All-In-One-Well AlphaLISA Assays for Direct Biomarker Quantification in Cell Cultures

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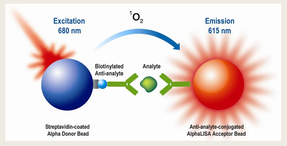
1 Abstract

The use of cultured cells for the assessment of compound activity offers many advantages over *in vitro* biochemical assays. Cellular models allow the simultaneous probing of various signaling pathways and the evaluation of a drug's permeability to cellular membranes. An End-Point Assay using Enzyme-Linked Immunosorbent Assays (ELISA) is the most widely adopted method for biomarker detection and quantification. ELISA offers high selectivity, sensitivity and assay versatility; however, it presents certain limitations such as a narrow dynamic range, low throughput and modest reproducibility due to its numerous wash steps. In contrast, homogeneous chemiluminescent bead-based AlphaLISA® assays allow measurement of biomarkers in a high throughput mode in the absence of any wash step. In the present work, various cytokines (TNFα, IL1ß, IL6, and IL8) and an integral plasma membrane protein (EGFR) were measured on stimulated cells using an All-In-One-Well AlphaLISA assay format. Biomarker production was measured from the adherent cell lines A431 or A549, and from suspension THP-1 cells directly in 384-well culture plates, in the absence of any transfer or wash step. This technology simplifies cellular assays, significantly reduces hands-on time and costs associated with plastic ware, and improves reproducibility. Moreover, results show excellent assay performance with wide dynamic range, low interference from cells or cell culture media, and high sensitivity. Indeed, AlphaLISA technology is a user-friendly and versatile tool for generating immunoassays for cellular models.

2 Introduction



AlphaLISA assay

The Streptavidin-coated Alpha Donor bead (blue) is used to capture the biotinylated anti-analyte antibody. The AlphaLISA Acceptor bead (red) is coated with anti-analyte specific antibody. The beads are brought into proximity through binding to the analyte. When excited by laser at 680 nm, the Alpha Donor bead generates singlet oxygen molecules which travels to the nearby AlphaLISA Acceptor bead where it induces emission of light at 615 nm.

3 Materials & Methods

AlphaLISA kits:
TNFα, PerkinElmer cat# AL208C
EGFR, PerkinElmer cat# AL212C
IL1β, PerkinElmer cat# AL220C
IL6, PerkinElmer cat# AL223C
IL8, PerkinElmer cat# AL224C

Equipment:
EnVision® Multilabel Plate Reader

Cell culture:
RPMI, Invitrogen cat# 11875
DMEM, Thermo scientific cat# SH30022
FBS, Wisent cat# 080150
2-mercapto-ethanol, Sigma cat# M6250
PBS 1X, Invitrogen cat# 10010
LPS, Sigma cat# L2018
IL1β, R&D Systems cat# 201-LB
CulturPlate™-384, PerkinElmer cat# 6007688
CulturPlate-96, PerkinElmer cat# 6005688

Protocol:

Standard curve	Inducer dose response
Plate 25 µL of non stimulated cells (in CulturPlate) or Add 25 µL of medium	Add inducer to the medium containing cells
	Plate 25 µL of stimulated cells (in CultuPlate)
	Incubate 4 or 24 h
Add 5 µL of analyte	Add 5 µL of AlphaLISA Immunoassay buffer

Harvest cells

Add 15 µL of mixture
(anti-analyte Acceptor beads at 10 µg/mL final and biotinylated anti-analyte Ab at 1 nM final)

Incubate 60 min at 23 C

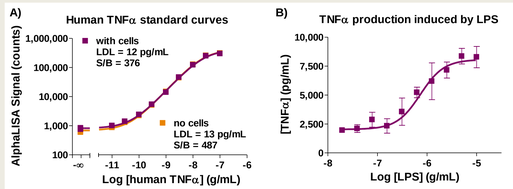
Add 5 µL of Streptavidin Donor beads at 40 µg/mL final

