Homogeneous, Bioluminescent Cytokine Assay Applied to a Monocyte Activation Test for Pyrogen Detection

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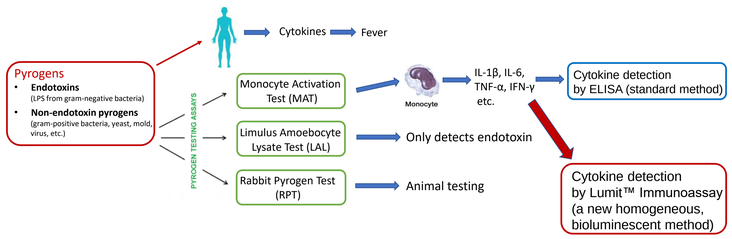
Abstract # 2020-A-3876-SOT

1. Introduction

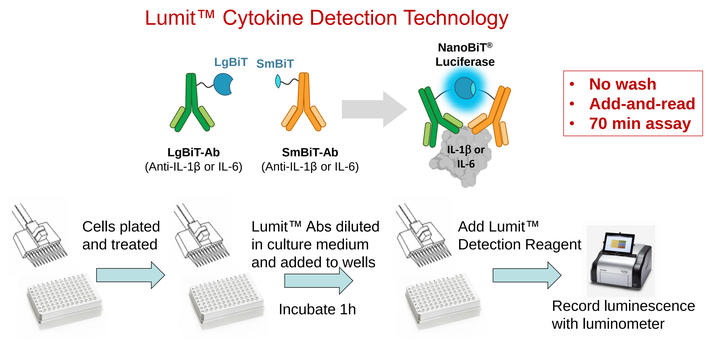
Pyrogens cause cytokine release from cells such as monocytes as a protective response to infection and injury. However, an exaggerated pyrogen response can cause systemic inflammation, shock, organ failure and death. Injectables and implanted devices are tested for pyrogens, especially endotoxins from gram-negative bacteria, because they can be left as residuals after manufacturing and disinfection processes. Non-endotoxin pyrogens are also a concern, and increasingly so with the expanded use and complexity of biologic drug formulations that may carry pyrogen contamination. Standard pyrogen tests include the endotoxin-selective, limulus amebocyte lysate (LAL) test and the broad-spectrum rabbit pyrogen test (RPT). Monocyte Activation Tests (MAT) were recently approved as an alternative pyrogen test to detect endotoxin and non-endotoxin pyrogens and avoid animal use. MATs measure IL-1β or IL-6 cytokines released from monocytes by ELISA. To simplify the MAT workflow, we developed a homogeneous cytokine assay method, termed Lumit, without transfer or wash steps to replace the ELISA. For a given target, two selective antibodies are respectively labeled with complementary subunits of Nanoluc® luciferase. When labeled antibody pairs bind their target, the subunits are brought into proximity to reconstitute a bright luciferase that generates light with the substrate furimazine. Appropriate antibody pairs for either IL-1β or IL-6 were selected and applied to 4-donor pools of peripheral blood mononuclear cells (PBMCs). Release of IL-6 or IL-1β from PBMCs was detected after treatment with several pyrogens, including LPS, R848, lipoteichoic acid (LTA), Pam3CSK4, and heat-killed Staphylococcus aureus (HKSA). Both assays are sensitive and have large linear dynamic ranges of greater than 3 logs. However, the IL-6 assay had greater sensitivity for all tested pyrogens and a larger response. The IL-6 immunoassay had a limit of detection of ~0.03 endotoxin units/ml. This homogeneous IL-6 assay enables a sensitive MAT in an add-and-read format that reduces ELISA workflow steps and facilitates assay automation. This simplified MAT is an attractive RPT alternative that detects a broad spectrum of pyrogens.

2. Monocyte Activation Test (MAT)

The monocyte activation test (MAT) has the advantages of detecting both endotoxin and non-endotoxin pyrogens while avoiding animal testing. Current MATs monitor either IL-1β release using whole blood or IL-6 release from PBMCs or the monomac 6 cell line. The cytokines are quantified by ELISAs in these current MAT kits. We aim to replace the ELISAs with our bioluminescent method that does not require any washes or transfers, creating an add and read format.

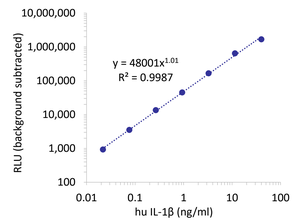


3. Simplicity of Homogeneous Cytokine Assay

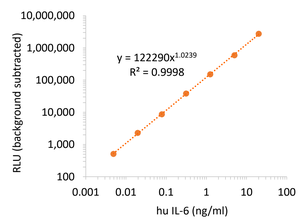


4. Broad Dynamic Range for Lumit™ Cytokine Assays

The Lumit™ homogeneous cytokine immunoassays are sensitive with linear ranges > 3 logs for both IL-1β and IL-6.



