APPLICATION NOTE

# AlphaLISA human IL-6 kit can quantify pyrogens in monocyte activation test.


## Author

Bagna Bao, Ph.D.,

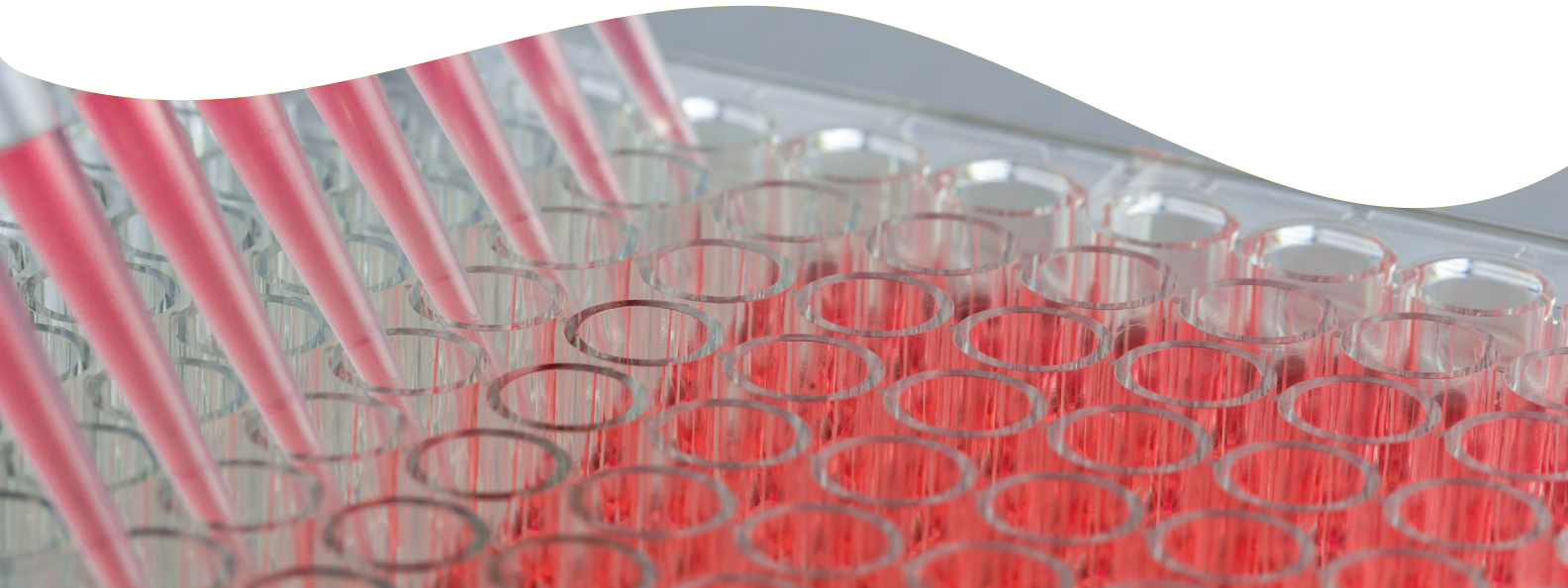
Revvity, Inc.


## Introduction

Pyrogens are substances that induce fever in human and animals and can be either endogenous or exogenous. Endogenous pyrogens such as interleukins are produced by the body's immune system while the exogenous pyrogens are produced by microorganisms such as bacteria, viruses or fungi. The exogenous pyrogens are classified into two groups, endotoxin and non-endotoxin pyrogens. Endotoxins, potent fever inducing substances in human and in animals, originate from gram-negative bacteria while the Non-Endotoxin Pyrogens (NEPs) originate from gram-positive bacteria, viruses or fungi. It is an important regulatory requirement to test pharmaceutical products for pyrogens to ensure product quality and patient safety. The purpose of the test is to show that the amounts of pyrogens contained in a product do not exceed the contaminant limit concentration.

There are two methods that are widely used for pyrogen testing[1], the Rabbit Pyrogen Test (RPT) and the Bacterial Endotoxin Test (BET). The RPT test involves the intravenous injection of pharmaceutical products and measuring the body temperature in rabbits. Although it detects all types of pyrogenic materials, the test can miss human-specific pyrogens, does not allow for quantification, and requires the use of animals. BET test, also called Limulus Amebocyte Lysate (LAL) test, requires the use of blood from the horseshoe crabs and does not detect NEPs. Both tests have their limitations, such as qualitative results, requiring the use of animals, or collection of the blood from horseshoe crabs, and potential for missing human-specific pyrogens.





