An automated 96-well-plate loader for the FACScan[®]

Harald Illges *

*University of Konstanz, Faculty of Biology,
Department of Immunology, Konstanz, Germany*

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Staining multiple samples for data acquisition with a flow cytometer is done in 96-well plates to save time and make large data assessment in one working day possible. However, the inability of the FACScan[®] to take up the samples from 96-well plates is a major drawback. In order to avoid the individual transfer of samples to tubes, we have developed a system, which allows using the FACScan[®] with 96-well plates. The machine consists of a programmable control module and a loader which moves the 96-well plate in 3 axis well by well along the sample collector. The machine is equipped with a wash buffer tank to avoid cross contamination of samples and a shaking option to avoid sedimentation of cells during acquisition. The machine can be further developed into a full automatic loader if connected to the FACS Station. In 24 hours about 7,000 samples with 10,000 cells each can be acquired.

1. Introduction

Flow cytometry is a widely used method. With the introduction of commercially available cytometers many researchers developed and used a variety of different methods [1,2]. While the speed of the machines is enhancing quickly the time required for staining and sample collection is still very high. Staining of multiple samples for data acquisition with a FACScan[®] is time-consuming when done in single tubes. Therefore, investigators use 96-well plates for analytical staining. However, for sample collection with the FACScan[®],

cells have to be transferred to single tubes. To avoid this time consuming and sample-limiting step we have constructed a 96-well-plate loader for the FACScan[®]. Mechanic and electronic units are separated in 3 modules to allow for further development and individual set up. A programmable microprocessor allows setting of all parameters and controls the loader. Therefore the loader can be used with any type of multi-well plate. Furthermore, the loader is capable of shaking samples, either for staining or to avoid sedimentation of the cells during sample collection. To avoid cross contamination of samples, the loader is equipped with 2 wash buffer tanks underneath the multi-well-plate holder. The tanks have openings allowing the FACScan[®] sample collector needle to be washed automatically, or operator mediated, after each sample acquisition. We have constructed a simple hand held device to control the progression of sample collection. The microprocessor control module has a serial interphase to be connected to the FACS Station for full automation.

2. Material and methods

2.1. Mechanical equipment

Stepping motors are from Isel, Eiterfeld, Germany (Y- and Z-axis). The stepping motor moving the plate holder (X-axis) was purchased from Bürklin, Düsseldorf, Germany. The X-, Y- and Z-axis motors drive spindles, the plate holder motor drives with a belt the plate for rotation. The wash buffer tanks are made from plastics and were cut at our mechanic workshop. All plates and the holders for the motors are aluminum and cut and put together at our mechanic workshop.

2.2. Electronic parts

The controller was build into a standard electronic box (Schroff, Straubenhardt, Germany). The heart of the controller is a microprocessor unit (80535) pur-

*Correspondence to: Harald Illges, University of Konstanz, Faculty of Biology, Department of Immunology, P.O. Box 5560, M662, 78457 Konstanz, Germany. Tel.: +49 7531 882255; Fax: +49 7531-883102; E-mail: Harald.Illges@uni-konstanz.de; <http://www.immunologie.uni-konstanz.de>.

chased from Rafi, Ravensburg, Germany. An EPROM on the microprocessor was used to program the controller. Programming was done on a PC using C. All materials used were standard parts available from electronic shops. A handheld control module with 1 switch for forwarding samples, one switch to change to programming mode and 2 switches to move within different modes and programming steps was connected to the controller to save space and make use of the loader more easy. The microprocessor unit contains a serial interphase and the hardware for the optional use with the FACS Station computer.

2.3. Reagents

Standard round bottom 96-well-plates were from Greiner, Germany. Sample collection and washing buffer: For both, a single buffer containing PBS, 2% fetal calf serum and 0.01% azid was used. Staining, antibodies, and cells: anti-CD21 monoclonal antibody BU33-FITC was purchased from Harlan Sera-Lab (UK) and Dako-CD19-PE from Dakopatts (Denmark).

