Measuring antibody-mediated complement-dependent cytotoxicity using flow cytometry

Immuno-oncology research applications for the BD Accuri™ C6 Plus flow cytometer

Features

- Assess complement-dependent cytotoxicity (CDC) mediated by a therapeutic monoclonal antibody, rituximab
- Measure CDC in cancer cell lines and heterogeneous primary cell populations
- Determine cell viability by measuring absolute cell counts

The therapeutic efficacy of anti-tumor monoclonal antibodies (mAbs) depends on their capacity to recognize the tumor-associated antigen and trigger immune defense mechanisms against the tumor. Rituximab is a chimeric murine/human mAb targeting CD20, a cell surface antigen expressed primarily by most B cells. Rituximab binding to CD20 leads to a rapid and sustained depletion of peripheral B cells, making it a potent cancer therapeutic to treat a broad variety of CD20-positive malignancies as well as eliminate pathogenic B cells in certain autoimmune disorders.

Rituximab-mediated B-cell cytotoxicity is in part driven by activation of proteins from the complement system, leading to the formation of the membrane attack complex (MAC) and subsequent cytolysis of the target cell. The efficacy of rituximab therapy depends on CD20 antigen density as high levels of CD20 expression permit more rituximab cell binding and subsequently more MAC deposition and cell lysis.

In this data sheet, we demonstrate the use of flow cytometry to determine CD20 antigen density and receptor occupancy by the therapeutic antibody rituximab. We also demonstrate that rituximab induces the lysis of CD20-expressing cells in the presence of normal human serum. The BD Accuri™ C6 Plus personal flow cytometer used in these experiments features volumetric cell counting, which uniquely enables assessment of cell viability by measuring absolute cell numbers instead of counting beads.

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Figure 1 shows that rituximab induced the lysis of CD20-expressing cells in a dose-dependent manner. Incubation of Daudi cells (Burkitt's lymphoma, ATCC-CCL-213) with rituximab and normal human serum led to rapid necrotic cell death, characterized by reduction in cell size (Figure 1A) and loss of cell viability, as indicated by 7-AAD staining (Figure 1B). The number of live (7-AAD$^-$) Daudi cells decreased upon treatment with rituximab and normal human serum, but not when the serum was heat inactivated, suggesting the involvement of temperature-sensitive components such as complement in the rituximab-mediated tumor cell killing.

