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Determination of the Inhibitory Activity of Ibrutinib on Cytokine Secretion by Primary Monocytes Using a Bead-Based Multiplex Assay

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Introduction

Flow cytometry is an established method used in bead-based multiplexing assays. It allows the simultaneous measurement of multiple targets in a single sample. Quantitative measurements of secreted and intracellular proteins, including cytokines, chemokines, growth factors, and phosphorylated cell signaling proteins, have broad applications in basic research. This application note describes how to use the Agilent NovoCyte flow cytometer with a commercially available bead-based multiplexing assay, designed to measure cytokine production.

Bead-based flow cytometry-based detection of cytokines provides a method for measuring multiple soluble analytes in a single sample using a mixture of bead populations with varying fluorescence intensities. Each bead population in the kit has been conjugated with a capture antibody, specific for a single human cytokine. Cytokine-specific phycoerythrin (PE)-conjugated antibodies serve as detection reagents, generating fluorescent signals proportional to the amount of bound analyte. After the capture beads and detection antibodies are incubated with samples containing soluble cytokines, the complexes are then measured using flow cytometry to resolve particles with fluorescence characteristics of both the bead and the detection antibody.

Agilent NovoCyte flow cytometers combine sensitive detection and accurate resolution with multiple fluorescence detection channels, making quantification of soluble cytokines using a bead-based multiplex assay a straight-forward approach. The Becton-Dickinson (BD) CBA Human Inflammatory Cytokines kit was used to determine the cytokine secretion by primary human monocytes and the inhibitory activity of Ibrutinib on cytokine production. Ibrutinib is a highly selective Bruton's tyrosine kinase (BTK) inhibitor, primarily used for the treatment of a rare and aggressive leukemia, mantle cell lymphoma. Ibrutinib irreversibly binds BTK in various immune cells, which inhibits cytokine secretion. The BD CBA Human Inflammatory Cytokines kit used in this research study provides detection antibodies specific for the pro-inflammatory cytokines IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12p70.

Cytokine standard curves

Representative plots identifying the cytokine-specific beads based on APC fluorescence are shown in Figure 1. Detection reagents with PE fluorescence are used to detect cytokine abundance. To determine cytokine concentrations in unknown samples, a standard curve for each cytokine was generated using 1:3 serially diluted standards covering a concentration range of 20 to 5,000 pg/mL. Differing concentrations of bound analyte based on PE fluorescence are shown in Figure 2. A five-parameter curve fit was applied and the fitted curve was then used for extrapolating the concentration of each analyte in the sample. PE fluorescence intensity was determined to be proportional to the concentration of the cytokine standard, and the R^2 value of each standard curve was above 0.999.
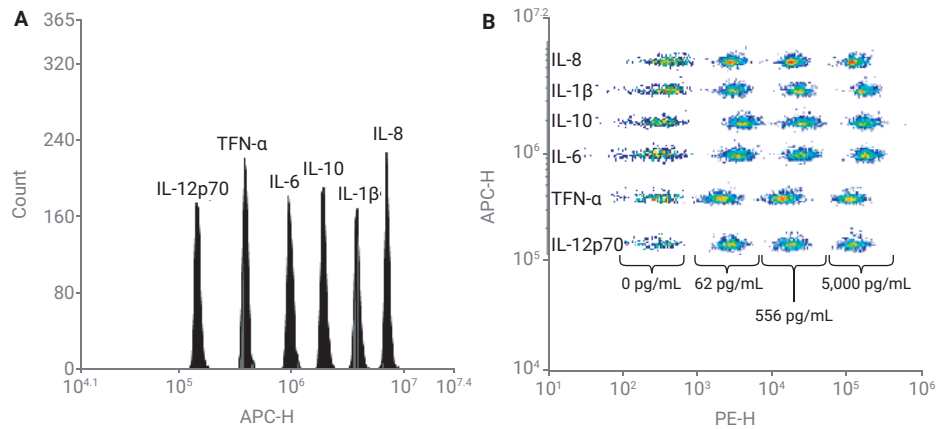

