



# Quantitation of mHLA-DR and nCD64 by Flow Cytometry to Study Dysregulated Host Response: The Use of QuantiBRITE™ PE Beads and Its Stability

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Accepted: 31 December 2021

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## Abstract

Quantitation of mHLA-DR and nCD64 is useful in understanding the dysregulated host response. The down regulation of HLA-DR expression on the circulating monocytes (mHLA-DR) is associated with anti-inflammatory response, and an increased expression of CD64 on neutrophil surface (nCD64) is associated with pro-inflammatory response. Quantitation of these antigen expression using beads (QuantiBRITE™ PE) is a precision technique. These beads are reported to be stable for 24 h after reconstitution. We report the results of our investigation examining the stability of QuantiBRITE PE beads over a period of 4-week post-reconstitution. The data suggest that reconstituted QuantiBRITE PE beads, if stored in dark at 2–8 °C, can be effectively used for up to 2 weeks for determining nCD64 and mHLA-DR antibody bound per cell (ABC) values.

**Keywords** Antibody bound per cell · mHLA-DR · nCD64 · QuantiBRITE PE · Antigen expression

## Introduction

Quantitation of antigen expression using QuantiBRITE™ PE beads (BD Biosciences, USA) is a precision technique extensively used in immunology research and also in diverse clinical settings such as AIDS, sepsis, and more recently, COVID-19. In research as well as in diagnostics, its applications include the flow cytometric estimation of antibodies bound

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per cell (ABC). Each QuantiBRITE™ PE tube contains lyophilized pellet of beads conjugated with four known levels of phycoerythrin (PE). These tubes are designed to use with PE-labelled monoclonal antibodies for the estimation of ABCs by flow cytometry. When QuantiBRITE™ PE beads are used in conjunction with PE conjugates with a PE to monoclonal antibody ratio of 1:1, PE molecules can be converted to ABCs (1).

Our recent study used this technique for the quantitation of mHLA-DR and nCD64 in diagnosing sepsis post-cardiac surgery [1, 2]. Sepsis is due to the dysregulation of immune response to infection [2, 3]. An increased expression of CD64 on neutrophil surface (nCD64) is associated with proinflammatory response, and a down regulation of HLA-DR expression on the circulating monocytes (mHLA-DR) is associated with anti-inflammatory response. Analysing these altered surface antigenic expressions can reveal the dysregulated host response.

Quantitation of these antigen expressions can be done using QuantiBRITE™ PE beads. The product datasheet specifies that the QuantiBRITE PE beads are stable for 24 h after reconstitution in 0.5 mL buffer when stored in dark between 2 and 8 °C. The usage of beads for a longer period of time post-reconstitution could lead to the saving of time as well as the overall cost involved per test. Thus, in this study, we looked at the stability of beads post reconstitution. Here, we report the results of our investigation examining the stability of QuantiBRITE PE beads over a period of 4-week post-reconstitution.

## Methods

### Collection of sample

Blood sample from healthy volunteer was collected in EDTA vacutainer (BD Biosciences, USA) after obtaining the Institutional Ethics Committee approval (IEC-AIMS- 2018-NANO-031). Fifty microlitre of the blood sample collected was stained with QuantiBRITE CD64PE/CD45PerCP (BD 340768) and CD14FITC (BD 347493) and with QuantiBRITE HLA-DRPE/anti-monocyte (BD 340827) and CD45APC-H7 (BD 641399) in two separate tubes. The sample was processed with a standard stain-lyse-wash method, acquired on a BD FACSCanto II flow cytometer and analysed for MFI measurements of nCD64 and mHLA-DR using FACSDiva v8.0.1 software as described previously [2, 4].

### Quantitation of antigen expression per cell with QuantiBRITE beads

QuantiBRITE PE beads consist of four levels of pre-calibrated lyophilized pellets. Each level of bead has a known value of PE molecules per bead. Therefore, a standard curve can be constructed relating the number of PE molecules and the median fluorescence intensity (MFI) obtained by acquiring the beads. Using this standard curve, the number of PE molecules can be computed for any unknown MFI. This quantification technique can be used for instrument-independent calibration, with spectrally matched antibody conjugate. By using a PE conjugated monoclonal antibody (1:1 conjugation), with a known stoichiometry of antibody-antigen binding, the number of antigens per cell can be determined as antibody bound per cell (ABC) [2].

