

Tracking inflammasome activity via measuring IL-1 β levels with homogeneous (no-wash) immunoassay technologies AlphaLISA and HTRF.

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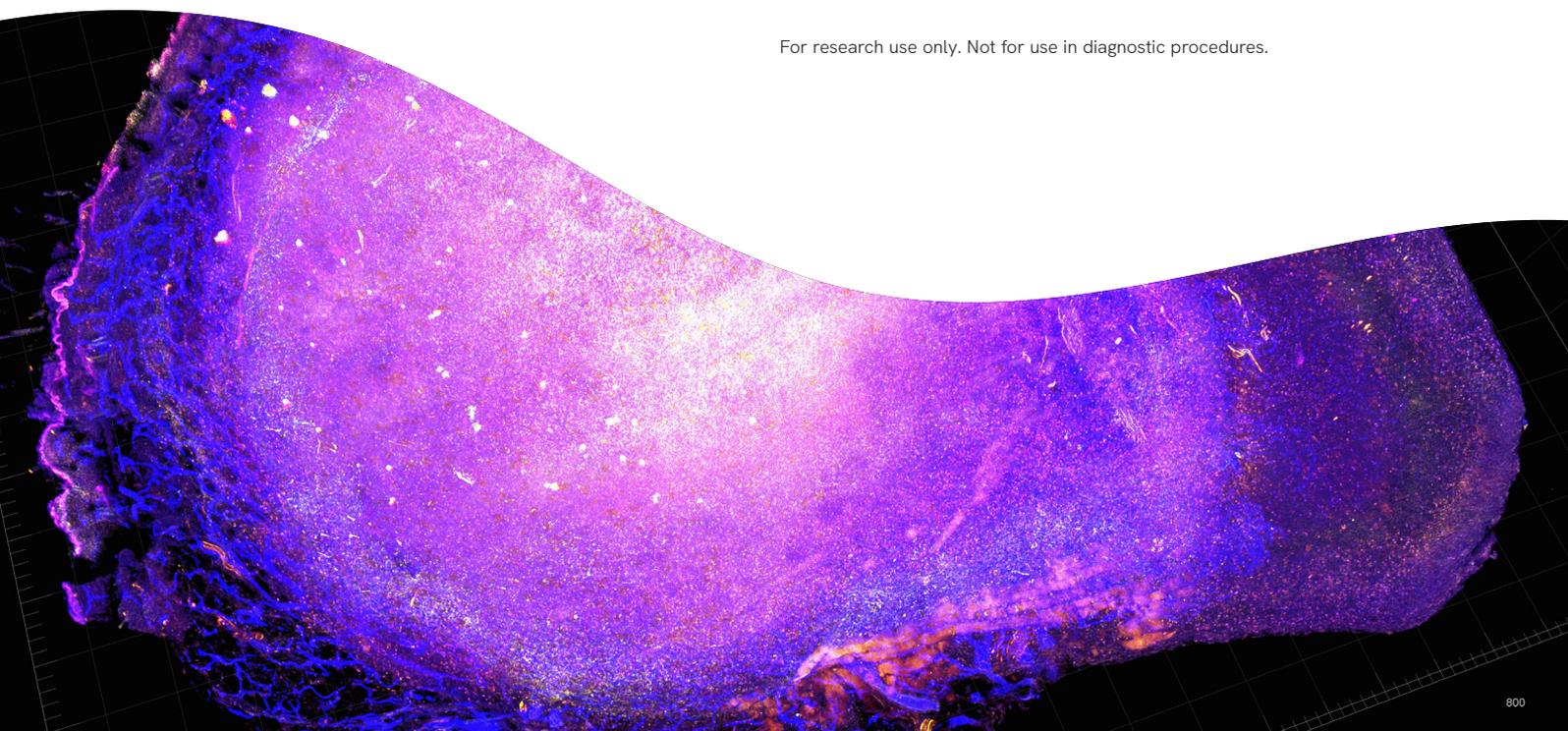
Adam Carlson
Hopkinton, MA

Introduction

It has been suggested that inflammation promotes malignancy via proinflammatory cytokines, such as IL-1 β , which enhance immune suppression through the induction of myeloid suppressor cells (MSC), thereby counteracting immune surveillance and allowing the outgrowth and proliferation of malignant cells.¹ IL-1cc is produced as an inactive 31 kDa precursor called pro-IL-1 β predominantly by inflammatory cells of myeloid lineage. Production of pro-IL-1 β is rapidly induced upon exposure of inflammatory cells to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs).⁷ An additional PAMP or DAMP stimulation is required to activate cytosolic pattern recognition receptors to form large multiprotein complexes called inflammasomes.⁴ Following activation, inflammasomes cause the activation of caspase-1 and the processing of pro-IL-1 β to a 17 kDa mature form of IL-1 β that is released extracellularly.