Figure 1. Resolution of bead populations on an Agilent NovoCyte flow cytometer. (A) Histogram plot of antibody-coated capture beads using the APC detection channel (Ex 640 nm/Em 675 nm). From left to right (lowest to highest APC MFI), beads coated with antibodies against IL-12p70, TNF-α, IL-6, IL-10, IL-1β, and IL-8, respectively. (B) Two-parameter plot of the indicated cytokines (APC channel) versus increasing concentrations of the corresponding standard cytokines (PE channel, Ex 488 nm/Em 572 nm). Four different standard concentrations are overlayered where the PE MFI corresponding to different concentrations of diluted standards versus APC fluorescence of the different bead populations is shown for a few standard concentrations. The fluorescence ranges of capture beads and analytes had good separation using the NovoCyte flow cytometer.
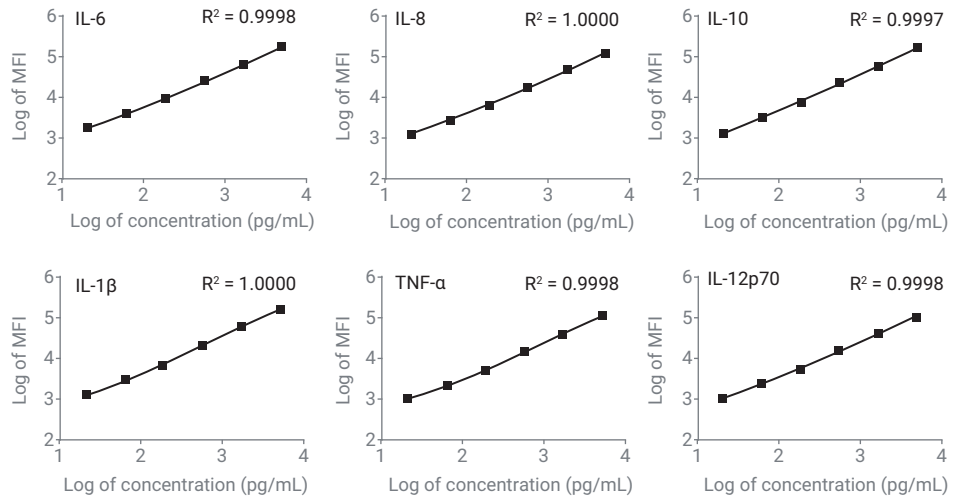

Figure 2. Cytokine standard curves generated using the Agilent NovoCyte flow cytometer. Initial data analysis was performed using Agilent NovoExpress acquisition and analysis software. A standard curve for each cytokine was obtained by plotting the concentration of cytokine standard versus the MFI (mean fluorescence intensity) obtained using the NovoCyte. A curve was fitted to the data points, and R^2 values are reported on each plot.

Effect of Ibrutinib on cytokine secretion

Monocytes were obtained from primary PBMCs using magnetic bead sorting (EasySep Human CD14 Positive Selection Kit, StemCell Technologies). Purified monocytes were then seeded in IgG-coated plates to stimulate pro-inflammatory cytokine secretion and treated with different concentrations of the BTK inhibitor Ibrutinib. After 18 hours, supernatants were processed following the CBA protocol. Briefly, supernatants were mixed with the pool of antibody-coated beads, washed, then added to the antibody-PE cytokine detection reagent. They were then washed again, and analyzed on a NovoCyte flow cytometer to quantify secreted cytokines under different treatment conditions. As shown in Figure 3, Ibrutinib inhibited the secretion of IL-8, IL-1b, IL-6 and TNF-α in primary monocytes in a dose-dependent manner. The IC_{50} values for IL-8, IL-1β, IL-6 and TNF-α were at 2.5 µM, 0.65 nM, 0.28 nM, and 0.69 nM, respectively (Figure 3A).

