

# Making the invisible visible: Rare event detection by flow cytometry using 10 colors in 65 million events

Matthias Schiemann<sup>#</sup>, Erico von Bueren<sup>&</sup> and Dirk H. Busch<sup>#</sup>

<sup>#</sup>Institute for Medical Microbiology, Immunology and Hygiene, Technical University Munich, Germany, <sup>&</sup>Clinical Cooperation Group "Immune-Monitoring", GSF - Institute of Health and Environment, Neuherberg, Germany, and Dako Colorado, Inc., Fort Collins, USA

## ABSTRACT

As a kind of 'standard procedure' for the detection and characterization of antigen-specific T cells, the acquisition of approximately 10 million events of total PBMCs or splenocytes has been recommended. However, there is increasing need to acquire significantly larger numbers of events, especially to improve statistical relevance of low frequency populations. We therefore established a method that for the first time allows to acquire/save up to 65 million multi-parameter events with the required high sensitivity and population separation needed for detection of rare event populations (<1/1000; here: CMV-antigen-specific T cell population within PBMC identified by MHC multimer staining) for immune status determination. The sample data were acquired in 19 minutes at 70,000 eps with a CV < 3% using a protocol with 10 antibodies and 10 colors / 11 parameters performed on the high-speed flow cytometer CyAn™ ADP (Dako) with 3 lasers and 9 colors, without any amplification or enrichment steps, directly *ex vivo*.

## INTRODUCTION

Rare event analysis in flow cytometry is a prerequisite to investigate and answer many immunological and clinical questions. The need to detect small cell populations is based on the fact that several immune relevant cells are very rare, e.g. antigen-specific T cells, tumor cells or disease/health-related markers. The precise detection of rare events using currently available flow cytometry analyzers is limited by their capability to acquire and save the needed large amounts of total events.

The size of a rare event population is defined by being lower than five percent (Poisson distribution). The two critical parameters in rare events analysis are the number of rare events to be detected and their CV (see figure 1), which define how many events need to be acquired in order to reach statistical significance (see figure 2).

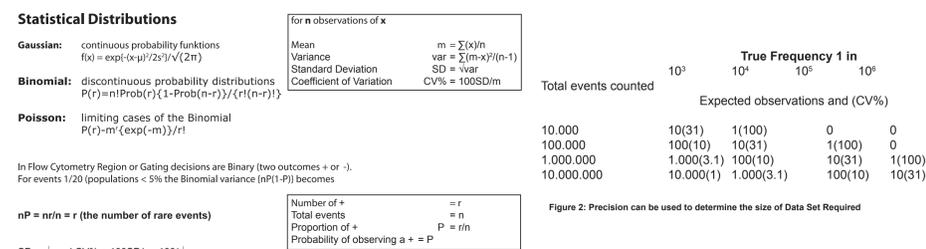


Figure 2: Precision can be used to determine the size of Data Set Required

Figure 1: Statistical Distribution used in flow cytometry

## MATERIALS

For rare event detection the hardware capacity of the commercially available CyAn™ ADP (Dako Colorado, Inc., Fort Collins, USA) was reconfigured using the new PC Dell 390 (Intel® Core 2 Duo E6600, 2.40GHz / 1066 MHz FSB / 4 GB DDR2 SDRAM Memory) and a special built of Summit software. The theoretical limitations of analysis limits are shown in figure 3. To obtain a higher number of cells of interest, PBMCs from an HLA-A\*0101-positive/HLA-B\*0801-negative, CMV seropositive adult individual was used for the presented experiments. As controls, unstained and "single color controls" (SCC), "fluorescent minus one" controls (FMO - to set gates by determining positive vs. negative expression) and "MHC multimer mismatch controls" to determine unspecific binding and reagent-derived background noise were used.

For SCC and FMO controls, 200,000 cells were stained for 20 minutes with titrated antibodies/multimers: no staining, CD62L/FITC, CMV-specific multimer HLA-A1/pp50<sub>245-253</sub>/PE, CD8/PE, CD19/PE-Alexa610, CD14/PerCP-Cy5.5, CD4/PE-Cy7, CD11b/PB, CD16/PB, CD3/AmCyan, CD45RA/APC, CD8/APC-Cy7. The FMO controls were prepared for FITC, PE, PE-Alexa610, PerCP-Cy5.5, PE-Cy7, PB, CY, APC and APC-Cy7 as shown in figure 4.

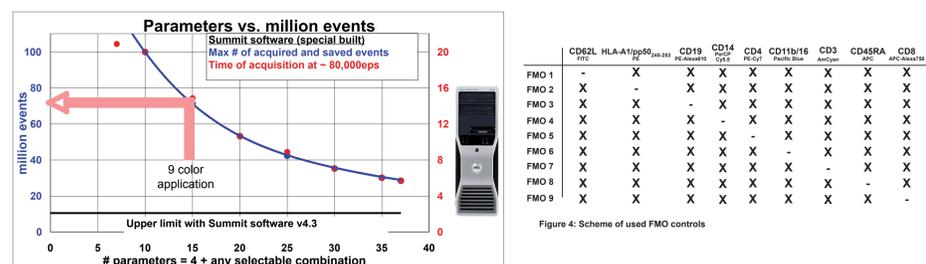


Figure 4: Scheme of used FMO controls

Figure 3: Theoretical limitations of analysis limits in Summit 4.3

The CMV multimer HLA-B8/IE-1<sub>88-96</sub>/PE was used as MMC; it was tested for functionality and specificity on different samples prior to this analysis. All controls were incubated for 20 min in the dark on ice. The main analysis samples were stained with a 9 color cocktail of 10 titrated antibodies/multimers (CD62L/FITC, CMV-specific multimer HLA-A1/pp50<sub>245-253</sub>/PE, CD19/PE-Alexa610, CD14/PerCP-Cy5.5, CD4/PE-Cy7, CD11b/PB, CD16/PB, CD3/CY, CD45RA/APC, CD8/APC-Cy7). HLA multimers were added 20 min prior to the other staining reagents; total incubation time was 45 minutes in the dark on ice. All samples were stored at 4°C (39°F) until use. Propidium iodide (PI) was added shortly before acquiring the samples with a CyAn™ ADP 9 Color. The special built of Summit software and FlowJo v8.5.x (Treestar) were used for data analysis.