Incubate 60 min at 23 C in the dark and Read on EnVision

Analytes	Cells	#cells/well	Cell culture media	Stimulation
TNFα	THP-1	10 000	RPMI, 10% FBS, 0.05mM 2-ME	LPS, 4h
IL1β	THP-1	10 000	RPMI, 10% FBS, 0.05mM 2-ME	LPS, 4h
IL6	THP-1	10 000	RPMI, 10% FBS, 0.05mM 2-ME	LPS, O/N
IL8	THP-1	10 000	RPMI, 10% FBS, 0.05mM 2-ME	LPS, 4h
	A549	100	DMEM, 10% FBS	IL1β, O/N
EGFR	A431	0 to 30 000	DMEM, 10% FBS	Constitutively over-expressed
	HEK293	0 to 30 000	DMEM, 10% FBS	Weakly expressed

The final volume of cytokine assays was 50 µL in CulturPlate-384. For the EGFR assays, the final volume was 70 µL in CulturPlate-384 in A) or 300 µL in CulturPlate-96 in B). For all experiments, plates were read on an EnVision Multilabel Plate Reader.

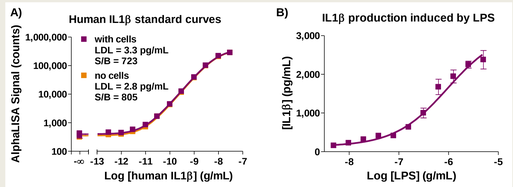
4 Tumor Necrosis Factor alpha



TNFα measurement. A) For the standard curves, TNFα was spiked at different concentrations in wells containing only medium or non stimulated THP-1 cells, and B) cells in log phase were plated and stimulated with different concentrations of LPS. All AlphaLISA reagents were added to the same well for the detection and TNFα concentrations were determined from the standard curve.

- The LPS-induced TNFα was efficiently detected by AlphaLISA in a single well format.
- The assay was not affected by the presence of cells.
- Although RPMI contains free biotin, a good sensitivity was obtained.

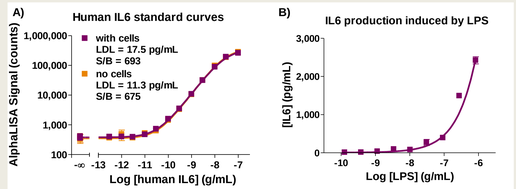
5 Interleukin 1 beta



IL1ß measurement. A) For the standard curves, IL1ß was spiked at different concentrations in wells containing only medium or non stimulated THP-1 cells, and B) cells in log phase were plated and stimulated with different concentrations of LPS. All AlphaLISA reagents were added to the same well for the detection and IL1ß concentrations were determined from the standard curve.

- The LPS-induced IL1ß was efficiently detected by AlphaLISA in a single well format.
- The assay was not affected by the presence of cells.
- Although RPMI contains free biotin, a good sensitivity was obtained.

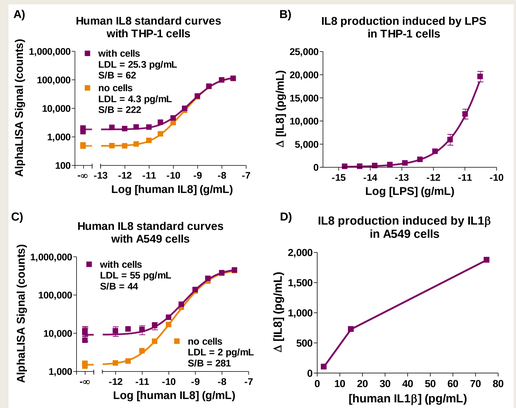
6 Interleukin 6



IL6 determination. A) For the standard curves, IL6 was spiked at different concentrations in wells containing only medium or non stimulated THP-1 cells, and B) cells in log phase were plated and stimulated with different concentrations of LPS. All AlphaLISA reagents were added to the same well for the detection and IL6 concentrations were determined from the standard curve.

- The LPS-induced IL6 was efficiently detected by AlphaLISA in a single well format.
- The assay was not affected by the presence of cells.
- Although RPMI contains free biotin, a good sensitivity was obtained.