For this experiment, we used three new QuantiBRITE PE bead tubes from the same lot. The tube was reconstituted using 0.5 mL of PBS and was vortexed before acquiring the events. Each tube was run three times per week for 4 weeks. After every run, the tubes

were immediately covered with aluminium foil and stored at 4 °C. A total of 2500 events were acquired for every run. The MFI of the four different level beads were measured, and the standard curve was plotted on each day separately for all the tubes, as described by Pannu et al. (2001) [5]. Instrument performance was monitored daily using Cytometer Setup and Tracking beads. The MFI values obtained for the same healthy volunteer were converted into ABC values with the slope (m) and y-intercept values obtained from each of the 36 standard curves, which were generated from the three QuantiBRITE bead tubes over the 4-week period.

## Results and Discussion

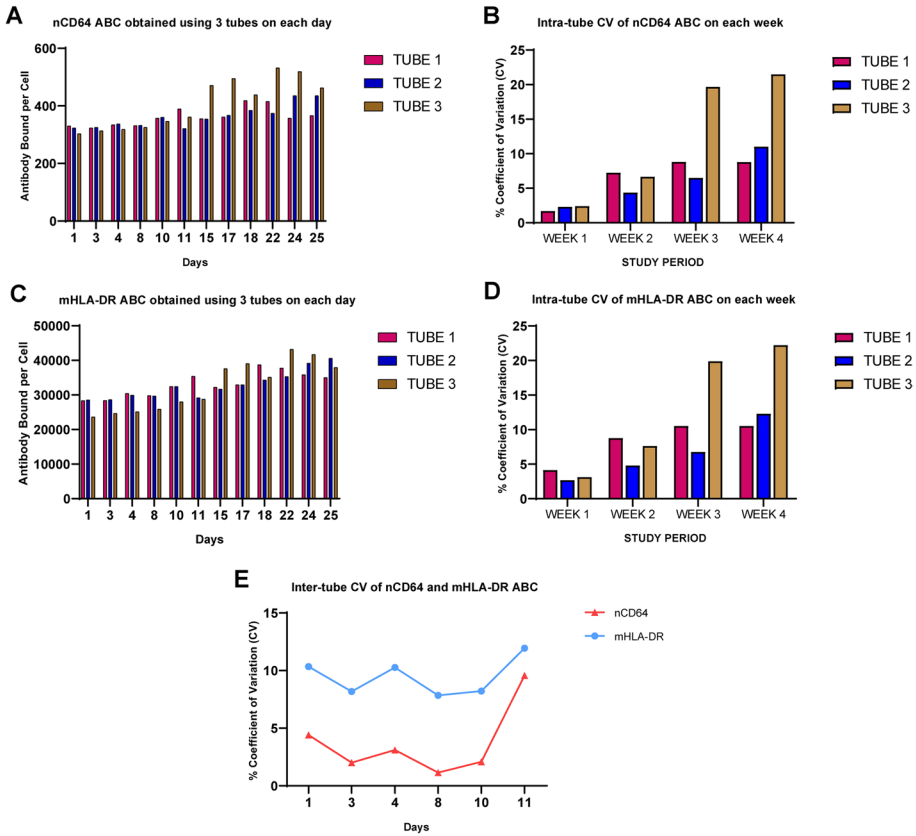
The calculated ABC values of both nCD64 and mHLA-DR are plotted in Fig. 1A and C. Average ABC values of nCD64 and mHLA-DR from 'Day 1' data were 319 and 26,859, respectively. These were similar to the values reported from healthy individuals in previous studies [3]. The intra-tube percentage coefficient of variation (CV) of nCD64 ABC values generated from standard curves of QuantiBRITE PE tubes 1, 2, and 3 were 1.7, 2.3, and 2.4, respectively, over the first week ( $n=3$ ); 7.2, 4.4, and 6.6 over 2 weeks ( $n=6$ ); 8.8, 6.5, and 19.7 over 3 weeks ( $n=9$ ); and 8.8, 11.0, and 21.5 over 4 weeks ( $n=12$ ). Similarly, intra-tube percentage CV for mHLA-DR ABC values were 4.1, 2.7, and 3.1, respectively, over first week; 8.8, 4.8, and 7.6 over 2 weeks; 10.5, 6.8, and 19.9 over 3 weeks; and 10.5, 12.3, and 22.2 over 4 weeks (Table 1). The computed CV is plotted in Fig. 1B and D. It was observed that the intra-tube CV of ABC measurement of both antigens was less than 10% for all QuantiBRITE tubes for up to 2 weeks.