Figure 1A

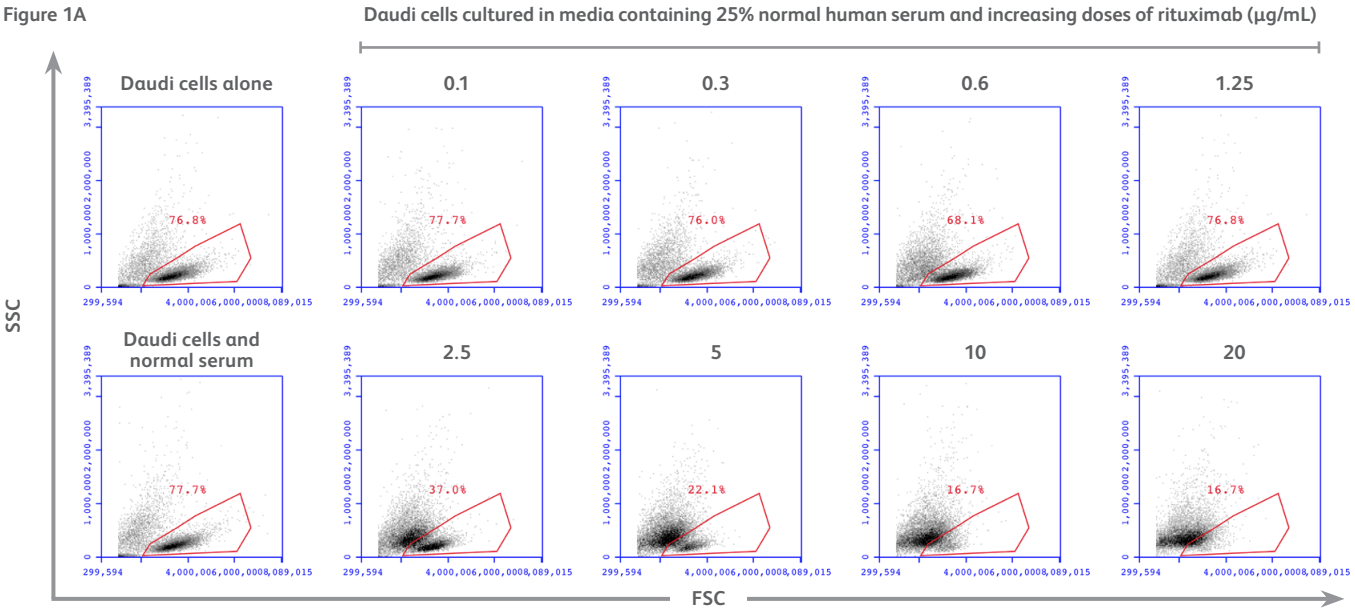

Figure 1B

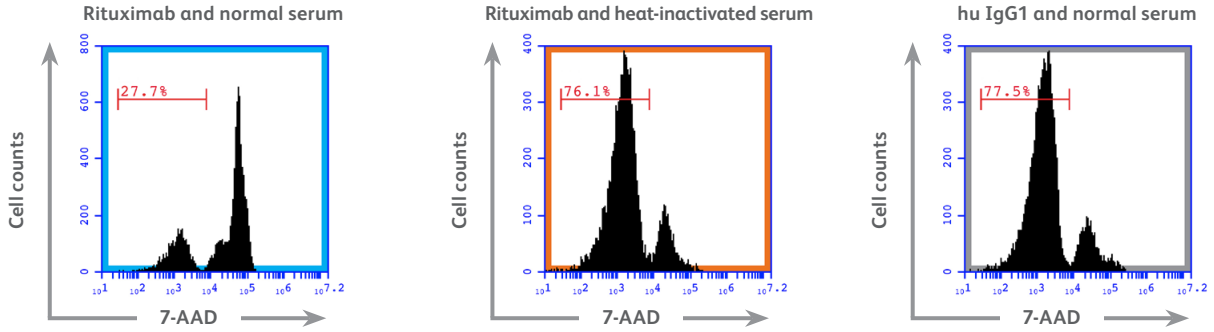

Figure 1C

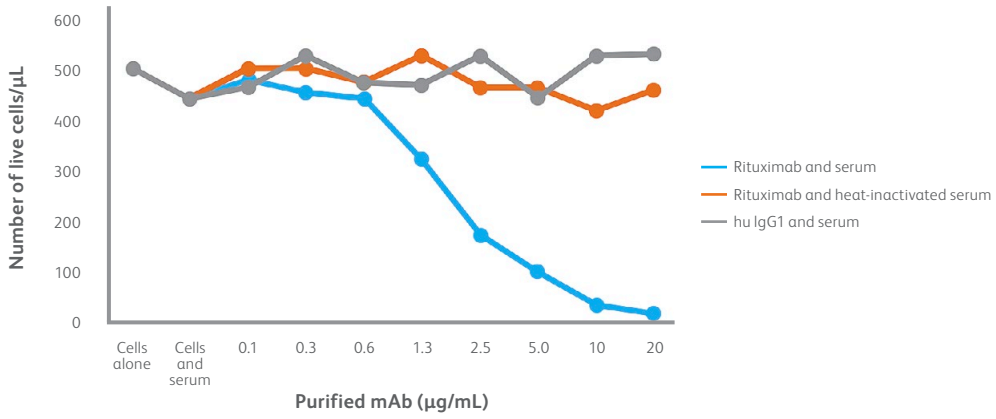

Figure 1. Rituximab-mediated complement-dependent cytotoxicity
Daudi cells were maintained in media containing heat-inactivated fetal bovine serum (56°C for 30 min). The cells were counted using the BD Accuri C6 Plus flow cytometer, resuspended at 1x10^6 cells/mL and 100 µL of cells were transferred to a 96-well U-bottom plate. Serial twofold dilutions (ranging from 0.1 µg/mL to 20 µg/mL) of the monoclonal therapeutic antibody rituximab (BioVision) or human IgG1 isotype control (BioLegend) were added to the cells. Cell cultures were supplemented with 25% normal or heat-inactivated human serum and the plates were then incubated at 37°C for 5 hours. The plates were spun down and the cells resuspended in BD Pharmingen™ Stain Buffer (FBS) containing BD Pharmingen™ 7-AAD solution. **A.** Representative data showing percentages of cells based on analysis of cell size, in which decreased forward scatter corresponds to dead cells or debris. **B.** After gating on all cells, 7-AAD$^+$ dead cells were excluded as shown in the representative histograms, in which the cells were treated with 2.5 µg/mL of rituximab. **C.** The graph shows the concentration of live 7-AAD$^-$ cells in the samples, measured by the BD Accuri C6 Plus as number of events/µL. **Results:** Rituximab-induced dose-dependent lysis of Daudi cells in the presence of normal human serum