IL-1 β can therefore be used as a biomarker for inflammasome activity in cell supernatant or serum samples and be detected via immunoassay technologies. While there are multiple immunoassay options to choose from including ELISA, this application note demonstrates the utility of AlphaLISA[®] and HTRF[®] for the detection of IL-1 β in cell supernatant and serum samples. Both technologies are homogeneous (no-wash) immunoassays, require minimal sample to perform, are high throughput, and can be used in cell-based assays to screen for inhibitors of IL-1 β exemplified with MCC950, a known small molecule inhibitor of the NLRP3 inflammasome.²

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Materials and methods

Matrix samples: For matrix testing, the following materials were used outside of kit sourced material: RPMI (ATCC, #30-2001), DMEM (ATCC, #30-2002), Fetal Bovine Serum (FBS) heat inactivated (Thermo Fisher, #10082147), and Normal Pooled Human Serum (Innovative Research, #ISER50ML).

Cell-based materials: THP-1 cells (ATCC, #TIB-202), RPMI (ATCC, #30-2001), Fetal Bovine Serum (FBS) heat inactivated (Thermo Fisher, #10082147), Lipopolysaccharides (LPS, Sigma #L4516-1MG), Phorbol 12-myristate 13-acetate (PMA, Sigma #P1585-1MG), MCC950 inflammasome inhibitor (Selleckchem, #S8930), CellCarrier-96 Ultra Microplate (Revvity, #6055302).

AlphaLISA assays: Alpha technology is a fast, easy, highly sensitive, homogeneous (no-wash) assay platform that can be performed in a microplate format. Alpha 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 then 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 of a Donor bead, little to no signal is produced over background. In the AlphaLISA IL-1 β assay (Revvity, #AL220C) a biotinylated anti-IL-1 β antibody binds to the streptavidin-coated Donor bead whereas the Acceptor bead is conjugated to another anti-IL-1 β antibody. Antibody binding to IL-1 β brings the Donor and Acceptor beads into proximity such that excitation of the Donor bead leads to emission from the nearby Acceptor bead (Figure 1 assay schematic). The AlphaLISA signal is therefore proportional to the amount of IL-1 β present in the sample. The assay plates (AlphaPlate-384, Revvity, #6005350) were read on an EnVision 2105 Multimode Plate Reader with the stock AlphaLISA settings. The concentration of IL-1 β present in experimental samples was interpolated from an IL-1 β standard curve run with each assay.

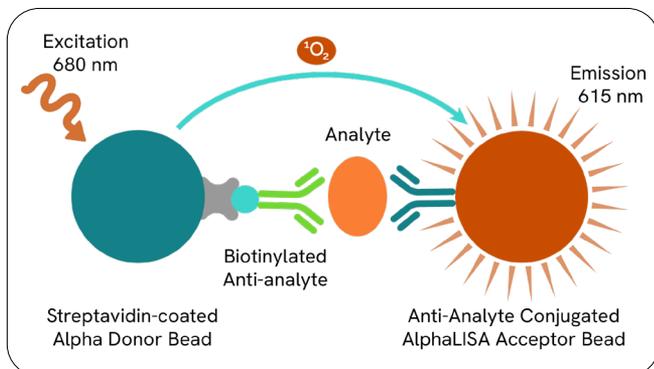


Figure 1: AlphaLISA Assay Schematic.