A third method, the Monocyte Activation Test (MAT), has been developed in recent years to detect and quantify endotoxin and NEP contaminations without using animals.[1-5] The assay is based on human immune cell responses to pyrogens in drug products by measuring the cytokine production from human Peripheral Blood Mononuclear Cells (PBMCs). Monocytes are the main cells within the PBMC population that produce cytokines in the presence of pyrogens, hence the name Monocyte Activation Test. Monocytic cells are known to play a critical role in the innate immune system. These cells express receptors (e.g. toll-like receptors) that bind specifically to exogenous pyrogens resulting in the release of cytokines (e.g. interleukins IL-1β, IL-6, IL-8, and TNFα), which act as endogenous pyrogens to initiate inflammatory responses that includes fever induction.

MAT involves the incubation of the test samples and a reference standard endotoxin with human monocytes followed by the analysis of IL-6 release using PyroMAT IL-6 ELISA Kit. A reference standard was used as a standard to quantify the amount of endotoxin in the samples. The MAT takes two full days to complete using the traditional high-volume wash-based ELISA method. This application note demonstrates the utility of the AlphaLISA™ IL-6 Kit to quantify pyrogens and the ease of using a homogeneous no wash assay with low sample volumes.

AlphaLISA technology allows for the detection and quantification of molecules of interest in buffer, cell culture media, serum, plasma, and other biological matrices in a homogeneous (no wash), highly sensitive, easy-to-use, and reproducible manner.[6,7] In an AlphaLISA assay, a biotinylated anti-analyte antibody binds to the streptavidin-coated Alpha Donor beads, while another anti-analyte antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into proximity. The excitation of the Donor beads at 680 nm converts ambient oxygen into singlet oxygen molecules that trigger a cascade of energy transfer within the Acceptor beads, resulting in light emission at 615 nm which can be detected on an Alpha-enabled reader, such as the EnVision™ Multilabel Plate Reader. The amount of light emission is proportional to the level of analyte present in the test samples. Figure 1 illustrates the AlphaLISA IL-6 kit in which IL-6, the analyte of interest, is recognized by two antibodies, a biotinylated anti-IL-6 antibody and anti-IL-6 antibody conjugated AlphaLISA Acceptor beads.

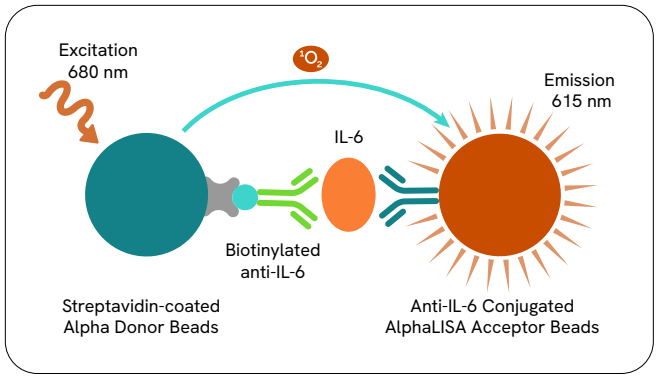



Figure 1. Schematic of AlphaLISA IL-6 Assay Principle.

## Materials and methods

### Reagents and assay kits

Cryopreserved Mono-Mac-6 cells (Millipore Sigma, Cat# PYROMAT), International Reference Standard Endotoxin (Millipore Sigma, Cat# 144160001), Endotoxin (Sigma, Cat# 1235503), Non-Endotoxin Pyrogen (NEP) Control (Millipore Sigma, Cat# MATHKSA), and RPMI 1640 (ATCC, Cat # 30-2001) were bought and stored at the recommended temperature until they were used for the assays. The kits evaluated were AlphaLISA Human IL-6 kit (Revvity, Cat# AL223) and PyroMAT IL-6 ELISA kit (Millipore Sigma, Cat# PYROMATKIT) using TopSeal™-A Plus Adhesive Sealing Film (Cat # 6050185), and 96-well ½ AreaPlate (Revvity, Cat# 6002350).