was demonstrated by the reduction in the percentages (Figure 1A) and numbers of live (7-AAD$^-$) cells (Figures 2B–C, blue histogram and curve). This effect was inhibited upon heat-inactivation of the serum (orange histogram and curve), suggesting a role for complement in rituximab-mediated cell death.

For the receptor-occupancy assay, we first tested and verified that PE Mouse anti-human CD20, clone L27 (PE-L27) competed with rituximab for binding to CD20 (data not shown). This fluorochrome-conjugated antibody was then used in combination with the BD Quantibrite™ PE Phycoerythrin Fluorescence Quantitation kit to estimate the number of rituximab molecules binding to the target cells (Figure 2A).

The quantity of rituximab bound per cell was calculated in two steps. First, Daudi cells were stained with PE-L27 only and the BD Quantibrite PE Fluorescence Quantitation beads were used to quantify the total number of CD20 receptors expressed per cell (represented by vertical bars). In a separate experiment, cells were first incubated with increasing doses of rituximab, followed by staining with the PE-L27 antibody. In this setup, only CD20 receptors unoccupied by rituximab were available for binding to the PE-L27 antibody (gray bar segments). The number of rituximab-occupied receptors was then calculated by subtracting the level of unoccupied CD20 receptors from the total CD20 expression (blue bar segments). Figure 2B demonstrates the inverse correlation between rituximab occupancy and cell viability.