HTRF assays: HTRF[®] (Homogeneous Time Resolved Fluorescence) is a no-wash TR-FRET technology. It combines standard Fluorescence Resonance Energy Transfer (FRET) technology with time-resolved measurement of fluorescence, eliminating short-lived background fluorescence. For a sandwich assay, two antibodies that recognize a protein of interest are used, with one antibody coupled to a Donor, and the other with the Acceptor. If the two antibodies recognize the analyte, the Donor will emit fluorescence upon excitation and the energy will be transferred to the nearby Acceptor, giving specific Acceptor fluorescence (Figure 2 assay schematic). In the HTRF IL-1 β assay (Revvity, 62HIL1BPEG) an anti-IL-1 β antibody is labelled with Donor Europium and another anti-IL-1 β antibody is labelled with the XL Acceptor. The assay plates (HTRF 96-well low volume plate, Revvity, #66PL96025) were read on an EnVision 2105 Multimode Plate Reader using laser excitation with APC 665 nm and Cy5 620 nm filters and the LANCE/DELFI Dual bias mirror. Both the 620 nm Acceptor and 665 nm Donor signal were collected, and the HTRF Ratio shown in the results was calculated as (665 nm/620 nm) $\times 10^4$. The concentration of IL-1 β present in experimental samples was interpolated from an IL-1 β standard curve run with each assay.

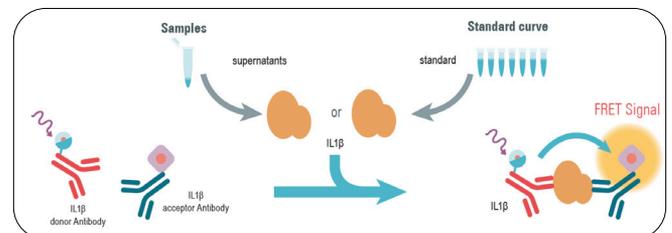


Figure 2: HTRF Assay Schematic.

Cell-based experiments: Differentiated THP-1 cells are known to release the mature form of IL-1 β into the cell supernatant upon stimulation with LPS.⁵ For the LPS dose-finding experiments, 50,000 cells/well were plated in a 96-well plate and treated with 50 nM PMA for three days to induce differentiation. THP-1 cells change morphology from suspension to adherent after differentiation making it possible to carefully aspirate the differentiation media and add fresh stimulation media. Cells were treated with a dose range of LPS-containing media as noted for four hours and supernatant was collected for testing. For the MCC950 inhibitor experiments, optimal LPS stimulation conditions were taken from the dose-finding experiments: 50,000 cells/well differentiated for three days with 50 nM PMA and stimulated with 1 EU/mL (2 ng/mL) of LPS. An MCC950 inhibition serial dilution curve was added and incubated concurrently with LPS stimulation for four hours after which supernatant was collected for testing.

Results and discussion

Assay performance in multiple matrices

For many studies, it is important to accurately quantify or detect low levels of IL-1 β in a variety of samples. These samples may be supernatants from treated cells or serum taken from patients. Therefore, assays that can detect and quantify IL-1 β in complex matrices are highly desirable. Here, IL-1 β was detected across multiple matrices using both AlphaLISA and HTRF. IL-1 β was diluted in the provided kit buffer for standard detection and quantification, in RPMI cell media (with 10%FBS) and in DMEM cell media (with 10%FBS) for quantification in cell-based work. Additionally, as IL-1 β can be found in human serum samples and used as a biomarker for disease monitoring,⁶ IL-1 β was diluted in FBS and compared to human serum in both assays. Typically, when testing human serum samples in AlphaLISA or HTRF, analyte depleted serum or FBS is recommended as the standard curve diluent for interpolation from a serum sample.

Figure 3 shows the AlphaLISA standard curve in Immunoassay buffer (IAB), cell media, and serum samples. The high concentration of biotin present in RPMI cell media alters the standard curve shape slightly and reduces the signal to background due to the interference with streptavidin Donor bead binding to the biotinylated antibody in the assay. However, RPMI cell media remains a viable option for IL-1 β detection in supernatants as the lower limit of detection (LDL) and dynamic range are not affected as seen in Table 1. LDL is calculated by taking three times the standard deviation of the blank values and adding that to the average of the blanks and interpolating the result from the standard curve. The increased AlphaLISA baseline value for the pooled normal human serum sample may be indicative of IL-1 β present in the human serum sample at a low level. In general, IL-1 β levels are low in normal serum samples; however, they have been reported up to ~200 pg/mL in some normal patients.⁸

Table 1: Assay metrics.

