APPLICATION NOTE

Simultaneous detection of IL-6 and IL-8 secretion by cell lines using AlphaPlex technology.

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Introduction

Cells react to various stimuli in the body by secreting modulator proteins called cytokines. These proteins bind to specific receptors to generate a response from the targeted cell. These responses range from cell growth, mobility, to alterations in differentiation and function, and even cell death. Cytokines are involved in many pathological pathways, including inflammation and cancer. As such, they are interesting research targets.

Immunoassays are the primary method used to measure production and modulation of cytokines by cells. However, the majority of these technologies are work-intensive, require large amounts of sample and can only analyze one cytokine per assay.

In this assay, we show how the AlphaPlex™ Technology can be used to perform the analysis of two different cytokines in the same sample. The combination of beads based on europium (AlphaLISA) and terbium (AlphaPlex 545) chemiluminescence allows for a fast, homogenous assay using as little as 5 µL of cell supernatant. We demonstrate the value of this assay for the detection of both IL-6 and 8, key cancer biomarkers, from two cell lines stimulated by IL-1b.

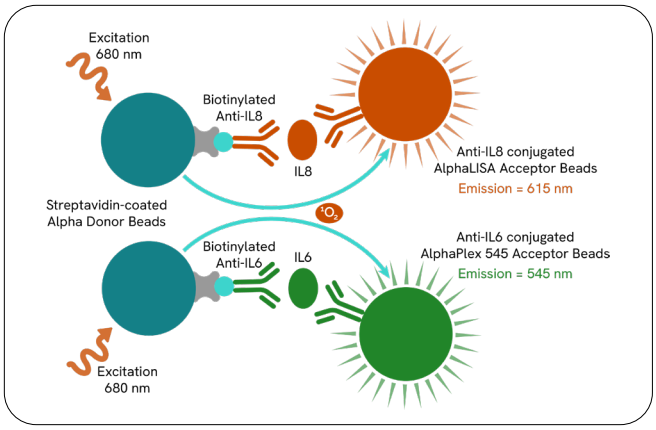

Figure 1: AlphaPlex assay principle: Biotinylated anti-analyte antibodies of both IL-6 and IL-8 bind to the Streptavidin-coated Alpha Donor beads, while other anti-analyte antibodies are conjugated directly to AlphaLISA (IL-8) or AlphaPlex 545 (IL-6) Acceptor beads. In the presence of the analyte(s), the beads come into close proximity. This proximity causes a series of events to occur: the 680 nm laser excitation of the Donor beads converts ambient oxygen to singlet oxygen molecules which causes a cascade of energy transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm for AlphaLISA beads and 545 nm for AlphaPlex 545 beads.

Materials and methods

The AlphaPlex assay for IL-6 and IL-8 contains the following components: Acceptor beads (europium) coated with an anti-human IL-8 antibody, Acceptor beads (terbium) coated with an anti-human IL-6 antibody, biotinylated anti-IL-6 and anti-IL-8 antibodies, Streptavidin Donor beads, recombinant IL-6 and IL-8 and AlphaLISA immunoassay buffer. The cell experiments use human colorectal adenocarcinoma HT-29 or human cervical cancer HeLa cells, DMEM culture media (with or without FBS) and human IL-1b for stimulation. Additional materials include AlphaLISA immunoassay buffer.

For the cellular assay, the cells are plated in 96-well cell culture plates at 80,000 to 625 cells per well. Each concentration is made in triplicate. The cells are left to adhere overnight. The cells are then starved by washing with PBS and replacing the media with serum-free culture media overnight. Next, they are stimulated by washing with PBS then supplementing with serum-free culture media containing 5ng/mL of IL-1b and overnight incubation. Finally, the cell supernatant is harvested for the immunoassay.

Protocol

Stock solutions of all the required reagents (analyte, Acceptor beads, biotinylated antibody, Donor beads) are prepared.
The following are mixed in final 1X AlphaLISA immunoassay buffer in three wells of a 384-well plate:

- 5 µL of analyte (cell supernatant or recombinant IL-x) with 10 µL of AlphaLISA and AlphaPlex 545 anti-analyte Acceptor beads (25 µg/mL)
- 10 µL of biotinylated anti-analyte antibody (2.5 nM) (incubate 60 min. at 23 °C)
- 25 µL Streptavidin Donor beads in each well (80 µg/mL (incubate for 30 min. at 23 °C in the subdued light conditions)
- Read using EnVision® Multilabel Plate Reader (with appropriate filters)

Results

Data show that the HT-29 cell line can produce high amounts of IL-8 even with a very low number of cells, but not any measurable IL-6. HeLa cells produce both cytokines at high levels with detectable amounts at 500 cells per well or below. Both cell lines were starved then stimulated 24 hrs with 5 ng/mL of IL-1b. Each cell line was tested with its own standard curve to insure that the matrix of the standard curve and the samples were the same.