### Cell cultures

Mono-Mac-6 cells were cultured in RPMI without FBS following the quantitative test protocol (Method A) provided in PyroMAT System. The cells, Reference Standard Endotoxins (RSE), and NEPs (HKSA= Heat-Killed Staphylococcus Aureus, a Gram-positive, round-shaped bacterium) were prepared as instructed in the PyroMAT System. To stimulate the cells, 50 µL/well of endotoxin standard concentrations (0.00, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 EU/mL; n=3 per EU/mL) or NEP (0x, 1x, 10x, 100x ; n=3 per dose) were added to cell culture plates first and then 200 µL of cells (7500 live)/well were added. The cell culture plates were incubated for 22 ± 2 hours at 37 °C in a humidified atmosphere (a reservoir with purified water was placed in the incubator in advance) without $CO_2$. In presence of pyrogens, the Mono-Mac-6 cells produce cytokines. Upon the completion of the cultures, the cell culture supernatants were transferred to 96-well sample storage plates that were sealed and stored at -20 °C until the assays were performed.

**Assays protocols**

AlphaLISA IL-6 Assays. AlphaLISA IL-6 assays were performed following the protocols provided in the kit technical data sheet (TDS) with modifications. All reagents were prepared in AlphaLISA immunoassay buffer (kit) except the IL-6 analyte standard dilutions that were prepared in RPMI. The highest concentration (100 ng/mL) of analyte standard was prepared by mixing 20 µL of reconstituted IL-6 analyte and 180 µL of RPMI to ensure the volume is enough for the assay. The assays were performed in 96-well ½ AreaPlate with the total assay volume of 100 µL/well. In the assay, the volume of analyte standards and cell supernatant samples was changed from 10 µL (recommended in TDS) to 40 µL. The volume of acceptor and biotin antibody mix was reduced from 40 µL (recommended in TDS) to 20 µL and streptavidin Donor beads was reduced from 50 µL (recommended in TDS) to 40 µL. The final concentrations of beads and antibodies remained the same as in the kit manual. Briefly, to a 96-well ½ Area Plate, 40 µL standards (n=2 per dilutions) or 40 µL cell supernatant samples were added first and then 20 µL 5x mix of biotinylated anti IL-6 antibody (1 nM final) and anti IL-6 Acceptor beads (10 µg/mL final) was added to each well. The plate was sealed using adhesive TopSeal film, gently tapped to mix the samples, and incubated 60 minutes at 23 °C. After incubation, 40 µL of freshly prepared 2.5x streptavidin Donor beads (40 µg/mL final) were added followed by an additional incubation for 30 minutes at 23 °C. The plate was then read on an EnVision 2105 Multilabel Plate Reader.

PyroMAT IL-6 ELISA Assays. The ELISA assay was completed using the standard protocol in PyroMAT IL-6 ELISA kit. Briefly, 100 µL/well of cell culture supernatant samples was added to a 96-well full area plate that was pre-coated with anti-IL-6 antibody followed by adding 200 µL/well of IL-6 conjugate. The plate was covered with adhesive sealing film and incubated for two hours at room temperature. The plate was then washed four times with 1x wash buffer and 200 µL/well of substrate solution was added, protected from light using black lid, and incubated for 30 minutes. Following the incubation, 50 µL/well of stop solution was added. The plate was read within 30 minutes after the addition of the stop solution to measure the optical density (OD) at 450 nm and 630 nm (reference wavelength) using an EnVision 2105 Multilabel Plate Reader. Color development is proportional to the amount of IL-6 secretion in the supernatant. The assay is designed to use the IL-6 ELISA assay OD signal to determine the levels of endotoxin units (EU/mL) in the test products by interpolating OD signals of test products to the endotoxin standard curve that was plotted using IL-6 ELISA OD signals and the endotoxin concentrations used to stimulate the cells. In PyroMAT test system, IL-6 analyte standard is not provided in PyroMAT IL-6 ELISA kit and therefore the concentrations of IL-6 cannot be determined in the cell culture samples.

