New LANCE™ Assays for the High Throughput Quantitation of Cytokine Biomarkers

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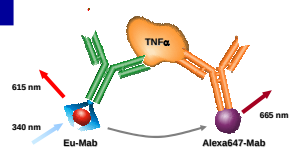
Introduction

TNF-α and IL-1β are pro-inflammatory cytokines produced primarily by activated monocytes, macrophages and phagocytic cells. They are implicated in both acute and chronic inflammation. TNF-α is selectively cytotoxic for many transformed cells *in vitro* and *in vivo*, leading to necrosis. Both IL-1β and TNF-α regulate growth and differentiation of a variety of cell types.

IL-2 is an immune modulator produced by T cells. It causes the proliferation and differentiation of various cell types including T cells, B cells and natural killer (NK) cells. IL-2 enhances the ability of the immune system to eliminate microorganisms as well as certain kinds of cancer cells.

We have developed LANCE™ assays for the detection of the IL-1β, TNF-α and IL-2 cytokines. The assays, in 384-well plate format, measure the amount of cytokine produced by differentiated lymphoid cell lines in response to immunological challenges. We will present data from three different cellular models.

1 Assay Principle



When a cytokine is captured by an Europium-labeled monoclonal antibody (Eu-Mab) and an Alexa 647-labeled monoclonal antibody (Alexa 647-Mab), an energy transfer complex is formed. Excitation of the Eu chelates in the complex generates signal at 615 and after energy transfer also at 665 nm.

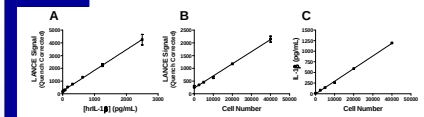
2 Methods

IL-1β. U937 cells were differentiated for 48 to 72 h with phorbol 12-myristate 13-acetate (PMA; 100 ng/mL), then transferred to 384-well plates and challenged for 18-24 h with lipopolysachharides (LPS; 10 µg/mL) in 30 µL. Anti-IL-1β LANCE antibodies were added to the cells in 10 µL at the same time as the LPS. Signal was measured with an EnVision™ multilabel plate reader using factory settings for LANCE.

TNF-α. THP-1 cells were differentiated in 384-plates for 24 h with 100 ng/mL PMA and 0.1 µM 1,25-dihydroxyvitamin D3 (1,25-OH_2D3) in 30 µL. LPS (0.1 µg/mL) were then added in 5 µL and plates were further incubated for 3 h. Anti-TNF-α antibodies were added after LPS stimulation in 5 µL of Reagent Buffer (RB) and signal was detected after 2 h with a VICTOR™ multilabel plate reader using the factory-set LANCE protocol.

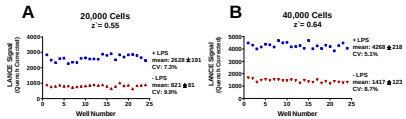
IL-2. Jurkat E6-1 cells were differentiated for 24 h in 384-well plates with 50 ng/mL PMA in the presence of the calcium ionophore A23187 to stimulate IL-2 production. Anti-IL-2 antibodies were then added in 10 µL of RB. Signal was detected after 2 h with a VICTOR™ multilabel plate reader using the factory-set LANCE protocol.

3 LANCE IL-1β Assay



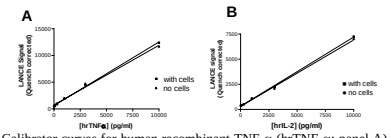
The U937 cell line is derived from a patient with diffuse histiocytic lymphoma. It has monocytic-like characteristics and undergoes differentiation along the macrophage pathway when treated with phorbol esters. Differentiated cells produce IL-1β when challenged with LPS. **A)** Calibrator curve of human recombinant IL-1β (hrIL-1β). **B)** Signal generated by cells differentiated for 72 h with PMA and treated with LPS for 18 h. **C)** Extrapolation of the amount of IL-1β secreted by U937 cells based on the calibrator curve data. Data points are means of quadruplicates

4 LANCE IL-1β Intraplate Variability



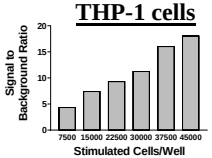
Intraplate variability of IL-β secretion by PMA-differentiated U937 cells was determined by comparing quench-corrected LANCE signal of cells treated or not with LPS for 20 h. Cells were plated at 20,000 (panel A) and 40,000 (panel B) cells per well in a final volume of 40 µL.