## EXPERIMENTAL DESIGN AND RESULTS

In order to further extend analyses of low frequency populations with a well-established protocol that was so far limited to the acquisition of up to 10 million events, we developed a procedure allowing to increase the number of acquired cells up to 100 million events – figure 5 (as FCS 3.0 file stored 7 parameters/fluorescence channels - FSC, SCC, HLA-A1/pp50<sub>245-253</sub>/PE, propidium iodide/PETxR, CD3/APC and CD8/Pacific Blue).

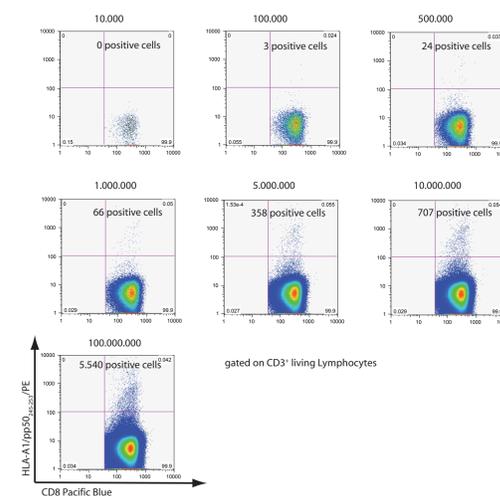


Figure 5: Percentages of antigen-specific T cells depend on number of events acquired

As shown in figure 5, the frequency of MHC multimer-positive antigen-specific T cells strongly changes with the total number of acquired events. The number of positive events upon acquisition of a total number of 1 million events still is with 66 positive events still relatively low, which affects the reliability and accuracy of the data. This can be significantly improved by enhancing the number of total events to 100 million; here, the population of positive events increased to 5540 events, which strongly improves the resolution of analysis. In order to control for and to reduce possible background staining, we designed a multi-color panel with 9 parameters and acquired up to the boundaries of the software's possibility (figure 6).

65 million events were acquired for each sample: analysis sample (stained with HLA-A1 pp50<sub>245-253</sub>/PE multimers), mismatch control (HLA-B8 IE-1<sub>88-96</sub>/PE multimer), and 'no multimer' (figure 7). Samples were acquired at high-speed mode of 70,000 events per second in approx. 19 minutes. 3884 HLA-A1-restricted pp50<sub>245-253</sub>-specific T cells were found in the acquired 65 million events, equivalent to 0.004% (frequency of rare events 4/100,000) of the analyzed cells.

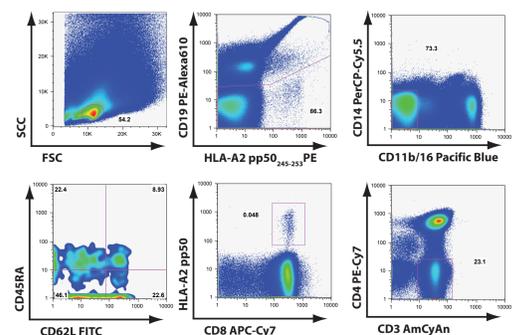


Figure 6: Acquisition of 65 million events and the gating strategy

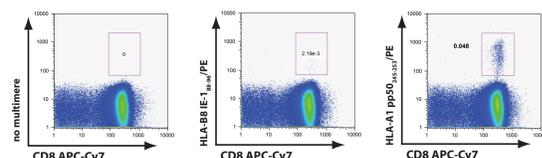


Figure 7: Controls to determine background and unspecific bindings

## CONCLUSIONS

The detection and measurement of antigen-specific T cells against CMV has gained specific importance for several fields in clinical research, like organ transplantation, hemodialysis, cancer therapy, immunosuppressive treatment, or HIV-infection, since these data might provide important information for further therapeutic decisions.

The big challenge in rare event analysis is the discrimination between background and truly positive cells. It is, therefore, indispensable to use specific controls for protocols without amplification. For instrument setup and definition of the gating strategies, unstained cells or stained isotypes are improper controls in multi-color experiments, especially while detecting very small subpopulations. Currently, the best controls are stained cells with all reagents except the one of interest (FMO). FMO controls should be used whenever accurate discrimination is essential or when antigen expression is relatively low

With two new options of Summit software, which allow to acquire large number of events and automatically determine the compensation matrix (Auto Compensate), it is now possible to determine *ex vivo* the size of rare populations of antigen-specific T cells with high statistical significance and speed.

Prof. Dirk H. Busch, M.D.  
Project Leader Clinical Cooperation  
Group Antigen Specific Immunotherapy  
(TUM/GSF - National Research Center  
for Environment and Health)  
Institute for Microbiology, Immunology  
and Hygiene

Technical University Munich  
Trogerstrasse 30  
81675 Munich  
e-mail: dirk.busch@lrz.tum.de  
Tel.: +49 89 4140-6191 (office)  
+49 89 4140-6244 (lab)  
Fax: +49 89 4140-4139