust amount of antibody but do not increase total volume of 100 µl.
10. Wash cells two times in 2 ml staining buffer and centrifuge (500 x g, 5 minutes).
11. Resuspend at 2 x 10^6/ml in cell buffer and load onto the chip.

Reagent	Supplier	Order No.
Extracellular antibody staining		
Calcein-AM:	Molecular Probes http://www.probes.com	C-3099
Anti hCD3 APC antibody	Pharmingen http://www.pharmingen.com	30119X
Anti hCD3 CY5 antibody	Pharmingen	30118X
Anti hCD4 APC antibody	Pharmingen	30159X
Paraformaldehyde: 16 % Solution EM Grade	Electron Microscopy Sciences http://www.emsdiasum.com	15710-S
Intracellular anti-ratGR staining		
SYTO16:	Molecular Probes http://www.probes.com	S-7578
BD FACS Permeabilizing Solution 2 500 Tests	BD Biosciences http://www.bd.com	340973
Anti rat GR antibody	Affinity Bioreagents http://www.bioreagents.com	MA1-510
Phycoprobe AP Anti Mouse IgG	Biomeda http://www.bioreagents.com	P68

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

Conclusion

Monitoring cellular expression of protein targets is a critical step for quality control, characterization of cell populations or assay optimization. In this Application Note we demonstrated that the Agilent 2100 bioanalyzer is a versatile tool to detect expression levels of surface as well as intracellular protein targets. Protocols and a list of recommended reagents for detection of two cell surface proteins and one intracellular protein are given. Excellent reproducibility of results from different chips is demonstrated. The Agilent 2100 bioanalyzer shows a high sensitivity, as demonstrated by the detection of small subpopulations expressing the target proteins with only 5% of all cells. Data from the 2100 bioanalyzer compares well with that of a conventional flow cytometer in spite of a 5- fold lower cell consumption. Data acquisition is done automatically and data analysis is achieved by an intuitive software package which does not require manual setting of instrument related parameters.

References

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