**Data analysis**

AlphaLISA assay plates were read on a Revvity 2015 EnVision Multilabel Plate Reader equipped with the ALPHA option using the AlphaScreen™ standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%). Data analysis was performed using Microsoft® Excel® and GraphPad Prism. Sigmoidal dose-response (variable slope) was used for AlphaLISA IL-6 standard curve fitting to obtain the concentrations of IL-6 cell culture supernatant samples. Endotoxin standard curves for PyroMAT IL-6 ELISA and AlphaLISA IL-6 assays were plotted using the [Agonist] vs. response - Variable slope (four parameters) curve fitting method in GraphPad Prism to calculate the Lower Limit of Detection (LOD).

## Results and discussions

**AlphaLISA IL-6 assay performance and quantification of endotoxin stimulated IL-6 secretion**

IL-6 AlphaLISA assays were performed to quantify IL-6 from Mono- Mac-6 cells stimulated with increasing concentrations of endotoxin standard. IL-6 analyte standard curve was included to demonstrate the AlphaLISA IL-6 assay performance under the modified conditions and to determine if the assay is suitable to measure IL-6 concentrations in cell media samples collected from Mono-Mac-6 cells stimulated with endotoxin that was used in PyroMAT. The data show the dynamic range of the AlphaLISA assay, demonstrating how it can be used with a wide range of endotoxin concentrations. Figure 2A illustrates the IL-6 analyte standard curve produced and AlphaLISA assay performance under modified conditions in RPMI showing the assay performed well with Lower Detection Limit (LDL) of 1.86 pg/mL, which is identical to the assay performance in AlphaLISA Immunoassay Buffer, LDL = 1.3 pg/mL (TDS of AlphaLISA Human IL-6 kit).

As expected, the assay performances were not altered by changing the sample volumes, adjusting the reagent volume accordingly, and running the assay in 96-well ½ Area Plates. The dynamic range of the IL-6 AlphaLISA assay is 1.86 to 100,000 pg/mL (Figure 2A). Quantifications of IL-6 concentrations in endotoxin stimulated Mono- Mac-6 cell culture supernatant samples indicated that IL-6 was readily detected (Figure 2B) and the measured levels of IL-6 (3 to 3000 pg/ mL for 0.0125 to 0.8 EU/mL) in endotoxin stimulated cell media samples fell within the dynamic range of the AlphaLISA IL-6 assay. These results demonstrate that the AlphaLISA IL-6 kit can be used to detect and measure effects of the standard concentrations of endotoxin (0.0125 to 0.8 EU/mL) used in PyroMAT. The data also suggests that concentrations of endotoxin for cell stimulation could be increased (e.g. up to 2 EU/mL) to expand dynamic range of endotoxin standard curve that could be measured with the AlphaLISA IL-6 kit.

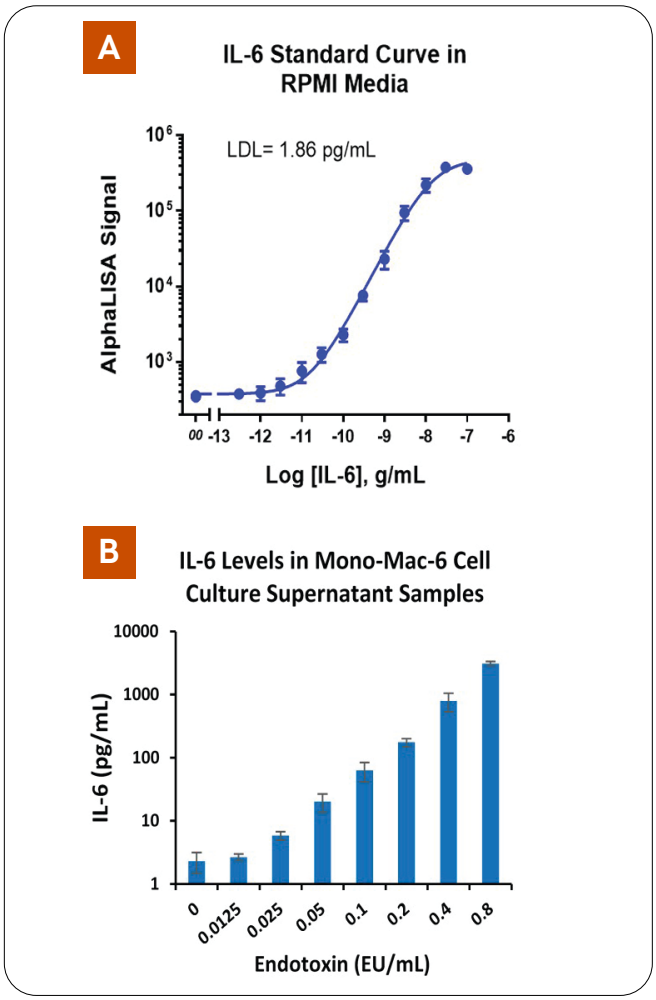



Figure 2. AlphaLISA IL-6 assay performance **(A)** and AlphaLISA IL-6 kit quantified IL-6 levels (pg/mL) in cell culture supernatant samples collected from endotoxin stimulated Mono-Mac-6 cells **(B)**.

