

HIGH-SPEED QUANTITATION OF CMV SPECIFIC CYTOTOXIC T-CELLS IN WHOLE BLOOD

Lene Have Poulsen, Kivin Jacobsen, Ian Storie, Tina Jakobsen, Jesper Laursen. Dako Denmark A/S, Department of Flow Cytometry Work Flow, Glostrup, Denmark.

Abstract

There is a high interest in accurate, fast and reliable assays for quantitation of antigen specific T cells. On the flow cytometer platform, T-cell specific fluorescently labeled MHC molecules loaded with the peptide of interest can be applied. Here we show that MHC Dextramers, MHC reagents built with a dextran backbone, can be used for identification and single platform enumeration of CMV antigen specific T cells in whole blood at detection levels below 1 cell per μL blood. Because this assay renders lysis of red blood cells obsolete, sample preparation was shortened to 20 minutes. Whole non-lysed blood was diluted with PBS and counting beads added before analysis on a CyAn ADP flow cytometer. Acquisition time was typically 5 minutes for 20,000 CD8+ events. The same method could be applied for further multicolor immunophenotyping of activation markers.

Background

Studies of antigen specific T-cell responses can be performed with a number of methods including MHC based reagents such as Tetramers and more recently MHC Dextramers. These methods have very different characteristics. However, the MHC based method has the valuable characteristics of simplicity, speed and single cell detection level and are generally recognized as highly amenable for standardization of procedures.

Whole blood analysis for antigen specific cytotoxic T-cells have been published previously and shown to be both sensitive and accurate [2,3]. In the study from Wöflf et al. (2004), the number of antigen specific T-cells were determined with a single-platform assay using a lyse/no-wash procedure and TrueCount beads. A very low number of false positives were found, typically 1-2 cells per 100 μL blood.

A two-step procedure to obtain the absolute count of antigen specific T-cells was used in the study by Heijnen et al. (2004). They first obtained the absolute CD8+ count using single-platform flow cytometry. Then the percentage of antigen specific T-cells of the CD8+ cells was obtained allowing the absolute count to be calculated. This methodology is advantageous when the blood contains low numbers of lymphocytes but prolongs the overall assay time significantly.

We have previously found (unpublished data) that the CyAn ADP™ analyzer is capable of processing diluted whole blood when the trigger decision is applied to one of the fluorescence parameters. If this capability could be extended to MHC assays, it seemed that the speed of the assay including acquisition could be increased without compromising the ability to analyze for multiple parameters simultaneously.

Fast and reliable MHC assays are of high value for numerous applications in the field of immunology such as cancer vaccine research.

In this study we therefore tested whether similar results concerning cell counting as well as immunophenotyping could be obtained with a fast no-lyse assay in comparison to lyse/no-wash and lyse/wash procedures, respectively.

Results

Simplified Work Flow

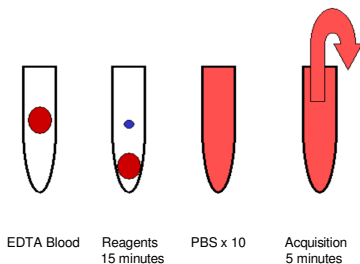


Figure 1. 100 μL EDTA stabilised human peripheral blood was incubated for 15 minutes at room temperature with a combination of fluorochrome conjugated Mouse Anti-Human Antibodies as well as with a relevant MHC Dextramer reagent(s).

The whole un-lysed blood was then diluted 1:10 in phosphate buffered saline (PBS) followed by acquisition on a 9 color CyAn ADP™ flow cytometer at 150 μL per minute. FL6 (CD45/Pacific Blue) or FL7 (CD45/Cascade Yellow) was used as trigger parameter.

Detection level

The detection level for antigen specific T-cells was well below 1 cell per μL blood when MHC Dextramer reagents were applied with the No-Lyse protocol described in Figure 1.

