AlphaLISA Assays are Homogeneous Sensitive Immunoassays for Detection of Analytes in a Variety of Biological Matrices

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1 Abstract

The AlphaLISA® assay is a homogeneous immunoassay alternative to classical ELISA. AlphaLISA assays were originally utilized to detect analytes in cell culture supernatants or serum/plasma samples. More recently, AlphaLISA has been applied to a wider variety of biological matrices including lysates from cultured cells, or fluids and tissue homogenates from animals. We report the development of an assay to measure active caspase-3 in cell lysates from both suspension and adherent cell models (Jurkat and HeLa, respectively). For compound screening, cells are treated with test material and subsequently lysis buffer is added. After a short incubation, target-specific AlphaLISA reagents are directly added, providing a highly efficient all-in-one-well assay format. Under optimal conditions, signal to background (S/B) values up to 17 and Z' values up to 0.8 were obtained with staurosporine-treated Jurkat cells. Assays were also developed for biological samples derived from rodents or humans. Quantitation of analytes in animal tissue extracts or biological fluids requires an appropriate diluent so that samples can be accurately extrapolated from the calibration curve. Following optimization of the assay, recovery of spiked analytes was generally in the range of 70 to 130%. As examples, mouse interleukin 6 was measured in bronchioalveolar lavage fluid (BALF) at a level of 20 pg/mL, human amyloid beta 1-42 peptide was measured in cerebrospinal fluid (CSF) at a level of 0.3 ng/mL, and mouse vascular endothelial growth factor (VEGF) was detected in lung homogenates at a level of 1 ng/mL. In general, a major advantage of using AlphaLISA for analysis of biological samples is that sample volumes as low as 2.5 µL can be utilized. Also, the absence of wash-steps greatly reduces the assay time and improves the reproducibility of the data.

2 Materials and Methods

Analyte detection

AlphaLISA kits and buffers are all available from PerkinElmer. All calibration curves were done using analyte and reagents supplied in the kits, and using optimal protocols available in the Technical Data Sheets.

Biological samples or fluids

All biological samples or fluids were supplied as pools of individuals (for assay development), or separate samples from 10 to 20 individuals, non-medicated, non-immunized, from Bioreclamation LLC:

- Mouse bronchial lavage fluid (BALF): Strain CD-1, cat# MSE-BRONLEV
- Rat bronchial lavage fluid (BALF): Strain Sprague Dawley, cat# RAT-BROLAV
- Human CSF: cat# HMCSF
- Mouse lung homogenates: Strain CD-1, cat# MSE-LUNG-HOMOG

Diluents tested

AlphaLISA Immunoassay Buffer: PerkinElmer, cat# AL000C
AlphaLISA HiBlock Buffer: PerkinElmer, cat# AL004C
AlphaLISA NaCl Buffer: PerkinElmer, cat# AL007C
Fluids tested as diluents were supplied as pools of individuals, non-medicated, non-immunized, from Bioreclamation LLC:

- Beagle bronchial lavage fluid (BALF): cat# BGL-BROLAV

Biological samples or fluids pre-treatment and storage

Prior to being used in AlphaLISA assays:

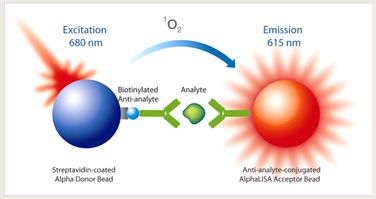
- BALF was centrifuged at 650xg for 15 min at 4°C to remove cells and cell debris, and supernatants were used in assays.
- CSF was not pre-treated.
- Mouse lung homogenates were generated by rinsing the entire lung (right and left) with PBS 1X, by homogenizing each entire lung on ice in 2 mL PBS 1X (containing protease inhibitors) using a polytron, and then centrifuging 10 min at 1,500xg at 4°C (to remove insoluble debris). Supernatants were used in assays.

Storage: BALF and CSF fluids were kept at -80°C. Mouse lung homogenates were kept at -20°C.