**AlphaLISA human IL-6 kit to quantify endotoxins (pyrogens)**

PyroMAT IL-6 assays were run following the kit protocol. As indicated in Figure 3A and in Table 1, the assays worked as expected. The absolute IL-6 concentrations in cell media samples are not quantified in PyroMAT test system. Instead, a Reference Standard Endotoxin (RSE) is used to stimulate cells to generate the endotoxin standard curves for pyrogen quantification. Mono-Mac-6 cells were cultured and stimulated with increasing concentrations of reference standard endotoxin (0.0125 to 0.8 EU/mL) and the resulting cell culture supernatant samples were assayed using PyroMAT IL-6 ELISA kit. IL-6 ELISA OD signals were plotted against the concentrations of RSE (0.0125 to 0.8 EU/mL) to generate the endotoxin standard curve (Figure 3A) and OD signals of unknown products (if included in cell cultures) could be interpolated to the standards curves to quantify the amounts of endotoxins (pyrogens) present in the test products or drugs. Although the AlphaLISA human IL-6 kit includes an IL-6 analyte standard, it is not necessary to quantify the IL-6 in the samples. Instead, an AlphaLISA Endotoxin standard curve was generated similar to that of the PyroMAT IL-6 ELISA kit. To compare the PyroMAT IL-6 ELISA results (Figure 3A) to the AlphaLISA IL-6 (Figure 3B), the signals from two assays (ELISA and AlphaLISA) in response to the endotoxin stimulation are summarized in Table 1. LOD (Lower limit of detection, EU/mL) for endotoxin standard curves was obtained by interpolating the results of Average of Blank + 3*SD. Table 1 contains the LOD (EUmL), MAX (maximum) and MIN (minimum) signals, and S/B (Signal to Background ratio) for each assay. LOD of PyroMAT IL-6 ELISA test is reported to be 0.05 EU/mL in the kit data sheet. In the current test, the LOD for PyroMAT IL-6 ELISA was 0.27 EU/mL with a good S/B ratio of 93 (Figure 3A; Table 1). The results of AlphaLISA IL-6 were excellent with LOD of 0.24 EU/mL and S/B ratio of 194 (Figure 3B; Table 1) which is 2 times greater than that of PyroMAT IL-6 ELISA. Figure 3C shows the dynamic range of IL-6 ELISA and IL-6 AlphaLISA are almost identical. However, the dynamic range, S/B and MAX signal for AlphaLISA IL-6 endotoxin standard curve can be increased if higher concentrations of endotoxins are included to the cell stimulation.