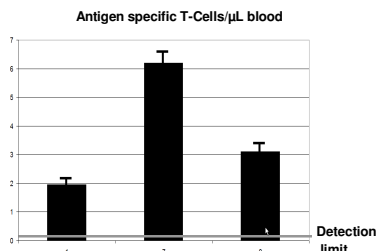
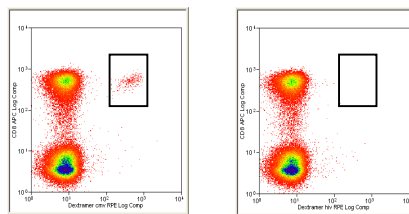


Figure 2. Four-color reagent CD3/Pacific Blue; CD8/APC; CD45/Cascade Yellow; MHC Dextramer/HLA-A*201 (ILKEPVHGV)/R-PE was used to estimate the background. The average number of false positive events per μL blood plus 2 x the standard deviation was used as detection limit (10 donors with 3 tests each). Samples 1, 7 and 9 were analysed with MHC Dextramer/HLA-A*0201 (NLVPMVATV)/R-PE. The average and SD (n=3) are depicted in the histogram. CytoCount™ beads were used to obtain the cell count directly on the flow cytometer platform.

No-Lyse procedure



Lyse/No-Wash procedure

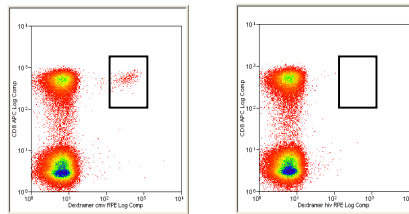


Figure 3. Typical result obtained with four-color reagent CD3/Pacific Blue; CD8/APC; CD45/Cascade Yellow; MHC Dextramer/HLA-A*201R-PE carrying either CMV derived peptide NLVPMVATV or HIV peptide ILKEPVHGV. The reagent was optimised individually for the No-Lyse procedure as well as for the EasyLyse™ Lyse/No-Wash procedure.

Sample stability

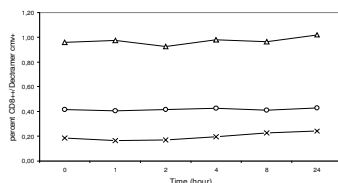
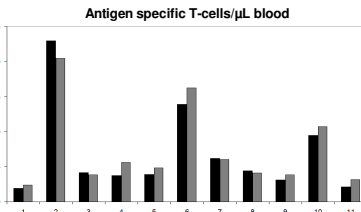


Figure 4. Antibody reagents and procedure as described in Fig. 2. Donor 1 (A), 2 (O) were analysed with HLA-A*0101(VTEHDTLLY) and donor 3 (X) with HLA-A*0201(NLVPMVATV). All samples were in duplicate and stored at 4°C before they were applied to the flow cytometer at the indicated time points.

Counting: No-Lyse compared to Lyse/No-Wash



Separation between CD8++ Dex+ and Dex- populations

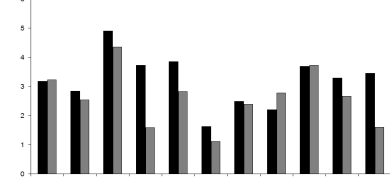


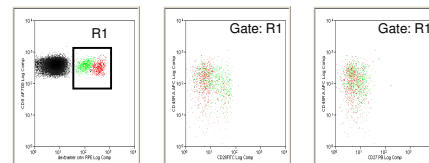
Figure 5. Black: no-lyse procedure. Grey: lyse/no-wash procedure. 11 comparisons were performed using 5 different combinations of HLA alleles and CMV peptides. The separation between the MHC Dextramer positive and negative CD8++ populations was quantitated in terms of the separation factor (see Materials & Methods). The No-Lyse procedure was as described in Figure 1. The Lyse/No-Wash procedure was performed with the EasyLyse™ kit.