5 LANCE TNF-α and IL-2 Assays



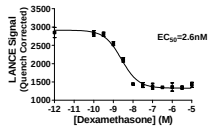
Calibrator curves for human recombinant TNF-α (hrTNF-α; panel A) and IL-2 (hrIL-2; panel B) were made in the presence or absence of 30,000 non-differentiated THP-1 cells and Jurkat E6-1, respectively. The recombinant cytokine and cells were dispensed in culture medium in a volume of 30 µL. The antibodies, diluted in Reagent Buffer, were added to the reaction mix in 10 µL. Signal was measured after 2- hour incubation.

6 TNF-α Secreted by THP-1 cells



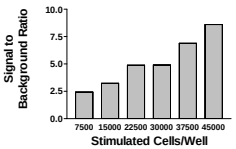
THP-1 cells are derived from the peripheral blood of a patient with human acute monocytic leukemia. These cells become phagocytic and secrete TNF-α upon differentiation with phorbol ester and vitamin D3. LANCE TNF-α assays were performed with different concentrations of differentiated THP-1 cells stimulated with LPS. Signal to background (S/B) ratios represent the signal ratio between LPS-stimulated and non-stimulated cell samples.

5 IL-1β Secretion Inhibition by Dexamethasone



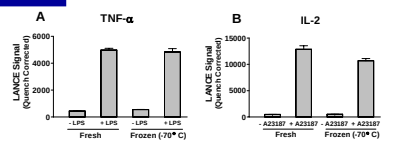
NF-kB controls transcriptional activity of the promoters of the proinflammatory cytokines IL-1β, TNF-α and IL-6. Glucocorticoids inhibit NF-kB activity and downregulate the transcription of IL-1β. The effect of the glucocorticoid dexamethasone on the secretion of IL-1β was determined. U937 cells were differentiated with PMA for 48 h and then treated simultaneously with LPS (10 µg/mL) and increasing concentrations of dexamethasone for 24 h. Data points are means of quadruplicates.

7 IL-2 Secreted by Jurkat Cells



Jurkat E6-1 cells are T cell lymphoblasts derived from a human acute lymphoblastic leukemia. They produce large amounts of IL-2 after stimulation with phorbol esters in the presence calcium ionophores, lectins, or anti-T3 antibodies. LANCE IL-2 assays were performed with different concentrations of cells stimulated with PMA and the calcium ionophore A23187. S/B ratios represent the signal ratio between A23187-stimulated and non-stimulated cell samples.

8 Comparison of Fresh and Frozen Cell Samples



Fresh and frozen cellular samples (30,000 cells/well) were compared in LANCE TNF-α (panel A) and IL-2 (panel B) cytokine assays. Fresh samples were measured right after stimulation treatments while duplicate 384-well plates were carefully sealed in plastic bags and frozen for two weeks at -70°C. Plates were let to thaw to room temperature before antibodies were added. Results are average values from six different samples.

Conclusions

- LANCE assays for the detection of cytokine biomarkers successfully detect cytokines secreted by stimulated cells in 384-well culture plates.
- Assays show minimal intraplate variability, are easy to set up, homogeneous and amenable to high throughput.
- Levels of IL-1β secreted by U937 cells can be extrapolated with as little as 5,000 cells per well.
- Inhibition of IL-1β secretion by a known inhibitor of inflammation (dexamethasone) was demonstrated.
- With TNF-α and IL-2, less than 10,000 cells per well are enough to provide a good screening window.
- TNF-α and IL-2 assays can detect at least up to 10 ng/mL of secreted cytokine, without needing sample dilution.

Color-Quench Correction of LANCE Signal

Color-quench correction of LANCE signal was performed using the formula:

$$\frac{\text{(Sample @ 665 nm – Buffer Blank @ 665 nm)} \times \text{Blank @ 615 nm}}{\text{Sample @ 615 nm}}$$

Sample @ 665 nm: signal from the sample measured at 665 nm
Buffer Blank @ 665 nm: wells with medium and possibly cells but not the antibodies
Blank @ 615 nm : wells with non-stimulated cells and antibodies or wells with medium and antibodies but no cells (no energy transfer)
Sample @ 615 nm : signal from the sample measured at 615 nm

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