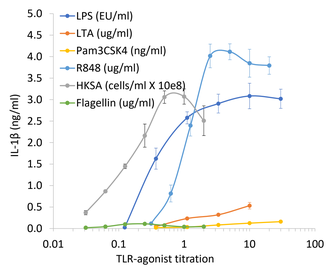
Linear range: 40 ng/ ml to 22 pg/ml
LOD (3 standard dev. above background): 20 pg/ml

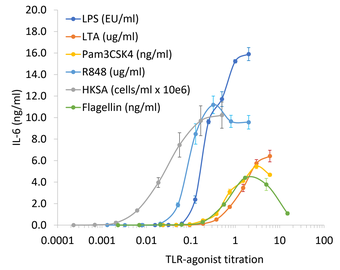


Linear range: 20 ng/ ml to 5 pg/ml
LOD (3 standard dev. above background): ≤ 4 pg/ml

5. MAT Results for Pyrogen-treated PBMCs

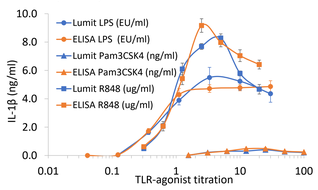
Pools of 4-donor Peripheral Blood Mononuclear Cells (PBMCs) (BioIVT) were thawed, plated in 96-well plates at 65,000 cells/well and treated overnight with titrations of various Toll-like Receptor (TLR) agonists. The next day, Lumit™ Abs were added directly to the wells for 1h, followed by addition of the Lumit™ Detection Reagent.



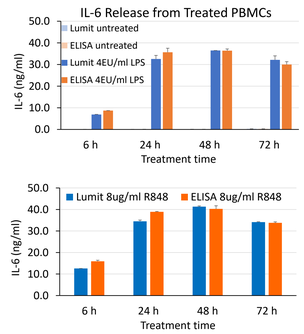


6. Comparison of Lumit™ Cytokine Assays with ELISA

PBMCs were treated overnight with titrations of various pyrogens and supernatants from the same well were tested in both the Lumit™ and ELISA assays for IL-1β release (below).

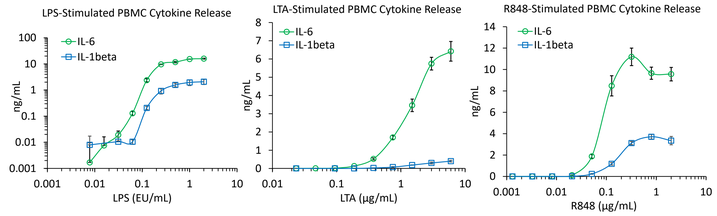


PBMCs were treated with LPS or R848 for various times and supernatants from the same sample were tested in both the Lumit™ and ELISA assays for IL-6 release (right).



7. IL-6 Lumit™ Assay is More Sensitive than IL-1β Lumit™ Assay

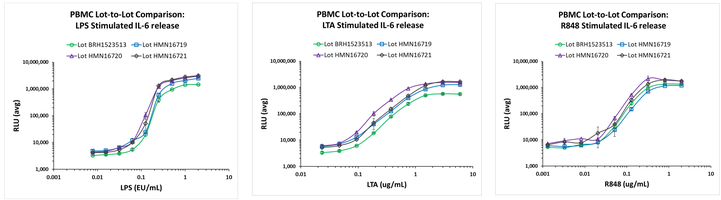
The IL-6 and IL-1β Lumit™ Assays were directly compared for PBMCs stimulated with all the pyrogens shown in Panel 5. In all cases, the IL-6 assay had a significantly larger signal window and was more sensitive than the IL-1β assay. Examples are shown below.



The LPS graph is shown on a log-log scale to show where the limits of detection are for the two assays. The IL-6 LOD is ~0.03 EU/ml and for IL-1β the LOD is ~0.1 EU/ml.

The LTA and R848 graphs are shown on a log-linear scale. Significantly more IL-6 than IL-1β is released from PBMCs for a given pyrogen concentration. In addition the IL-6 assay has a linear range with a lower limit of detection. These factors give the IL-6 assay a larger dynamic range and greater sensitivity, making it the preferred choice for the MAT.

8. Consistency Between 4-Donor Pools in the MAT



Four lots of different 4-donor pooled PBMCs (BioIVT) were thawed, plated in 96-well plates at the same cell number/well and tested with several pyrogens in the IL-6 assay. Three example pyrogens are shown above. Over the full titration range, the four lots gave similar results for all pyrogens, although there are some differences in RLU values.

The authors wish to thank BioIVT for providing multiple lots of PBMCs for testing.

9. Conclusions

Homogeneous, bioluminescent cytokine assay enables a simplified MAT
- After treating PBMCs, the assay requires 2 additions and takes just 70 min.
- The SmBiT and LgBiT-labeled Abs and Lumit™ Detection Reagent are added directly to the PBMCs in culture medium

The IL-6 assay is preferred over the IL-1β assay for the MAT
- The IL-6 assay has a lower limit of detection than the IL-1β assay
- PBMCs release significantly more IL-6 than IL-1β upon stimulation
- The IL-6 assay detects lower levels of both endotoxin and non-endotoxin pyrogens than the IL-1β assay

4-donor pools of PBMCs are used to mitigate human variation in the MAT
- 4 different lots of 4-donor pools were tested side-by-side and shown to give similar results with broad titrations of several different pyrogens
- Testing 4-donor pools of PBMCs with the Lumit™ IL-6 Assay simplifies the MAT

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