References

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- Wöflf M., Scalk S., Hellmich M., Huster K.M., Busch D.H. and Berthold F. (2004). Quantitation of MC Tetramer-Positive Cells From Whole Blood: Evaluation of a Single-Platform, Six-Parameter Flow Cytometric Method. *Cytometry Part A* 57A:120-130.
- Heijnen I.A.F.M., Barnett D., Aroz M.J., Barry S.M., Bonneville M., Brando B., Dhautcourt J.L., Kern F., Tötterman T.H., Marijt E.W.A., Bossy D., Prejers F.W.M.B., Rothe G. and Gratama J. (2004). Enumeration of Antigen-Specific CD8+ T Lymphocytes by Single-Platform, HLA Tetramer-Based Flow Cytometry: A European Multicenter Evaluation. *Cytometry Part B* 62B:1-13.
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Immunophenotyping: No-Lyse compared to Lyse/Wash

No-lyse staining procedure



Lyse/Wash staining procedure

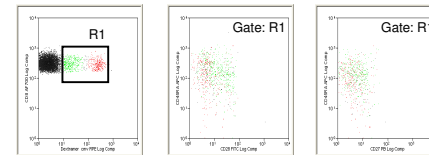


Figure 6. A multicolor reagent containing CD45/Cascade Yellow; CD8/Alexa700; MHC Dextramer HLA-B*0702(TPRVTGGGAM)/R-PE; CD27/Pacific Blue; CD28/FITC and CD45RA/APC was optimised individually for each procedure. The No-Lyse staining and acquisition was performed as described (Figure 1). The Lyse/Wash procedure was performed with EasyLyse™ according to specifications. The specificity of the stains was confirmed using FMO controls.

Methods and Materials

Human peripheral blood was obtained according to Danish regulations from anonymous healthy donors at a local hospital. All blood samples were collected by venous puncture in EDTA containing tubes.

Donors were screened for CMV reactive T-cells by multicolor reagents containing combinations of FITC, R-PE and APC labelled MHC Dextramers as well as CD45/Pacific Blue, CD3/Cascade Yellow and CD8/Alexa Fluor 700. For this initial screen, the No-Lyse procedure (see Figure 1) was applied.

Counting beads: 50 μL CytoCount™ beads were applied with each 100 μL blood sample after incubation with reagents but before addition of PBS. Mouse Anti-Human CD3/Pacific Blue, CD45/Cascade Yellow, CD8/APC, CD28/FITC and MHC Dextramers were from Dako.

Conjugation of Mouse Anti-Human CD27 with Pacific Blue, Mouse Anti-Human CD8 with Alexa Fluor 700 and Mouse Anti-Human CD45RA with APC were performed according to standard procedures.

Dextramer (R-PE)	HLA-A*0201 (NLVPMVATV)	HLA-A*0101 (VTEHDTLLY)	HLA-A*0702 (TPRVTTGGGAM)	HLA-A*0702 (RPHRNGFTVL)	HLA-A*0801 (ELRRKMYNO)
Test.No.	1, 7, 9	2, 4, 5, 10	3, 6	8	11

The MHC Dextramer control was R-PE labelled and carried HIV derived peptide ILKEPVHGV in HLA-A*0201 context. All samples were acquired on a CyAn ADP™ flow cytometer and analysed with Summit™ software.

The CD45 parameter was used as trigger channel for the no-lyse samples whereas FSC was used as trigger for lyse/no-wash and lyse/wash samples.

The detection limit was determined according to recommendations⁴.

The separation factor is calculated from:

$$\text{[mean (pos) - mean (neg)] / [SD(pos)^2 + SD(neg)^2]^{0.5}}$$

Conclusions

It is feasible to count CMV antigen specific T-cells with fast and simple whole blood flow cytometry on the CyAn ADP™ analyzer.

The whole blood procedure simplifies sample handling with one step and shortens the preparation time to 20 minutes.

Acquisition on the CyAn ADP™ analyzer can be performed with the trigger set on a fluorescence parameter allowing routine sampling rates of 20,000 CD8+ cells in 5 minutes.

The detection threshold was well below 1 cell per μL blood.

The percentage of the cytotoxic T-cells that are positive for the MHC Dextramer reagent appears stable for at least 8 hours after staining and dilution.

Interestingly, in the small sample set there appears to be a correlation between a relative higher percentage of CMV reactive T-cells with the lyse/no-wash procedure and a relatively higher separation between positive and negative cytotoxic T-cells with the no-lyse procedure. We speculate whether this may be caused by differences in unspecific staining induced by the lysing procedure.

CD45RA, CD27 and CD28 expression was qualitatively indistinguishable between samples analysed with the no-lyse and a standard lyse/wash procedure.