2.4. Sample preparation, flow cytometry

Purification of cells and staining were performed as described elsewhere [3]. In brief, human PBMC from buffy coats were purified by Ficoll-gradient centrifugation at 2300 rpm in a Heraeus minifuge (Heraeus, Osterode, Germany) for 15 min without brake. After washing $1-3 \times 10^5$ cells/well were transferred into 96-well-plates and stained with fluorochrom conjugated monoclonal antibodies for 20 min on ice. Cells were washed again, resuspended in sample collection buffer with Propidium Iodide and analyzed using a FACScan® and Cell-Quest software (Becton Dickinson, Mountain View, USA).

3. Results

3.1. Mechanic module of the loader

The mechanic module (Fig. 1a) is placed beside the FACScan®. Three stepping motors were used to move the 96-well-plate holder, each separately controllable by the microprocessor unit (Figs 1, 2). Because the space around the sample collection needle is very small we have replaced part of the FACScan® box with an easily exchangeable metal plate for standard use allow-

ing the plate holder to move into the box. The Calibur version of the FACScan has enough space around the sample needle to avoid this. In order to reach all wells of the 96-well plate a belt driven motor was introduced. The motor is built into the plate holder. The FACScan® switch indicating that a sample is inserted is replaced in our setup by a switch which is turned on by the controller whenever the plate holder reaches certain positions defined by the *X*-, *Y*- and *Z*-axis. We have constructed a tube with a rubber ring for sample acquisition with 96-well plates. This tube is pressed onto the holes of the 96-well-plate by movement of the plate holder allowing soft but closed touch such that the sample can be acquired with minimum pressure on the plate holder and the FACScan® needle. The loader needs about 5 min to move to all 96 wells and about 12 min if a washing step is introduced between the acquisition steps. Time for the actual sample acquisition is of course dependent on the cell concentration supplied by the operator. At a flow rate of 2000 cells/sec and 10,000 cells to be acquired a whole 96-well plate can be scanned in about 20 min.

3.2. Electronic controller module

The controller contains a programmable microprocessor unit directing cards targeting each motor separately (Figs 1b, 2). Programming was done in C using a personal computer. The controller has a LCD-display showing the current mode and positions of the plate. This display is also used to implement a new program, e.g., for use with different types of plates. To avoid sedimentation of cells 2 modes of shaking, unidirectional and circular, have been programmed. The controller is equipped with a serial interphase to be connected to the FACS Station for further development towards full automation. The microprocessor used already contains the hardware for this purpose. After each movement 2 light barriers indicate the position of the holder. This is necessary to make sure that samples are taken in a specific order in case acquisition gets accidentally interrupted. Switches are placed within the loader mechanic to indicate a stand-by position. After resetting, the loader moves to this position. The movement of the 96-well plate is controlled to 25 μm in each axis by the microprocessor unit.

Fig. 1. Set up of the Loader and the FACScan®. The loader consists of 3 parts; the mechanical unit (a), the electronic control module (b) and the hand held control for routine operation (c). The electronic module (b) consists of the microprocessor unit, four cards to control the 3 stepping motors and the motor for rotation of the 96-well plate, a connector for the hand held switch and a power supply. (c) The hand held control has a switch to change from normal use to programming and for changing mode, a switch for forwarding the holder and 2 switches to move through the programming steps and modes.

3.3. Hand-held control module

To make use easier, a small sized hand held controller is connected to the main controller (Fig. 1c). Using this, the sample acquisition from 96-well-plates is very simple and only requires the use of a single switch for forwarding the loader. In addition, the module can be used for programming and changing modes. We have introduced this device to simplify the use and to be able to put the controller beside the actual working space.

3.4. FACScan analysis using the loader

Applying the shaking option of the machine, sedimentation of the cells during sample acquisition is avoided, and mixing during staining procedures is done. The controller can induce unidirectional and circular rotation for shaking samples. Underneath the 96-well plate holder are 2 50-ml washing buffer tanks with openings at the small sides of the plate holder. These openings are made such that they can be used like the wells of the plates, that is the FACScan® sample needle can take up washing buffer not only to clean the needle but also to replace the fluids within the nozzle and tubing. This was done to avoid cross contamination when between wells. Most importantly, the sample itself is not wasted for washing purposes. The loader first moves wells A1–D12 along the acquisition needle, then turns the plate to collect from wells E1–H12.