IL-10 and IL-12p70 were not induced during IgG stimulation. Raw data plots for the different treatment conditions are shown in Figure 3B. Interestingly, IL-8 production was induced by DMSO treatment alone and even more so with DMSO and IgG stimulation. Even though it is difficult to visualize using the log scale and high IL-8 background levels with DMSO alone, there was a dose-dependent effect with increasing Ibrutinib concentrations (Figures 3A and 3B).
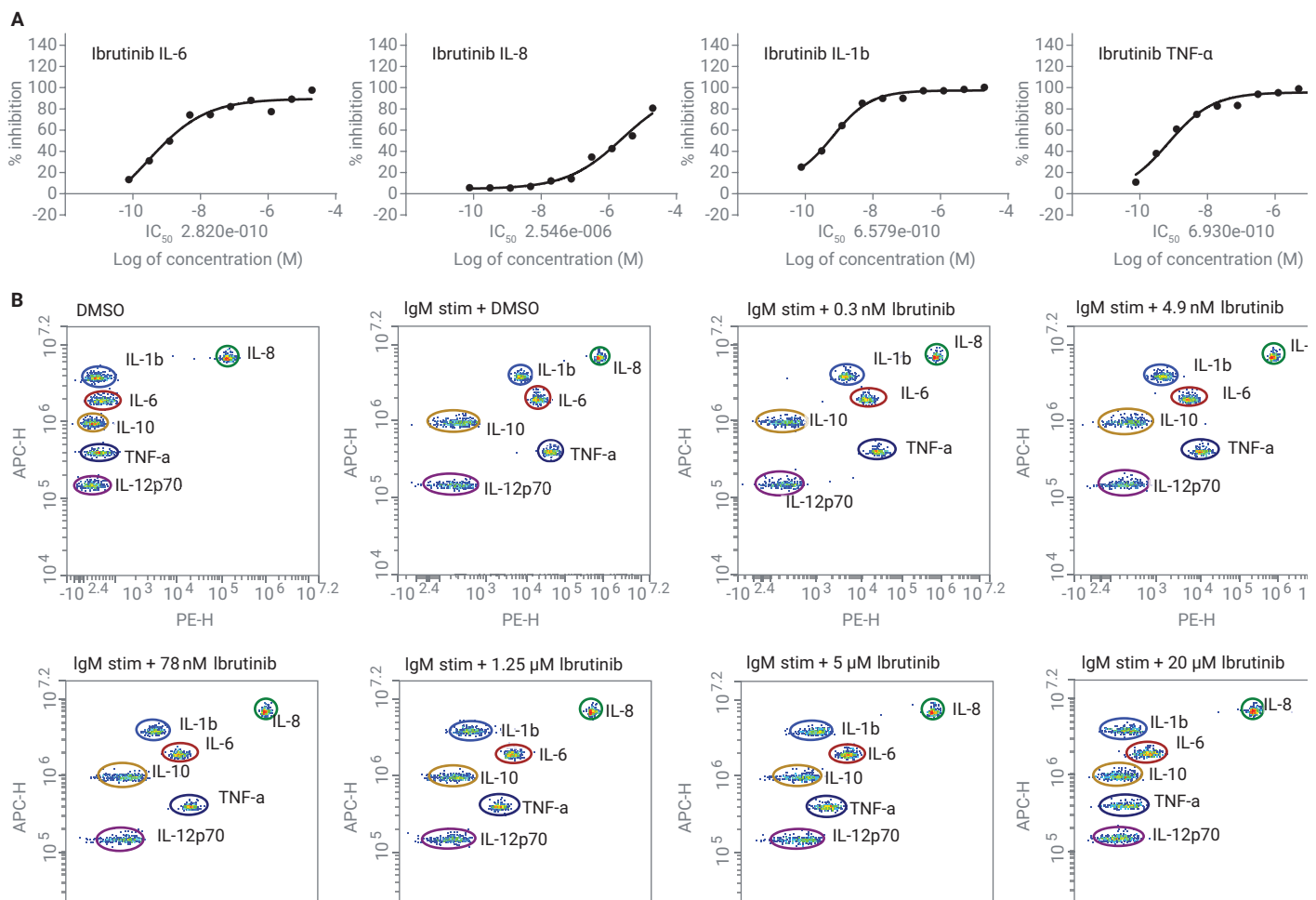

Figure 3. Ibrutinib inhibition of IL-8, IL-1b, IL-6 and TNF-α secretion in primary human monocytes. (A) Ibrutinib inhibited the secretion of IL-8, IL-1β, IL-6, and TNF-α in a dose-dependent manner. IC_{50} values were determined using Prism 6 software (GraphPad). (B) Two-parameter plots showing the effect of different Ibrutinib concentrations on the IgG-induced cytokine secretion profile in primary human monocytes. Bead populations specific to individual cytokines are identified by varying APC fluorescence, while the concentration of bound analyte is measured using PE fluorescence.

Conclusion

Flow cytometry is a sensitive, powerful, and quantitative tool for measuring analyte concentrations. This research study demonstrates the simultaneous analysis of six cytokines from cell culture supernatants and detects changes in concentration caused by the addition of a small molecule inhibitor. Using this method of analyte detection, research laboratories can increase their multiplexing capabilities and decrease required sample volumes. Current commercially available kits for this type of assay can include up to 30 different analyte measurments simultaneously. Combining multiplexed analyte detection with an easy-to-use flow cytometer, such as the NovoCyte, can provide faster and more accurate results.

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