We argued that if the intra-tube CVs of the nCD64 and mHLA-DR ABC values were lower than the respective inter-tube CVs obtained on 'Day 1 run' of the QuantiBRITE beads (which was run as per the product's instructions for use), then it might be reasonable to use the stored reconstituted beads, with potential time and cost savings for the lab. Results of the inter-tube CVs for the first 2 weeks are illustrated in Fig. 1E. We found that the intra-tube CVs observed in the first week for nCD64 (1.7, 2.3, 2.4) and mHLA-DR (4.1, 2.7, 3.1), and over the 2-week period for mHLA-DR (8.8, 4.8, 7.6) were lower than the day 1 inter-tube CVs of nCD64 (4.4%) and mHLA-DR (10.3%), respectively.

We used this technique for the quantitation of antigen expression per cell (mHLA-DR and nCD64) in diagnosing sepsis post-cardiac surgery [1, 2].

## Conclusion

In conclusion, these data suggest that reconstituted QuantiBRITE PE beads, if stored in dark at 2–8 °C, can be effectively used for a week for determining nCD64 and mHLA-DR ABC values, well within the limits of experimental variation inherent in the use of these beads as per the recommended protocol. Further, if the research hypothesis and the



**Fig. 1** A and C Day-wise measurement of nCD64 and mHLA-DR ABC. The measured nCD64 ABC (A) and mHLA-DR ABC (C) for each tube on each day of the experiment is shown. B and D Intra-tube coefficient of variation in the measurement of nCD64 and mHLA-DR. The coefficient of variation of nCD64 ABC (B) and mHLA-DR (D) among all the values obtained until the end of a week for each tube throughout the study period is displayed. The intra-tube CV remains less than 10% up to the second week for both nCD64 and mHLA-DR. E Inter-tube coefficient of variation in the measurement of nCD64 and mHLA-DR. The coefficient of variation of nCD64 ABC and mHLA-DR ABC, among the values obtained using the 3 tubes on the same day after reconstitution of the tubes until day 11 is shown

biological variation in the expression of analytes allow tolerating an experimental variation of 10%, these beads could be used for up to 2-week post-reconstitution.

**Acknowledgements** The authors are grateful for the support provided by Mr. Sugavanan K for the conduct of the study, BD Biosciences, India, for providing antibodies and QuantiBRITE™ PE beads as a part of “Amrita-BD center of excellence in clinical flow cytometry” and also for the infrastructure provided by Centre for Nanosciences and Molecular Medicine, Amrita Vishwa Vidyapeetham.

**Author Contribution** SS was responsible for acquisition of data, analysis, interpretation of data, and initial drafting of manuscript; PJ for critical revision of the manuscript and intellectual inputs; VVP for critical revision of the manuscript; PKV for critical revision of the manuscript, intellectual inputs and supervision of the study; UM for study concept and design, supervision of the study, analysis and interpretation of data, manuscript writing, and intellectual inputs.

**Table 1** Intra-tube coefficient of variation (CV) of measured ABC values: The ABC value of each antigen was computed for the same MFI value of a healthy individual , with the standard curves obtained for each day the experiment was done in a week. Thereafter the CV was calculated from the ABC values obtained till the end of each week, expressed as percentage and tabulated here

Week	Tube 1		Tube 2		Tube 3	
	nCD64 (ABC)	mHLA-DR (ABC)	nCD64 (ABC)	mHLA-DR (ABC)	nCD64 (ABC)	mHLA-DR (ABC)
<b>1</b> (n=3)	1.7%	4.1%	2.3%	2.7%	2.4%	3.1%
<b>2</b> (n=6)	7.2%	8.8%	4.4%	4.8%	6.6%	7.6%
<b>3</b> (n=9)	8.8%	10.5%	6.5%	6.8%	19.7%	19.9%
<b>4</b> (n=12)	8.8%	10.5%	11.0%	12.3%	21.5%	22.2%

**Data availability** Available upon reasonable request.

## Declarations

**Ethics Approval** Institutional Ethics Committee approved (IEC-AIMS- 2018-NANO-031).

**Consent to Participate** Not applicable.

**Consent to Publish** Not applicable.

**Competing Interests** The authors declare no competing interests.

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