	LDL [pg/mL]		LLOQ [pg/mL]		Signal to background		Dynamic range (logs)	
	Alpha	HTRF	Alpha	HTRF	Alpha	HTRF	Alpha	HTRF
IAB (Alpha Kit)	0.76	n/a	2.68	n/a	944.5	n/a	5	n/a
Diluent #5 (HTRF Kit)	n/a	8.53	n/a	38.7	n/a	34.9	n/a	2.7
RPMI Media	0.57	27.55	2.05	85.5	618.9	26.8	4.7	2.4
DMEM Media	0.46	35.98	1.67	122.1	931.6	27	4.8	2.3
FBS	0.31	114	1.18	301.6	912.4	9.5	5	1.7
Human Serum	3.41	550	13.8	1244	286.1	6.9	4	1

* Values in red indicate lower sensitivity and signal to background ratio in human serum

The HTRF matrix results are shown in Figure 4. RPMI and DMEM cell media perform well in the HTRF format with both media types having a slightly down-shifted HTRF Ratio standard curve relative to the kit provided Diluent #5. Assay sensitivity (LDL) is shown in Table 1. The results indicate that using RPMI and DMEM cell media containing 10% FBS as a diluent for standard curve and cell supernatant sample preparation reduces the sensitivity of the HTRF assay from three to four-fold compared to the Diluent #5 supplied with the kit. In general, AlphaLISA is the preferred assay format when working with serum samples as it is more compatible with the serum matrix when compared to HTRF (see Assay Metrics Table 1).

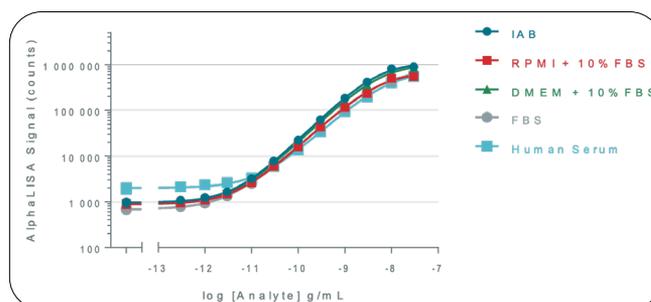


Figure 3: **AlphaLISA Matrix Testing.** IL-1 β standard was diluted in each matrix as noted in triplicate to generate each curve. Reduction in signal can be seen with RPMI cell media as well as serum samples when compared to the Immunoassay buffer (IAB) control effecting the overall Signal to Background value.

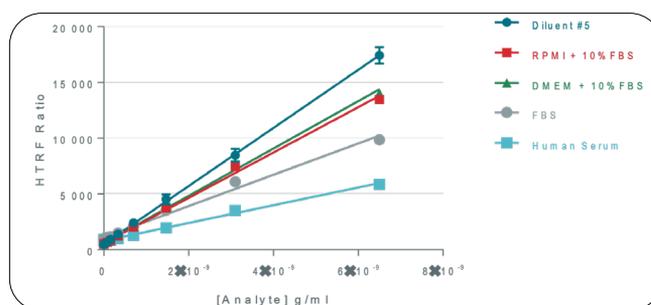


Figure 4: **HTRF Matrix Testing.** IL-1 β standard was diluted in each matrix as noted in triplicate to generate each curve. Reduction in signal can be seen with cell media as well as serum samples when compared to the buffer control (Diluent #5) effecting the overall Signal to Background value.

Testing inhibitors of mature IL-1 β release in a cell-based assay

To show the utility of AlphaLISA and HTRF assays for measuring inhibitors of IL-1 β release into supernatant samples, a cell-based assay using THP-1 cells was developed. THP-1 monocyte cells were grown in RPMI media and differentiated with PMA prior to treatment with LPS to induce mature IL-1 β release into the cell supernatant. Overtreating the cells with LPS can result in poor cell health and even cell death if the LPS concentration is too high. Therefore, the first experiment was performed to determine the optimal concentration of LPS to use in the inhibitor experiment (Figure 5). The amount of IL-1 β released into the supernatant at each LPS concentration was determined from the standard curve run alongside the samples for each assay type. Both assays showed almost the same amount of IL-1 β release at each LPS concentration tested. Based on these results, 1 EU/mL of LPS was chosen to give an optimal amount of IL-1 β release for subsequent experiments.

