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FLOW CYTOMETRY

CyAn™ ADP | Rare Event Analysis of Antigen-Specific T Cells using MHC Multimers

Analysis of rare events is defined as the identification of sub-populations with a size of less than five percent (Poisson distribution). The precise detection of rare events using currently available flow cytometry analyzers is often limited by their capability to acquire and save the needed large amounts of total events. As a consequence, conventional experimental designs require cell enrichment methods.[1]

High-speed flow cytometry analyzers like the CyAn ADP LX 9 Color offer excellent sensitivity and effective signal separation. With the new Summit software version 4.3 it is now possible to acquire and analyze rare events from blood without any amplification or enrichment steps directly ex *vivo*.

Materials and Methods

PBMC from an adult HLA-A*0101-positive/HLA-B*0801-negative, CMV seropositive individual was used for this experiment. Since controls are very important for rare event analysis, recommendations include:

1. Unstained and "single-color controls" (SCC) to properly set PMT voltages and compensations.
2. "Fluorescent minus-one" controls (FMO) to set gates by determining positive vs. negative expression.
3. "MHC multimer mismatch control" (MMC) to determine unspecific binding and reagent-derived background noise.

For each SCC 200,000, cells were stained for 20 minutes with titrated antibodies/multimers: no staining, CD62L/FITC, CMV-specific multimer HLA-A1/pp50$_{245-253}$/PE, CD8/PE, CD19/PE-Alexa610, CD14/PerCP, CD4/PE-Cy7, CD11b/PB, CD16/PB, CD3/CY, CD45RA/APC, CD8/APC-Alexa750. The FMO controls were prepared for FITC, PE, PE-Alexa610, PerCP, PE-Cy7, PB, CY, APC and APC-Alexa750 as shown in Table 1.

The CMV multimer HLA-B8/IE-1$_{88-96}$/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 minutes (in the dark on ice).

The main analysis samples were stained with a nine-color cocktail of 10 titrated antibodies/multimers (CD62L/FITC, CMV-specific multimer HLA-A1/pp50$_{245-253}$/PE, CD19/PE-Alexa610, CD14/PerCP, CD4/PE-Cy7, CD11b/PB, CD16/PB, CD3/CY, CD45RA/APC, CD8/APC-Alexa750). HLA multimers were added 20 minutes 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 LX 9 Color. Summit software version 4.3 was used for data acquisition. And Summit software version 4.3 and FlowJo v8.1.1 were used for data analysis.

Table 1

| | CD62L | HLA-A1/ pp50$_{245-253}$ | CD19 | CD14 | CD4 | CD11b/16 | CD3 | CD45RA | CD8 |
	FITC	PE	PE-Alexa610	PerCP	PE-Cy7	Pacific Blue	Cascade Yellow	APC	APC-Alexa750
FMO 1	–	✔	✔	✔	✔	✔	✔	✔	✔
FMO 2	✔	–	✔	✔	✔	✔	✔	✔	✔
FMO 3	✔	✔	–	✔	✔	✔	✔	✔	✔
FMO 4	✔	✔	✔	–	✔	✔	✔	✔	✔
FMO 5	✔	✔	✔	✔	–	✔	✔	✔	✔
FMO 6	✔	✔	✔	✔	✔	–	✔	✔	✔
FMO 7	✔	✔	✔	✔	✔	✔	–	✔	✔
FMO 8	✔	✔	✔	✔	✔	✔	✔	–	✔
FMO 9	✔	✔	✔	✔	✔	✔	✔	✔	–

Results

The CyAn ADP LX 9 Color was set up in three steps:

1. Adjustment of PMT voltages using unstained sample.
2. Acquisition of stained SCC saving 25,000 total events.
3. Setting compensation using Auto Compensation function in Summit software version 4.3 and SCC gated on live lymphocytes.

After setting up the CyAn ADP LX 9 Color, 100,000 total events of the FMO controls were acquired to validate compensation and to correctly set gates (Figure 1).

Ten million events from the analysis sample (stained with HLA-A1 pp50$_{245-253}$/PE multimers), mismatch control (HLA-B8 IE-1$_{88-96}$/PE multimer) and "no multimer" were acquired. High-speed mode of 70,000 events per second for 2:20 minutes was used for all samples.

A total of 1,459 HLA-A1-restricted pp50$_{245-253}$-specific T cells were found in the acquired 10 million events, equivalent to 0.01% (frequency of rare events 1/10,000) of the analyzed cells (Figure 2).