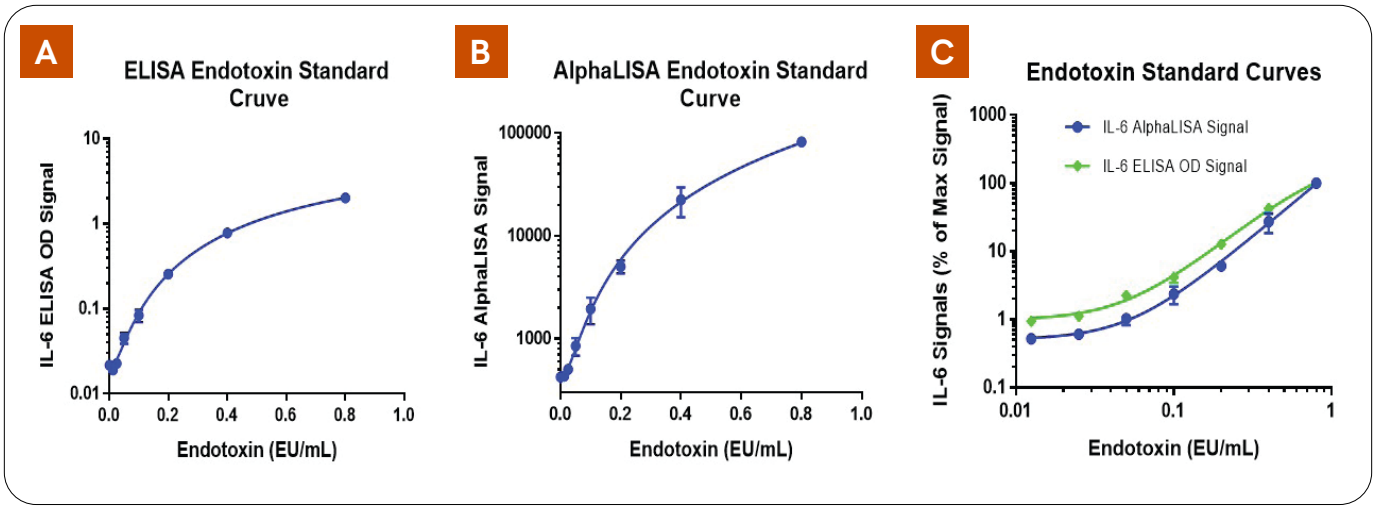



Figure 3. Endotoxin standard curves of IL-6 ELISA **(A)**, IL-6 AlphaLISA **(B)**, and Combined IL-6 ELISA and AlphaLISA in one plot using the percent maximum (%Max) signal to show dynamic ranges. Mono-Mac-6 cells were stimulated with the standard concentrations (0.0125 to 0.8 EU/mL) of Reference Standard Endotoxin (RSE). The signals of IL-6 in the cell culture supernatant samples were determined by PyroMAT IL-6 ELISA and AlphaLISA IL-6 kits. The signal intensity is proportional to the amount of endotoxin.

Table 1. Data summary and the performance of PyroMAT IL-6 ELISA and AlphaLISA IL-6 kit. Average signals for each assay were summarized with LOD, MAX and MIN signals, and S/B ratio.

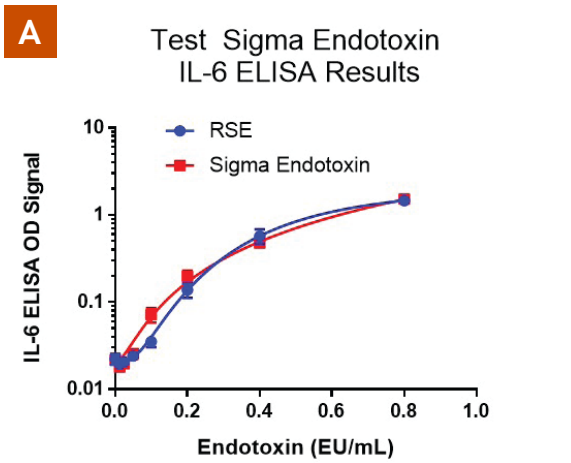
| Endotoxin (EU/mL) | PyroMAT IL-6 ELISA OD Signal (n=3) | IL-6 AlphaLISA Signal (n=3) |
|---|---|---|
| 0 | 0.022 | 422 |
| 0.0125 | 0.019 | 429 |
| 0.025 | 0.023 | 501 |
| 0.05 | 0.045 | 845 |
| 0.1 | 0.084 | 1941 |
| 0.2 | 0.255 | 5015 |
| 0.4 | 0.860 | 22376 |
| 0.8 | 2.019 | 82067 |
| LOD (EU/mL) | 0.027 | 0.024 |
| MAX Signal | 2.019 | 82067 |
| MIN Signal | 0.022 | 422 |
| S/B Ratio | 93 | 194 |