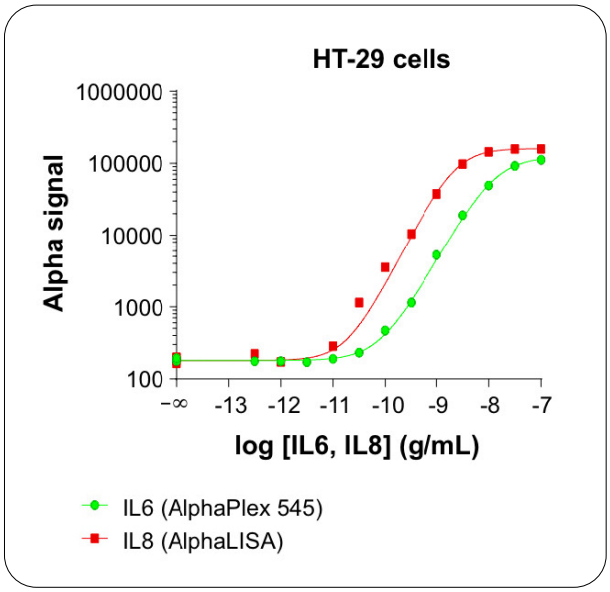

Figure 2

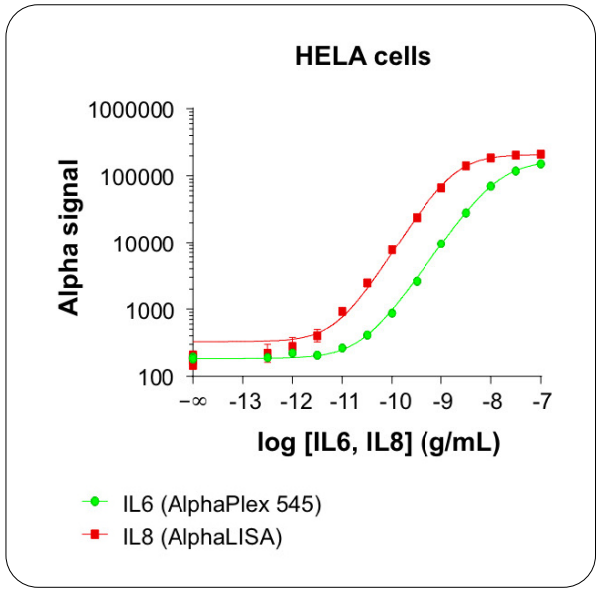

Figure 3

Table 1

Cytokines in supernatant of HT-29 stimulated with IL-1b (ng/mL)								
cell #	625	1250	2500	5000	10000	20000	40000	80000
IL-6	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
IL-8	0.71	1.48	5.9	7.43	>10	>10	>10	>10

Table 2

Cytokines in supernatant of HELA stimulated with IL-1b (ng/mL)								
cell #	625	1250	2500	5000	10000	20000	40000	80000
IL-6	0.38	0.62	1.85	3.5	11.1	20.9	34	49.6
IL-8	0.55	1.04	1.54	3.92	14.3	>30	>30	>30

Differential regulation of IL-6 and IL-8 in HT-29 and HeLa cells. Distinct standard curves for both IL6 and IL8 were performed with HT-29 (Figure 2) and HeLa (Figure 3) cell lines to guarantee matrix homogeneity. Both cell lines were starved then stimulated 24 hrs with 5ng/mL of IL-1b. Data show that the HT-29 cells can produce high amounts of IL-8 even with a very low number of cells but not measurable IL-6 (Table 1). HeLa cells produce both cytokines at high levels with detectable amounts at 500 cells per well or below (Table 2).

Summary

Here, we demonstrate the feasibility and value of using AlphaLISA and AlphaPlex 545 beads to create a duplex immunoassay in complex matrix. This allows for more meaningful experiments with more analytes tested per well and more accurate quantification of the analytes as well as significant savings in time and cost (amounts of reagents and plates used). With this newly-developed assay, we have created standard curves for both target cytokines in a single well with high sensitivity, dynamic range and specificity.

The IL-6/IL-8 duplex assay identified specific cytokines generated by two different cell lines, showing a very different secretion pattern for IL-6 between HT-29 (no measurable amounts) and HeLa (ng/mL amounts). IL-8 was secreted in large, comparable amounts by both cell-lines.

Finally, the assay showed very high sensitivity, with the capacity to work at 500 cells per well or less, while detecting amounts of cytokines well within the standard curve.

These various attributes make AlphaPlex technology a powerful and versatile method for measuring a variety of molecules and mediators in a complex biological setup.

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