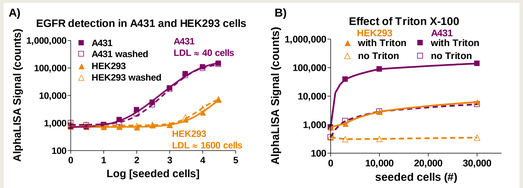
7 Interleukin 8



IL8 determination. A) and C) For the standard curves, IL8 was spiked at different concentrations in wells containing only media or non stimulated THP-1 (A) or A549 (C) cells, and B) and D) cells in log phase were plated and stimulated with different concentrations of LPS (B) or IL1ß (D). All AlphaLISA reagents were added to the same well for the detection assay. IL8 induction was determined from the corresponding standard curve without cells, subtracting the counts value from the non stimulated cells.

- Induced IL8 was efficiently detected by AlphaLISA in a single well in both THP-1 and A459 cell lines.
- Signal in the presence of non stimulated THP-1 or A549 cells is likely due to significant IL8 basal levels.
- IL8 can be detected in IL1β-stimulated A459 cells using as few as 100 cells/well.

8 Epidermal Growth Factor Receptor



EGFR measurement. A) The cells (A431 and HEK293) were plated at different concentrations and incubated 18h at 37°C, 5% CO2. Then, the cells were washed twice with PBS or not and AlphaLISA Immunoassay buffer was added to lyse the cells for 30 minutes at 23°C. The AlphaLISA assay was then performed in the same well. B) The cells (A431 and HEK293) were plated at different concentrations and incubated 40h at 37°C, 5% CO2. Then, the cells were washed twice with PBS after which AlphaLISA Immunoassay buffer with or without Triton X-100 (0.5%) was added for 30 minutes at 23°C. The AlphaLISA EGFR assay was then performed in the same well.

- EGFR can be detected in as few as 40 A431 or 1600 HEK293 plated cells. This difference is in line with the ~50-fold difference in EGFR expression levels reported for these two cell types (Kimura et al. 2007, Cancer Sci; Fabricant et al. 1977, PNAS).
- No significant difference was found between PBS-washed and non-washed cells after 18h of plating (without any interference from the cell culture media). However, at longer incubation times, the contribution of secreted EGFR-ECD in A431 cells should be evaluated.
- The use of an Immunoassay buffer without Triton X-100 proved to have a marked effect on assay sensitivity, causing a 30-fold and 15-fold drop in the signal with the A431 and HEK293 cells respectively, showing that the EGFR protein detected is indeed the membrane-bound form.

9 Summary

- Biomarker levels were measured directly in CulturPlates-96 and -384 in a simple, fast, all-in-one-well AlphaLISA assay format (also compatible with 1536-well plates).
- The elimination of transfer and wash steps simplifies cellular assays, reduces variability and significantly reduces hands-on time and costs associated with consumables.
- Integral plasma membrane (EGFR) and secreted soluble proteins (TNFα, IL1ß, IL6, IL8) were successfully determined, on adherent or suspension cells, using the standard AlphaLISA Immunoassay buffer .
- Excellent assay performance was observed, with wide dynamic range, low interference from cells or cell culture media (i.e., DMEM, RPMI, 10% FBS), high reproducibility and sensitivity.

AlphaLISA kits list

Angiogenesis	VEGF, VEGFB, VEGFC, VEGFD, TNFα
Biologics	hIgG, CHO-P, NS0-P
Cancer	EPO, EPOR, EGFR, PSA, AFP, MMP1, MMP9, β-NGF, ERBB2/HER2
Cardiovascular	Myeloperoxidase, NT-proBNP, Plasminogen, Renin/Prorenin, tPA
Inflammation	COMP, G-CSF, GM-CSF, CRP, IFN-γ, IL1α, IL1ß, IL2, IL3, IL6, IL8, IL10, IL12 (p70), IL13, IL17, IL18, CCL2/MCP1
Metabolic	Insulin, GH, Adiponectin, GLP-1, Leptin, Prolactin, IGF1, IGF2
Neurodegeneration	sAPPα, sAPPβ, Amyloid ß1-40, Amyloid ß1-42
Virology	HIVp24

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