Furthermore, the 2 washing buffer tanks are build underneath the 96-well-plate support such that during sample collection from wells A–H 1–6 and A–H 7–12 the washing buffer tank underneath the respective halves are used to save time moving the plate. To test the machine and to check whether we can avoid cross-contamination between wells we have purified PBMC from healthy donors and stained the B-cells with monoclonal antibodies directed against CD19 (PE) and CD21 (FITC). Staining and acquisition was done in the same 96-well-plate. Staining was done alternating (Fig. 3). CD19 was used for cells in wells A1, A3 and so on; CD21 was used in A2, A4 and so on. Cells were acquired at about 2000 cells/second. As can be seen in Fig. 3 relevant contamination between wells was not found whether or not the washing option was used.

4. Conclusion

The construction of a 96-well plate loader makes sample collection for the FACScan® easier and saves time substantial. We can acquire 96 samples using 96-well-plates within 20 min. This time can be achieved when 10,000 cells are collected at about 2000/second. Acquisition time at this rate and amount is about 5 seconds/well. Reducing cell numbers to be acquired to 5,000 results in a run of about 16 min. The time for moving the 96-well-plate could be further reduced (approximately by 50%) using a larger power supply al-

Fig. 2. Plan of the components of the loader. The drawing gives a rough overview about the setup of the 96-well plate holder. (A) The microprocessor unit gives the signal to acquire samples to the cytometer when the 96-well plate is in position. At this time the cytometer is displaying "ready". When the amount of signals to be acquired is reached the FACS Station beeps and the user forwards the 96-well-plate manually using the hand held controller module. (B) The drawing describes the fluidics of the system. A plastic tube is cut such that it fits like a usual acquisition tube. The tube is sealed onto the 96-well plate by a rubber ring made from silicon.

lowing about 17,000 samples to be acquired per day. Using 96-well plates for staining and the loader for sample collection allows staining and collecting more individual samples in a working day, e.g., for screening hybridomas or in drug discovery such as using the Rhodamine 123 test for multi drug resistance [4]. The modular set up allows individual development of the units. In particular the controller has already a serial connector to be used with the FACS Station for full automation. The hand held control is of palm size and allows simple usage. An operator using the hand held control could operate the loader without need to know the programming and set up of the controller. The controller can be programmed for use with different types of plates. This allows for future development of assays with different plates. For work in a routine biomedical lab, 96-well plates could also be marked for use with a bar code reader to automatically register the num-

ber of the plate when multiple plates are analyzed. The use of a programmable microprocessor makes any parameter changeable to the specific need of a particular lab or operator. The currently used stepping motors and the way the stepping motors are controlled is certainly far above the needs of accuracy ($25\ \mu\text{m}$). The sample volume in round bottom 96 well plates can be $200\text{--}250\ \mu\text{l}$, but should not be below $50\ \mu\text{l}$. Because of the introduction of a washing step it is not necessary to use this sample volume for cleaning of tubing and nozzle fluids from the previous sample. To reduce the volume needed for proper acquisition, it would be possible to shorten the length of the needle connected to the nozzle. However, because this part is not easily exchangeable we have currently not done so. The introduction of a 96-well plate loader will enhance the usage of 96-well plates for staining wherever this has not been done so far. Using 96-well plates

Fig. 3. To analyze whether contamination between wells takes place when using the washing option PBLs have been separately stained with anti CD19-PE and anti CD21-FITC monoclonal antibodies as indicated. Staining was done alternating, that is well A1 CD19-PE, well A2 CD21-FITC and so on. Panel A shows acquisition with a washing step, Panel B without washing between acquisition from different wells. No contamination between wells can be observed.

also allows staining with small volumes of antibody solutions. We currently use only 20 μ l for staining routinely. The loader can be adapted to other types of cytometers. The major advantage of the loader is saving substantial time, extending possible sample numbers, and further automation of staining and acquisition procedures towards application in high throughput screening.

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