APPLICATION NOTE

# High Performance AlphaLISA Cytokine Detection Kits in PBMC supernatants


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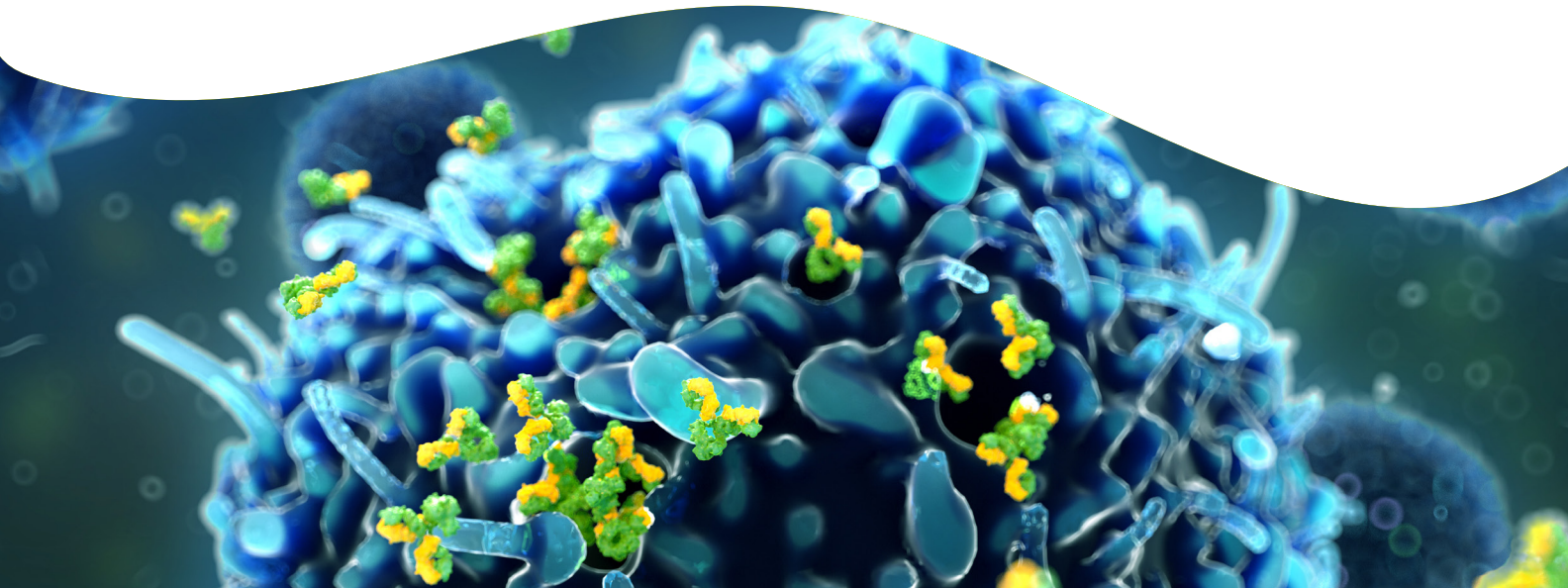
## Introduction

Detecting the presence of cytokines, small proteins that are secreted by cells to mediate an immune or inflammatory response, can help researchers better understand how the immune system responds to invading pathogens. The functions of cytokines are diverse and include roles in normal T-cell-mediated immunity, the inflammatory response, cancer, autoimmunity, and allergies. Therefore, various pathologic conditions are accompanied by changes in cytokine levels.[1] Cytokine production by peripheral blood mononuclear cells (PBMC) *in vitro* can be an important and reliable measure of immunocompetence. PBMC are a mixture of mononuclear immune cells containing monocytes, dendritic cells, and lymphocytes such as T cells, B cells, and NK cells. PBMC are often used as an *in vitro* model system for the screening and identification of new drug candidates aiming to target immune pathways. The ability to measure cytokine secretion from such cell models requires assays that are both sensitive and robust. A powerful immunoassay technology available to detect cytokines is AlphaLISA™.