Gating Strategy

Figure 1

Figure 2

100,000 events total

10 MEGA events total

HLA-A1 pp50$_{245-253}$

HLA-B8 IE-1$_{88-96}$
"HLA mismatch control"

No Tetramer
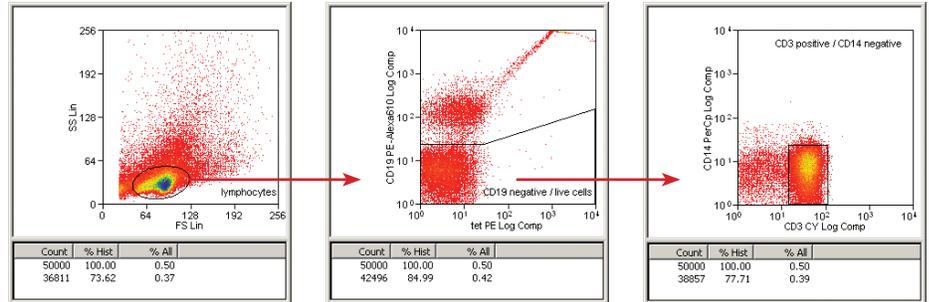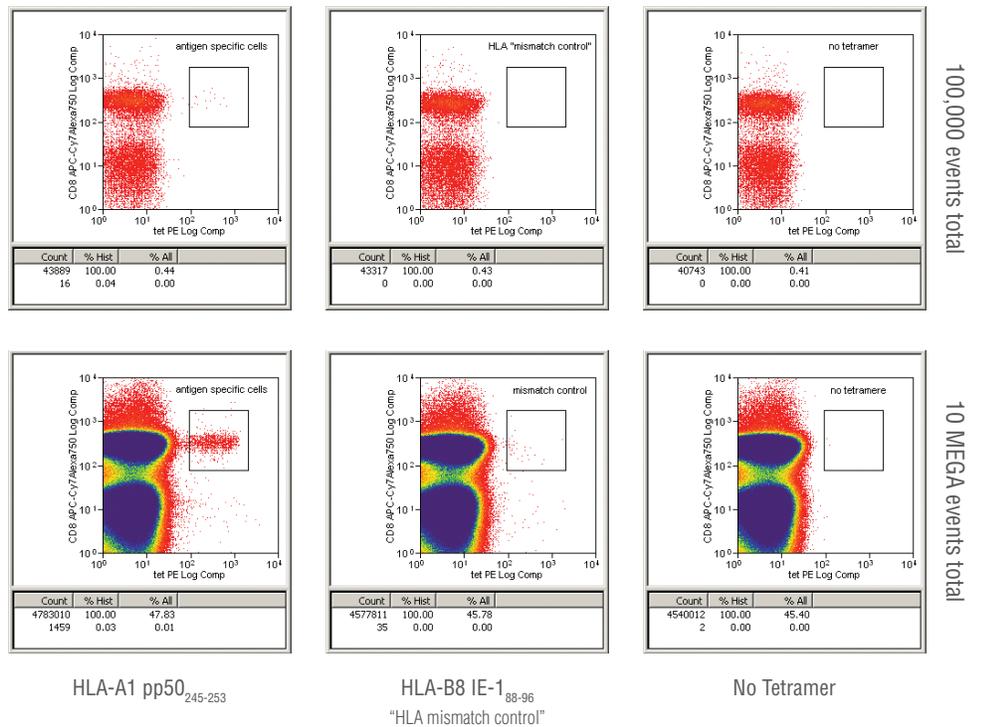
TECHNICAL TIPS

> It is important to use SSC and FMO controls to correctly set up the instrument and compensation prior to run the sample of interest.

> It is very important that the sample line be cleaned adequately prior to running the samples of interest: Start "sample clean" and run "Decontamination solution," then "Cleaning and Rinse Solution" and then "DI water."

Discussion

Antigen-specific T cells are crucial for protection against intracellular pathogens such as viruses and intracellular bacteria. Loss of antigen-specific T cells or their functional impairment, as observed in immunocompromised patients, can result in severe health conditions.

The herpes virus CMV is found universally throughout all geographic locations and socioeconomic groups, and chronically infects between 50% and 85% of all individuals. Primary CMV infection can become life-threatening for immunocompromised patients. However, the more common clinical problem is the reactivation of latent virus in patients during a longer phase of immuno-suppression, where CMV-related diseases contribute to a large number of severe clinical complications.

The detection and measurement of antigen-specific T cells against CMV has gained specific importance for several fields in clinical research (including 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 discriminating 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 the software options of Summit software version 4.3 to acquire large number of events and Auto Compensation to easily define compensation, it is now possible to determine *ex vivo* the size of rare populations of antigen-specific T cells with high statistic significance and speed.

Contributors

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Acknowledgements

Special thanks to Katleen Goetsch for the excellent technical assistance.

References

1. Hataye, J., J.J. Moon, A. Khoruts, C. Reilly, and M.K. Jenkins. 2006. Naive and memory CD4+ T cell survival controlled by clonal abundance. *Science 312:114-116*.

PRODUCT	CODE
CyAn ADP LX 9 Color	CY201
Sheath Fluid	S2322
SpectrAlign Beads	K0111
8-Peak-Beads	K0112
Cleaning and Rinse Solution	S2323
Decontaminating Solution	S2324
CD62L/FITC	F7085
CD3/CY	CA696
CD8/PE	R0806

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