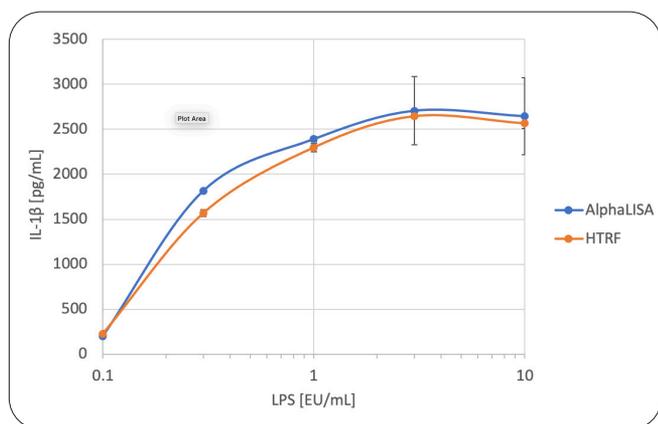


Figure 5: **AlphaLISA and HTRF LPS Dose-finding Experiment.** Increasing amount of LPS was added to PMA differentiated THP-1 cells. IL-1 β released into supernatant was measured by both AlphaLISA and HTRF with IL-1 β concentration determined from the standard curve from each assay technology.

Next, we wanted to showcase the specificity of the IL-1 β release stimulated by LPS by utilizing a known pathway inhibitor and looking for a decrease in IL-1 β in the supernatant. With this format in mind, either AlphaLISA or HTRF assays could be used to screen for inhibitors of IL-1 β activation (processing from the pro form to the mature form) and release by monitoring IL-1 β levels present in the supernatant. The inhibitor chosen to test for modulating the IL-1 β release was MCC950. MCC950 is a small molecule inhibitor of the NLRP3 inflammasome that effectively inhibits caspase-1 activity and processing of IL-1 β pro form (31 kDa) to the mature form (17 kDa) released from cells.³

Optimal IL-1 β stimulation conditions were taken from the first cell-based LPS experiment. Figure 6 shows the dose-response curve of the inhibitor MCC950 in both assay formats with comparable IC₅₀ values (AlphaLISA 24.4 nM, and HTRF 18.3 nM).

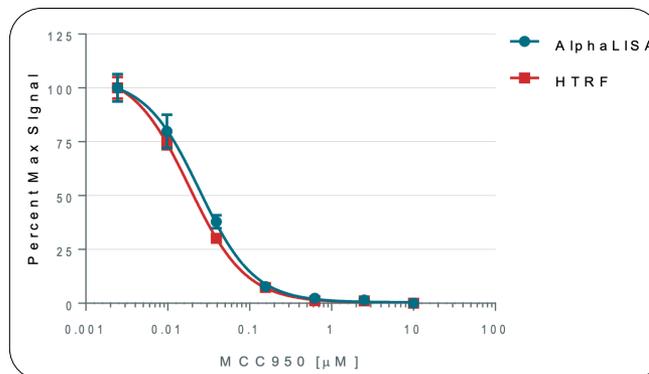


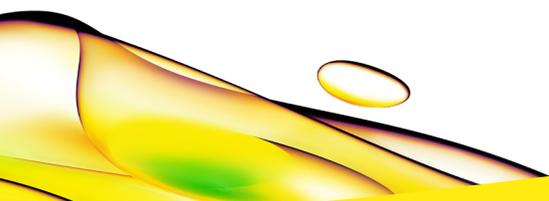
Figure 6: **MCC950 Inhibition Dose-response Curves.** AlphaLISA signal and HTRF Ratio were normalized to 100% maximal activity for each assay to directly compare the two readouts side-by-side. Detection of MCC950 activity by either assay yields comparable IC₅₀ result, AlphaLISA 24.4 nM and HTRF 18 nM.

Summary

IL-1 β can effectively be measured in both AlphaLISA and HTRF assays across multiple matrices with AlphaLISA showcasing superior performance of the two technologies as seen by the assay metrics displayed in Table 1 (LDL, LLOQ, Signal to Background and Dynamic Range). Both AlphaLISA and HTRF are homogeneous (no-wash) assays, easy to use, require minimal sample volume to run (just 5 μ L in AlphaLISA), and are amenable to automation (scaling to 384-well format). These assay formats allow assessment of IL-1 β and its role in inflammation-based cancer progression including screening for new inhibitors. The data here demonstrate specificity of the inflammasome response by way of a known pathway inhibitor MCC950 exerting its effect on IL-1 β release in cell culture supernatants. Both assays returned an IC₅₀ value for MCC950 that is consistent with that found in literature in other cellular models.² AlphaLISA and HTRF assay technologies provide an excellent tool for studying IL-1 β and the role of the inflammasome in disease progression and tumor growth.

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