AlphaLISA technology is a fast, no-wash assay platform that can be performed in a microplate format for both simple and complex matrices. AlphaLISA technology allows for the detection of molecules of interest such as cytokines in assay buffer, cell culture media, serum, and plasma, in a highly sensitive, quantitative, reproducible, and user-friendly workflow. AlphaLISA assays require two bead types: Donor beads and Acceptor beads. The Donor beads generate singlet oxygen upon illumination at 680 nm. The singlet oxygen can diffuse approximately 200 nm in solution. If an Acceptor bead is within



that distance, energy is transferred to the Acceptor bead, resulting in light production. If an Acceptor bead is not in proximity to a Donor bead, little to no signal is produced over background. The amount of AlphaLISA assay signal generated is proportional to the amount of cytokine present in the sample. An example of an AlphaLISA detection assay is shown in Figure 1 with streptavidin-coated Donor beads, a biotinylated anti-analyte antibody, and a different anti-analyte antibody conjugated to Acceptor beads combined into a sandwich assay format.

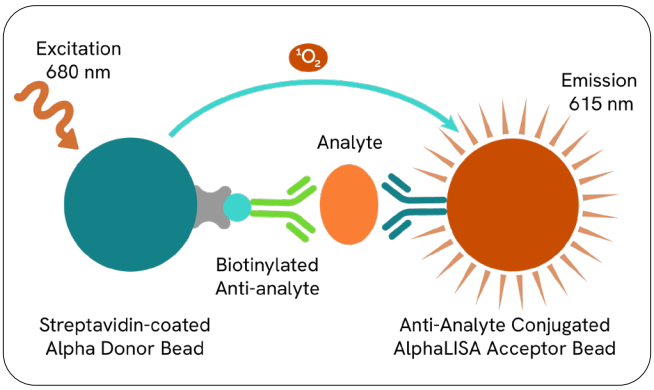



Figure 1. AlphaLISA Schematic. Anti-analyte sandwich assay format utilizing biotin-streptavidin interaction.

The AlphaLISA High Performance Cytokine Detection Kits are the newest generation of AlphaLISA kits for cytokine detection, developed by selecting high performing and highly specific monoclonal antibody pairs for each assay. In this application note, we evaluated the performance of the new AlphaLISA High Performance Cytokine Detection Kits relative to the original AlphaLISA cytokine kits. We assessed the sensitivity of detection by calculating the lower limit of detection (LDL), the lower limit of quantification (LLOQ), as well as the signal-to-background (S/B) ratio. In addition to performing the assays on recombinant proteins, we measured endogenous cytokine production from PBMC with various stimulation conditions. By evaluating the dilutional linearity of the endogenous samples, we were able to compare the sensitivity of each cytokine kit tested (High Performance vs original kit offering) by analyzing the lowest detectable dilution of the cytokine in the PBMC supernatant. This application note therefore demonstrates the further improvements to sensitivity and detection that were achieved by the next generation of AlphaLISA High Performance Cytokine kits across several biological targets.

## Materials and methods

### Cell culture and stimulation conditions

PBMC were isolated using Ficoll gradient (Cytiva, #17-5442-02) from Buffy Coat (French National Blood Service [EFS Pyrénées-Méditerranée], #B3111) following the procedure outlined in the Revvity Technical Note: Guidelines from PBMC Isolation to Cytokine Assay Optimisation.[2] Subsequently, isolated PBMC were grown in RPMI media (Thermo Fisher, #61870-036) supplemented with 10% Fetal Bovine Serum (Eurobio, #CVFSVF00-01). For stimulation of all cytokines, cells were counted and plated at a density of 200,000 cells/well in a 96-well assay plate (#6005680). To induce secretion of cytokines, PBMC were treated overnight with either lipopolysaccharides (LPS, Sigma Aldrich, #L4391) at 2 µg/mL or a combination of phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, #L1585) at 50 µg/mL with ionomycin (Sigma Aldrich, #I0634) at 1 µg/mL. After overnight stimulation, cell supernatant was carefully collected for subsequent measurement of cytokine levels present in the sample. AlphaLISA assays require just 5 µL of sample for testing in each well, allowing ample material for replicate testing.

### AlphaLISA kits

The following High Performance Cytokine Detection Kits were used: Human IFN-γ (#AL3153), Human IL-2 (#AL3155), Human TNF-α (#AL3157), Human IL-10 (#AL3159), Human IL-1β (#AL3160), Human IL-17A (#AL3161), Human CXCL10 (#AL3163), and Human IL-8 (#AL3164).

In addition, the results from the High Performance Cytokine Detection Kits were compared to the corresponding original product kits: Human IFN-γ (#AL217), Human IL-2 (#AL221), Human TNF-α (#AL208), Human IL-10 (#AL218), Human IL-1β (#AL220), Human IL-17A (#AL219), Human CXCL10 (#AL259), and Human IL-8 (#AL224).