Figure 2A

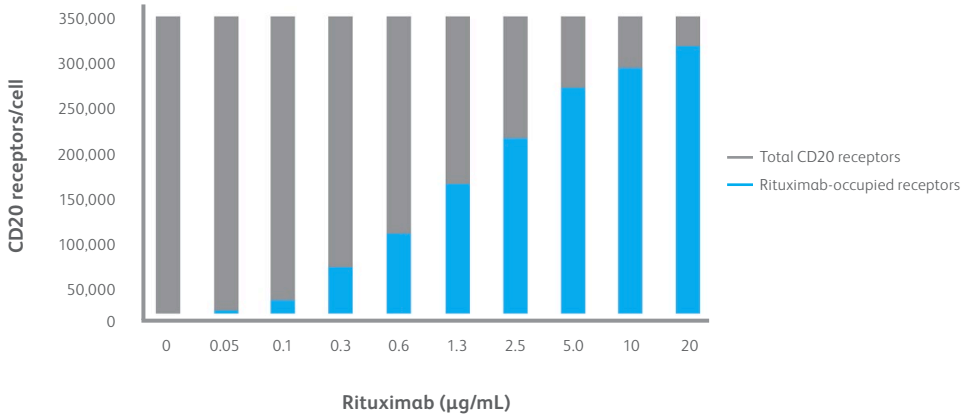

Figure 2B

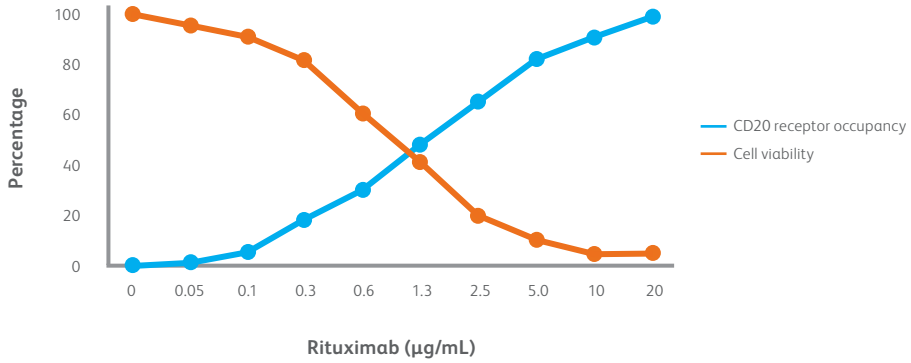

Figure 2. Correlation between rituximab-mediated Daudi-cell cytolysis and CD20 receptor density and occupancy
Daudi cells were counted using the BD Accuri C6 Plus and $1x10^5$ cells were incubated with twofold serial dilutions of rituximab (ranging from 0.05 µg/mL to 20 µg/mL) at 37°C for 30 min. The cells were washed and stained with PE Mouse Anti-Human CD20, clone L27 (PE-L27), which competes with rituximab for binding to CD20, as previously determined (data not shown). In parallel, the BD Quantibrite PE Phycoerythrin Fluorescence Quantitation kit was used to calculate the number of anti-CD20 molecules bound per Daudi cell. Additionally, rituximab-coated cells were cultured at 37°C for 5 hours in the presence of 25% normal human serum to analyze cell viability. **A.** The numbers of anti-CD20 (PE-L27) bound per cell appear gray, while the calculated numbers of CD20 receptors occupied by rituximab appear blue in the graph. **B.** The graph shows a correlation between CD20 receptor occupancy and cell viability. The blue curve represents the percentages of CD20 receptors occupied by rituximab, while the orange curve represents the percentages of 7-AAD$^-$ live cells relative to cells cultured without rituximab (100% cell viability). **Results:** The BD Quantibrite PE Phycoerythrin Fluorescence Quantitation kit combined with PE-L27 enabled the calculation of the total numbers of anti-CD20 bound per cell and the CD20 receptors bound to rituximab. Cell viability decreased in proportion to receptor occupancy, demonstrating that the level of cell surface CD20 is a critical factor in rituximab-driven target cell lysis.

Lastly, we analyzed rituximab-mediated and complement-dependent B-cell killing using peripheral blood mononuclear cells (PBMCs). As shown in Figure 3A, virtually all CD20 receptors were occupied by rituximab, and a minimal level of free receptors was detected by PE-L27. Therefore, to detect B cells treated with rituximab the cells were stained with anti-CD19. The volumetric counting capability of the BD Accuri C6 Plus system was used to determine the absolute numbers of $CD19^+$ B-cell and $CD3^+$ T-cell subsets. Rituximab treatment led to significant B-cell-specific cell killing, as demonstrated by the decrease in $CD19^+/CD3^+$ ratio with increasing doses of rituximab (Figure 3B). The same experiment performed with an isotype control did not affect the $CD19^+/CD3^+$ ratio, confirming the role of rituximab in mediating B-cell killing.