### Testing an additional endotoxin on stimulation of IL-6 release

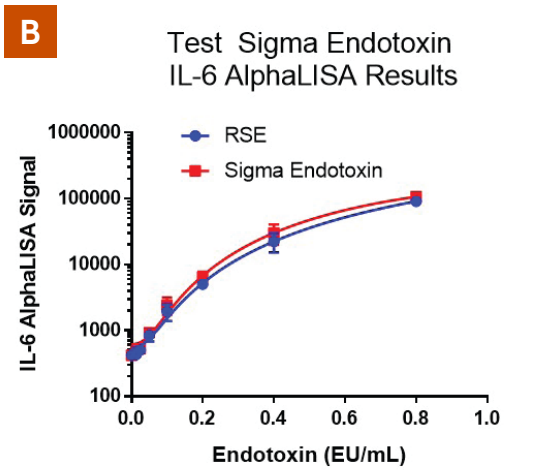
This experiment was conducted to test a commercially available endotoxin (Sigma, Cat # 1235503) to verify that other endotoxins can stimulate IL-6 release. The Sigma endotoxin was prepared following the same procedures to prepare reference standard endotoxin (RSE) that was included in the PyroMAT system. To compare the results, the Mono-Mac-6 cells were cultured and stimulated with the Sigma endotoxin. The range of Sigma endotoxin concentrations tested were the same as RSE (0.0125 to 0.8 EU/mL). Cell culture supernatant samples were assayed using the PyroMAT IL-6 ELISA kit and AlphaLISA IL-6 kit. The endotoxin standard curves generated using both the Sigma endotoxin and RSE are summarized in Figure 4A (PyroMAT IL-6 ELISA OD signal) and Figure 4B (AlphaLISA IL-6 signal). The results indicated that both endotoxins increased IL-6 secretion to the media with increasing concentrations and there was no significant difference between two endotoxins for the stimulation of IL-6 release from Mono-Mac-6 cells (Tables below the Figure 4A and Figure 4B).

### Detection of non-endotoxin pyrogen

It is known that the PyroMAT IL-6 ELISA kit can detect both endotoxin and non-endotoxin pyrogens. To test non-endotoxin pyrogens, cells were stimulated with a non-endotoxin control (HKSA = Heat-Killed Staphylococcus Aureus, a gram-positive, round-shaped bacterium) and the release of IL-6 in cell culture supernatant samples were assayed by PyroMAT IL-6 ELISA kit and AlphaLISA IL-6 kit. The results presented in Figure 5A and Figure 5B indicates that both kits detected IL-6 secretion (shown by the ELISA OD signal and AlphaLISA signal) in the cell media samples showing that AlphaLISA IL-6 kit detected non-endotoxin pyrogens.

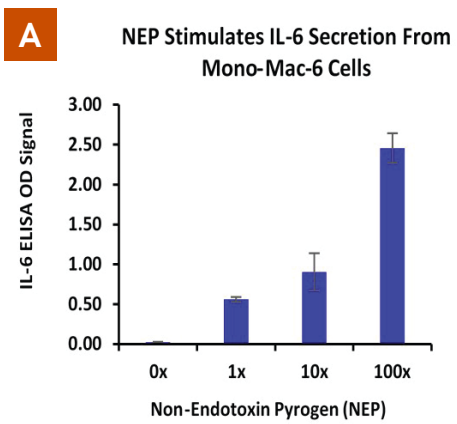



| IL-6 ELISA | RSE | Sigma Endotoxin |
|---|---|---|
| LOD (EU/mL) | 0.043 | 0.018 |
| MAX Signal (OD Signal) | 1.462 | 1.515 |
| MIN Signal (OD Signal) | 0.021 | 0.022 |
| S/B Ratio | 71 | 70 |




| IL-6 AlphaLISA | RSE | Sigma Endotoxin |
|---|---|---|
| LOD (EU/mL) | 0.028 | 0.022 |
| MAX Signal (Alpha Signal) | 90734 | 106676 |
| MIN Signal (Alpha Signal) | 422 | 421 |
| S/B Ratio | 215 | 254 |

Figure 4. Test of Sigma endotoxin. Mono-Mac-6 cells were stimulated with standard concentrations of Sigma endotoxin and RSE. Cell supernatant samples were tested using PyroMAT IL-6 ELISA kit **(A)** and AlphaLISA IL-6 kit **(B)**. IL-6 assay signals (ELISA OD or AlphaLISA Signals) are plotted against the endotoxin concentrations. Data summaries of RSE and Sigma endotoxin are shown in the tables below the graphs.