Cytokine kit performance was determined by comparing the standard curves generated with recombinant protein in immunoassay buffer or HiBlock buffer, as provided with each optimized kit. Performance was further analyzed by using the appropriate stimulated PBMC supernatant for each AlphaLISA target and testing 2-fold serial dilutions until the signal reached baseline. The lowest sample concentration that fell above the LLOQ of the assay was noted for each kit. This method of sample dilution can be used to demonstrate the sensitivity of detection on endogenous samples.

**Instrument and data analysis**

Assay data were collected on an EnVision™ multimode plate reader with instrument settings in place to measure the AlphaLISA signal. Data were analyzed using GraphPad Prism® software. The standard curve from each kit was plotted using a nonlinear regression with a four-parameter logistic equation (sigmoidal dose-response curve with variable slope and $1/y^2$ weighting). The LDL was calculated from 12 blanks and interpolated from the standard curve using the following equation: LDL signal = Average blanks + (3 x Standard Deviation blanks). The LLOQ was calculated from 12 blanks and interpolated from the standard curve using the following equation: LLOQ signal = Average blanks + (10 x Standard Deviation blanks). The LLOQ value is the more biologically relevant measurement of sensitivity as it marks the lowest level at which the analyte of interest can accurately be detected in a sample by the assay.

## Results

For each cytokine assay kit (High Performance or original kit), the standard curve was run in the supplied buffer from each kit for determination of sensitivity (LDL & LLOQ) and in cell culture media for interpolation of unknown sample concentration in the PBMC supernatants. Shown in Figure 2 is a comparison of the standard curve for Human IL-2 from each kit offering prepared in immunoassay buffer. With the change to a new pair of monoclonal antibodies selected for detection in the High Performance cytokine assay kit there is an improvement in the overall signal-to-background as seen by the higher AlphaLISA signal achieved with the High Performance kit.

With some cytokine targets gaining an increase in S/B relative to the original kits, this affected the amount of analyte required for the highest concentration of the standard curve. In a few cases, the increased sensitivity led

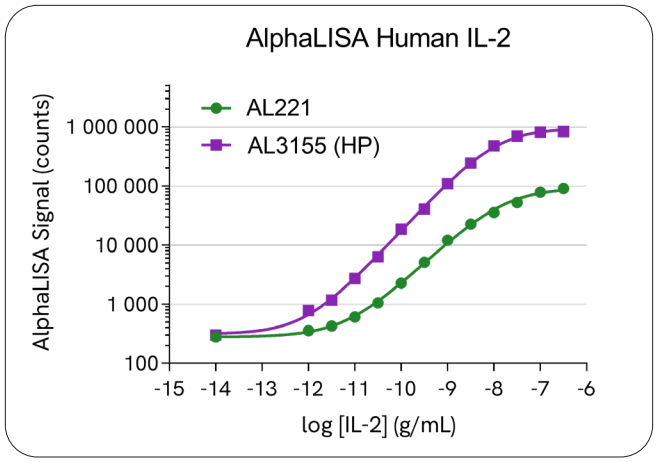



Figure 2. Comparison of the Recombinant Protein Standard Curve for Human IL-2 in Immunoassay Buffer. The standard curve was run for each cytokine kit (High Performance or original) for determination of changes to signal-to-background as well as improvement of the LDL and LLOQ. Human IL-2 results shown as a representation of the results for all kits tested.

to a hook point at the highest concentration and therefore the amount of supplied analyte for the standard curve was reduced. This resulted in the left-shift of the dose-response curve and the inclusion of an additional dilution point on the curve at the low end. Figure 3 shows an example of this with the standard curves for Human IL-8 from the High Performance cytokine kit and the original kit.

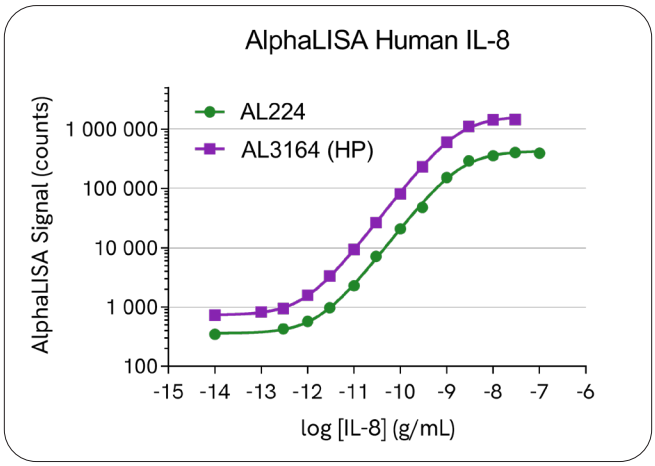



Figure 3. Comparison of Analyte Standard Curve for Human IL-8 in Immunoassay Buffer. The top concentration of the standard curve is reduced in the High Performance cytokine kit while an additional dilution is prepared at the low end of the curve to compensate for the increased sensitivity.