Figure 3A

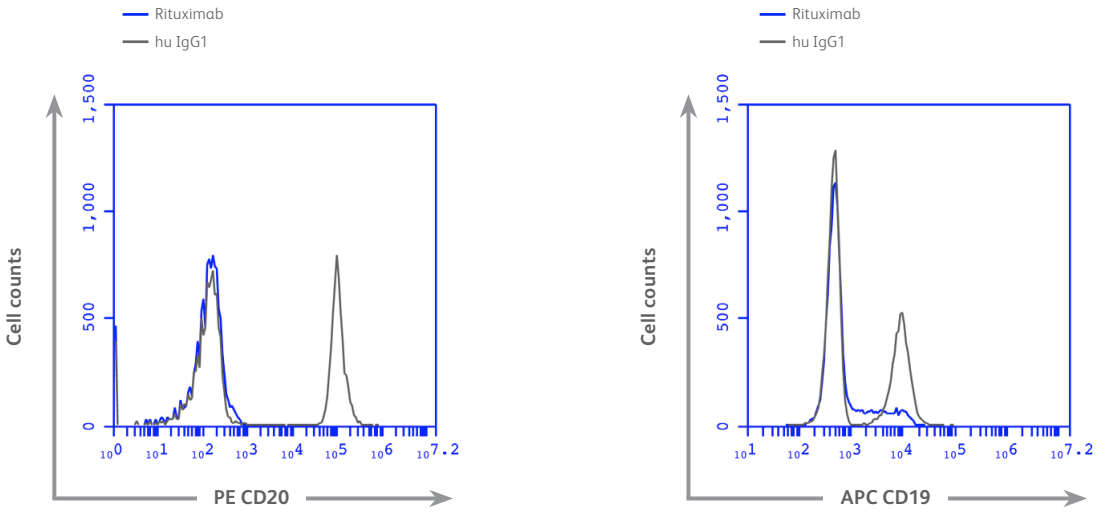

Figure 3B

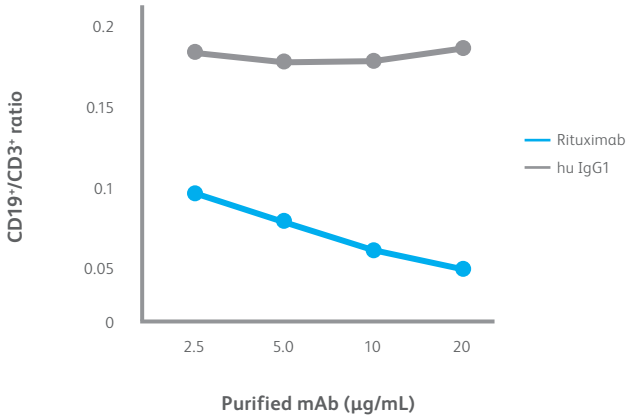

Figure 3. Rituximab-mediated CDC against primary B cells

PBMCs were cultured for 5 hours with different concentrations of rituximab or isotype control supplemented with 25% normal human serum, as described in Figure 1. The cells were stained with BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD3, BD Pharmingen™ APC Mouse Anti-Human CD19 and PE Mouse Anti-Human CD20, clone L27. Live cells were gated based on cell size discrimination and then $CD3^-$ cells were gated for analysis of either CD20 or CD19 expression. **A.** Histogram overlays show CD20 or CD19 expression in cells cultured with 10 µg/mL of isotype control (gray histogram) or rituximab (blue histogram). **B.** The graph represents ratios between absolute numbers of $CD19^+$ and $CD3^+$ cells in cultures with increasing doses of rituximab or isotype control. **Results:** Rituximab treatment led to the loss of $CD19^+$ B cells, demonstrated by lower $CD19^+/CD3^+$ ratios in the samples treated with rituximab (blue curve) compared to samples treated with isotype control (gray curve).

Ordering information

Systems and software
Description
BD Accuri™ C6 Plus Flow Cytometer System
BD Accuri™ C6 Plus Workstation Computer and Software
BD CSampler™ Plus Automated Sampling System (optional)

Reagents	
Description	Cat. No.
PE Mouse Anti-Human CD20 (clone L27)	346595
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD3	560835
BD Pharmingen™ APC Mouse Anti-Human CD19	555415
BD Quantibrite™ PE Phycoerythrin Fluorescence Quantitation Kit	340495
BD Horizon™ 7-AAD	559925

Class 1 Laser Product.

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