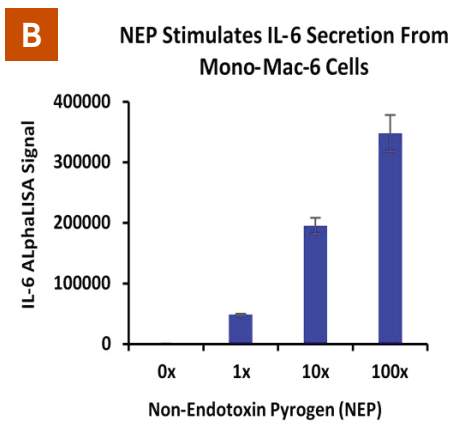




Figure 5. Non-Endotoxin Pyrogen (NEP) control was included in the cell cultures. 1000x NEP control (HKSA = Heat-Killed Staphylococcus Aureus) was prepared as instructed in PyroMAT System. The cells were stimulated with 100x, 10x, 1X, and 0x (Blank), n=3 for each preparation. The cell supernatants were assayed using the PyroMAT IL-6 ELISA kit **(A)** and AlphaLISA IL-6 kit **(B)**.

## Conclusion

In this application note, AlphaLISA human IL-6 kit was evaluated as an alternative to the PyroMAT IL-6 ELISA kit. Our experiments indicate that AlphaLISA human IL-6 kit can be used in PyroMAT to detect pyrogens as low as 0.025 EU/mL by using less than half the sample volume (40 µL/well) as the PyroMAT IL-6 ELISA kit (100 µL/well) offered in PyroMAT System. The AlphaLISA assay was completed in < 2 hours while PyroMAT IL-6 ELISA kit assay required > 3 hours to complete. Although measuring the absolute IL-6 concentrations is not required for pyrogen quantification in endotoxin-stimulated cell media samples, IL-6 levels that are quantified by AlphaLISA human IL-6 kit can be used to determine the range of endotoxin concentrations for Mono-Mac-6 cell stimulations.

In conclusion, the homogeneous no wash AlphaLISA human IL-6 kit can be used to measure the levels of pyrogens in endotoxin-stimulated Mono-Mac-6 cell culture supernatant samples. AlphaLISA IL-6 assay uses less sample materials and the results can be obtained in less than two hours.

## References


1. Evaluation of the monocyte activation test for the safety testing of meningococcal B vaccine Bexsero: A collaborative study. Lucy Studholme, Janet Sutherland, Trusha Desai, Jason Hockley, Rory Care, Ida Karin Nordgren, Caroline Vipond, Collaborative Study Group; Vaccine, 2019 Jun 27; 37 (29): 3761-3769. https://pubmed.ncbi.nlm.nih.gov/31202504/

2. Monocyte-activation test to reliably measure the pyrogenic content of a vaccine: An *in vitro* pyrogen test to overcome *in vivo* limitations. Sara Valentini, Giovanna Santoro, Federica Baffetta, Sara Franceschi, Marilena Paludi, Elisa Brandini, Leonardo Gherardini, Davide Serruto, Barbara Capecchi. Vaccine, 2019 Jun 27;37(29):3754-3760. https://pubmed.ncbi.nlm.nih.gov/31202503/

3. Development and validation of a monocyte activation test for the control/safety testing of an OMV-based eningococcal B Vaccine. Caroline Vipond, Janet Sutherland, Karin Nordgren, George Kemp, Alan Heath, Rory Care, Lucy Studholme. Vaccine, 2019 Jun 27; 37(29):3747-3753. https://pubmed. ncbi.nlm.nih.gov/31202503/

4. Evidence for the Detection of Non-Endotoxin Pyrogens by the Whole Blood Monocyte Activation Test. Nina Hasiwa, Mardas Daneshian, Peter Bruegger, Stefan Fennrich, Astrid Hochadel, Sebastian Hoffmann, Felix E. Rivera-Mariani, Christoph Rockel, Stefanie Schindler, Ingo Spreitzer, Sandra Stoppelkamp, Kranthi Vysyaraju, and Thomas Hartung, Altex 30, 2/13:169- 208. https://pubmed.ncbi.nlm.nih.gov/23665806/

5. Limitations of the rabbit pyrogen test for assessing meningococcal OMV based vaccines. Caroline Vipond, Lucy Findlay, Ian Feavers, Rory Care. https://pubmed.ncbi.nlm.nih. gov/26626274/


**Revvity, Inc.**
940 Winter Street
Waltham, MA 02451 USA
**www.revvity.com**

**For a complete listing of our global offices, visit www.revvity.com**