Similar changes were made to the amount of analyte required to generate the standard curve for the High Performance cytokine kits CXCL10, IL-17A, IL-1β and IFN-γ as shown in Table 1.

Table 1. Analyte Stock Provided with Each Kit. Adjustments to the analyte amount supplied were made for five kits.

| Cytokine Target | Previous Part Number | Previous Analyte (µg) | New Part Number | New Analyte (µg) |
|---|---|---|---|---|
| IL-8 | AL224 | 0.1 | AL3164 | 0.03 |
| CXCL10 | AL259 | 0.3 | AL3163 | 0.1 |
| IL-17A | AL219 | 1 | AL3161 | 0.3 |
| IL-1β | AL220 | 0.1 | AL3160 | 0.03 |
| IFN-γ | AL217 | 0.3 | AL3153 | 0.1 |

After collecting the data for all the cytokines tested, the LDL and LLOQ values were determined from the standard curve using the average of the blank replicates. Table 2 demonstrates the improvements seen in the sensitivity for the new High Performance cytokine kits relative to the original kits ranging from a 1.8 to 35-fold decrease in the LLOQ.

Table 2. Lower Limit of Detection and Lower Limit of Quantification for Both Kit types.

| Cytokine Target | Previous LDL (pg/mL) | New LDL (pg/mL) | Previous LLOQ (pg/mL) | New LLOQ (pg/mL) |
|---|---|---|---|---|
| IL-8 | 0.24 | 0.11 | 0.91 | 0.36 |
| CXCL10 | 2.42 | 0.26 | 7.27 | 2.39 |
| IL-17A | 6.2 | 1.78 | 15.06 | 6.17 |
| IL-1β | 25.25 | 0.89 | 92.1 | 2.58 |
| IL-10 | 1.06 | 0.34 | 3.3 | 1.1 |
| TNF-α | 1.2 | 0.86 | 4.7 | 2.62 |
| IL-2 | 0.78 | 0.12 | 2.85 | 0.97 |
| IFN-γ | 3.13 | 0.2 | 11.5 | 0.65 |

When looking for additional performance improvements of the High Performance cytokine kits, we compared the S/B results of the undiluted PBMC samples run in RPMI media. Figure 4 shows that four of the eight High Performance cytokine kits had improved performance with endogenous samples relative to the original kits with the largest increase in S/B seen with Human IL-2.

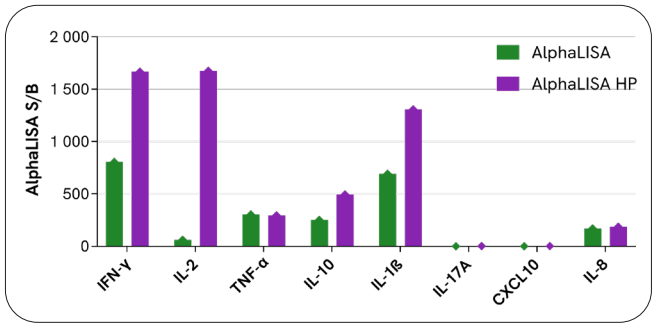



Figure 4. Signal-to-Background Results. S/B was calculated for each kit on the undiluted, stimulated PBMC sample. Increases in the S/B were seen with four of the eight kits tested.

Another method to gauge sensitivity of an AlphaLISA assay is to perform serial dilutions on the stimulated PBMC samples and determine the lowest dilution that yields signal above the LLOQ of the assay. PBMC were stimulated overnight with a combination of PMA and ionomycin to induce the production of IFN-γ. In the results shown with the IFN-γ kit comparison in Figure 5, the original kit loses sensitivity (dilution at which endogenous sample can be detected) at 1:128 (green arrow) whereas the High Performance cytokine kit retains sensitivity for five more serial dilutions reaching a final dilution of 1:4096 (purple arrow).

