Simultanous detection of IL-6 and IL-8 secretion by cell lines using Alpha technology

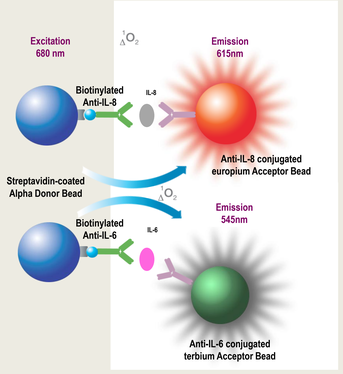
Philippe Roby, Kevin Groves, Weal Yared and Roger Bossé
Life Sciences and Technology, **PerkinElmer, Inc., Hopkinton, MA, USA**

1 Introduction

In the body, cells can react to various stimuli 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 as such interesting research targets.
Immunoassays are the primary methods 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.
A new Alpha technology approach has been developed to allow the analysis of two different cytokines in the same sample. Using a combination of beads based on europium and terbium chemiluminescence, this allows for a fast, homogenous assay using as little as 5µL of cell supernatent.
Here we demonstrate the value of this assay on the detection of both IL-6 and 8, key cancer biomarkers, from two cell lines stimulated by IL-1β

2 Alpha IL-6 and IL-8 Assays

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



The signals are read on PerkinElmer's flagship reader EnVision® Multilabel Plate readers using specific, appropriate filters for both wavelengths.

3 Material and Methods

Materials – The duoplex IL-6/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-1β for stimulation.
Additional materials include AlphaLISA immunoassay buffer.

Cell assays – 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.
Cells are then starved by washing the with PBS and replacing the media with serum-free culture media overnight.
Cells are stimulated by washing with PBS then supplementing with serum-free culture media containing 5ng/mL of IL-1β and overnight incubation.
Finally, the cell supernatent is harvested for the immunoassay.

Instruments – The only instrument needed is an EnVision plate reader Alpha enabled with 615nm and 545nm emission filters.

Immunoassay method

Stock solutions of all required reagents (analyte, acceptor beads, biotinylated antibody, donor beads) are prepared.
In 3 wells of a 384 well plate the following are mixed in final 1X AlphaLISA immunoassay buffer:
•5 µL of analyte (cell supernatant or recombinant IL-x) with 10 µL of AlphaLISA anti-analyte acceptor beads (25µg/mL) and
•10 µL of biotinylated anti-analyte antibody (2.5nM).

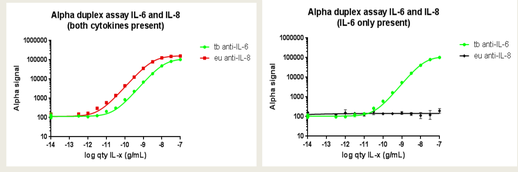
⬇ Incubate 60 minutes at 23°C

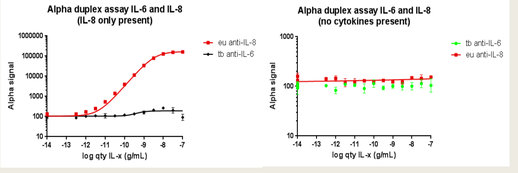
25 µL streptavidin alpha donor beads are added to each well (80µg/mL)

⬇ Incubate 30 minutes at 23°C in the dark

Read using EnVision (with appropriate filters) and analyze data using GraphPad Prism®

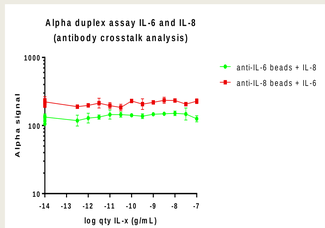
4 Duplex detection of IL-6 and IL-8

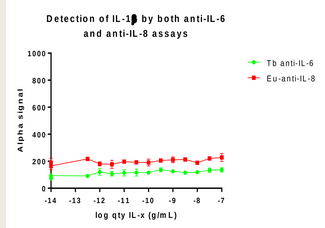




Duplex detection of IL-6 and IL-8 in AlphaLISA buffer. IL-6 and IL-8 were diluted and added to a mix of anti-IL-6 and anti-IL-8 detection systems. The assay was read sequentially at emission 545nm then 615nm.

5 Assays of antibodies specificity





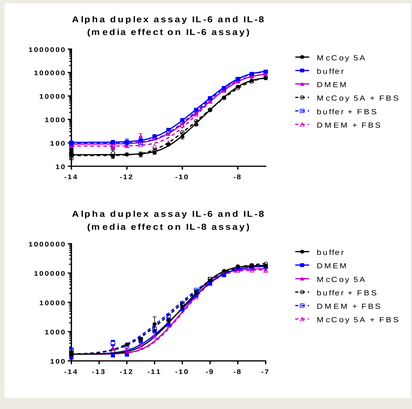
The assay is specific to each cytokine with no cross-talk.

Assay specificity was first tested by trying to detect IL-8 with the terbium anti-IL-6 assay or IL-6 by the anti-IL-8 assay. Neither test generates any measurable signal.

To ensure absence of interference, we also tested both assays with IL-1β. Neither assay generated any measurable signal.

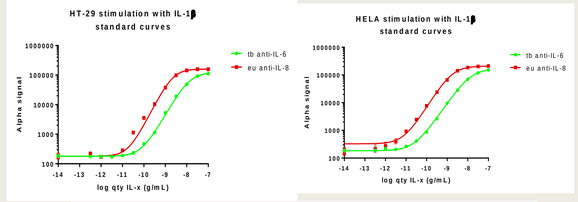
These results ensure that the assay is not subject to false positive due to cross-talk between the assays, and also that neither assay is detecting the cell-stimulating agent

6 Performance in various cell culture media



Detection of IL-6 and IL-8 in appropriate cell culture media. IL-6 and IL-8 were diluted in 3 different media (AlphaLISA buffer, McCoy's 5A and DMEM) with or without 10% FBS and analyzed using the respective acceptor beads and biotinylated antibodies. While FBS shows no significant effect on signal either for terbium or europium beads, McCoy's 5A media (normally used for HT-29 cells) shows a reduction in signal, due to the higher biotin content which interferes with the assay. For this reason, HT-29 were grown in DMEM media and HELA in MEM.

7 Detection of cytokines from IL-1β stimulated cell lines



Cytokines in supernatent of HT-29 stimulated with IL-1β (ngéml)								
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

Cytokines in supernatent of HELA stimulated with IL-1β (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. 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 hours with 5ng/mL of IL-1β. Each cell line was tested with its own standard curve to insure that the matrix of the standard curve and the samples are the same.

8 Summary

Herein, we demonstrate the feasibility and value of using AlphaLISA to duoplex assays in complex matrices. This allows for more meaningful experiments and significant savings in time, cost (amounts of reagents and plates used) as well as effort, leading to considerable savings.

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. Cross-talk between channels was negligible.

The IL-6/IL-8 duoplex 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.

All of this makes AlphaLISA duplexing a powerful and versatile approach to measure a variety of molecules and mediators in a complex biological setup.

PerkinElmer, Inc., 940 Winter Street, Waltham, MA USA (800) 762-4000 or (+1) 203 925-4602 www.perkinelmer.com