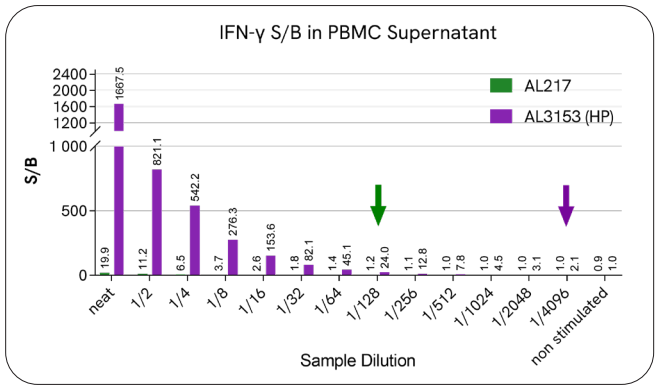



Figure 5. PBMC Serial Dilution Result for IFN-γ Detection. Increased sensitivity of the IFN-γ High Performance cytokine kit is demonstrated by improving on the lowest dilution tested that yields an AlphaLISA signal above the LLOQ of the assay. Arrows mark the LLOQ for each assay.

In another example of increased sensitivity of an AlphaLISA High Performance cytokine kit, Human IL-2 results for the PBMC sample dilution testing are seen in Figure 6. PBMC were stimulated overnight with a combination of PMA and ionomycin to induce the production of IL-2. The original Human IL-2 kit loses sensitivity at 1:512 (green arrow) whereas the High Performance cytokine kit retains sensitivity for two more serial dilutions reaching a final dilution of 1:2048 (purple arrow).

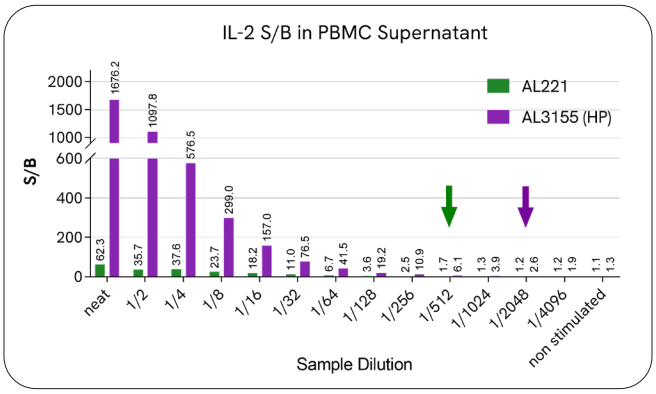



Figure 6. PBMC Serial Dilution Result for IL-2 Detection. Increased sensitivity of the IL-2 High Performance cytokine kit is demonstrated by improving on the lowest dilution tested that yields an AlphaLISA signal above the LLOQ of the assay. Arrows mark the LLOQ for each assay.

## Conclusion

Improvements in sensitivity were seen for the AlphaLISA High Performance Cytokine Detection Kits relative to the original kits when assessing both LDL and LLOQ calculated from the recombinant protein standard curve (Table 2) and increased ability to further dilute endogenous PBMC supernatant samples and detect signal above the LLOQ of the assay (Figures 5 and 6). Also, four of the eight High Performance cytokine kits tested displayed improved signal-to-background on the undiluted PBMC supernatants (Figure 4). This increase in sensitivity resulted in a change in some kits to the amount of analyte required to generate the top dose and subsequent standard curve (Table 1). In addition to the kits shown here, biotin-free kits which do not rely on the streptavidin-biotin reaction were further improved for Human IL-8, Human IL-17A, Human TNF-α, Human IL-2, and Human IFN-γ. Similar improvements to sensitivity (lower LDL and LLOQ) were seen. Biotin-Free kits are generally less sensitive than traditional AlphaLISA kits but are impervious to biotin interference from endogenous biotin in samples, such as that found in cell culture media, making them a good option when biotin levels are high. Another added benefit of the newly developed AlphaLISA High Performance Cytokine Detection Kits is the increased shelf life, with most kits improving from 12 months after date of manufacture to 24 months of stability. Taken together, improvements in assay sensitivity and stability make the AlphaLISA High Performance Cytokine Detection Kits attractive options for cytokine research, enabling the accurate detection of endogenous cytokines in simple to complex matrices with small sample volumes using AlphaLISA technology.

## References

1. Foster, JR (2001) The functions of cytokines and their uses in toxicology. *International Journal of Experimental Pathology;* 82(3): 171–192

2. Revvity Technical Note: Guidelines from PBMC Isolation to Cytokine Assay Optimisation.

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