Active caspase-3 cellular assay

Jurkat cells (ATCC TIB-152™) were grown in RPMI medium supplemented with 10% FBS. 2.5 µL of staurosporine (LC labs, Cat# S-9300) prepared in serum-free medium at a 2X concentration was disposed into a CulturPlate®-384. 15,000 cells/well were seeded in a volume of 2.5 µL in serum-free medium and the plate was incubated at 37°C, 5% CO_2 for 2-4 hours. 5 µL of 3X Lysis Buffer (1.5X final) supplemented with protease inhibitors were added to each well and the plate was gently agitated on a plate shaker for 10 minutes at room temperature. Then an AlphaLISA assay was performed by adding 5 µL of a 10X MIX (freshly prepared) AlphaLISA Anti-Caspase-3 Acceptor beads (10 µg/mL final) and Biotinylated Antibody Anti-Caspase-3 (1 nM final), and by incubating for 60 minutes at 23°C. 35 µL of 1.43X Streptavidin Donor beads (40 µg/mL final) were added and plates were incubated 30 minutes at 23°C in the dark. Reading was performed using an EnVision Multilabel Plate Reader with Alpha HTS option.

3 Alpha Technology Assay Principle



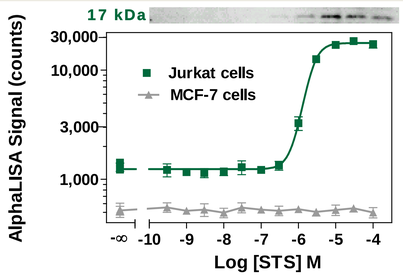
The biotinylated anti-analyte antibody binds to the Streptavidin-coated Donor beads while another anti-analyte antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads resulting in a sharp peak of light emission at 615 nm.

4 Expanding Applications

Cat. no.	Analyte	Species	BALF	Cell Lysate	CSF	Lung tissue
AL254	sAPPα	Human			√	
AL255	sAPPß	Human			√	
AL271	Tau	Human			√	
AL274	Aß 1-15 / 16	Human			√	
AL275	Aß 1-40	Human			√	
AL276	Aß 1-42	Human			√	
AL278	Caspase-3 active	Human		√		
AL288	Amyloid ß 1-x	Human			√	
AL504	IL6	Mouse	√			
AL505	TNFα	Mouse	√			
AL509	MCP-1	Mouse	√			
AL516	CCL5/RANTES	Mouse	√			
AL517	GM-CSF	Mouse	√			
AL518	IL1-ß	Rat	√			
AL520	VEGF A	Mouse	√			√

Data can be found in the corresponding Technical Data Sheets for these kits (website), except for AL504, AL505, and AL509, which were presented in a poster at the Cytokines 2010 conference, held in Chicago on October 03-07, 2010 (Michaud *et al.*, 2010).

5 Active Caspase-3 Cellular Assay



Dose-response curve of caspase-3 activation by increasing concentrations of staurosporine in Jurkat and MCF-7 cells. A Western blot for active caspase-3 (p17 subunit), which uses a rabbit monoclonal antibody against amino acids 171-175 of human caspase-3, is illustrated above the dose-response curve.

MCF-7 cells were included in the test as a negative control, since they do not express caspase-3. The AlphaLISA results correlate well with the relative amounts of cleaved caspase-3 detected by Western blot.

6 Active Caspase-3 Assay Precision

A. Intra-assay precision.
The intra-assay variability was evaluated by performing a Z' factor determination.

B. Inter-assay precision
Inter-assay precision was determined using a total of two independent determinations with 48 measurements for each control sample.

15,000 cells + 30 µM staurosporine

Assay	Z'	S/B	CV max	CV min
1	0.69	16	9.3%	8.3%
2	0.71	17	8.8%	6.3%

15,000 cells + 30 µM staurosporine

Counts Max	SD Max	CV Max	Counts Min	SD Min	CV Min
22842	2057	9.0%	1395	103	7.4%

15,000 cells + 1 µM staurosporine

Assay	Z'	S/B	CV max	CV min
1	0.55	8	12.0%	8.3%
2	0.46	9	15.3%	6.3%

15,000 cells + 1 µM staurosporine

Counts Max	SD Max	CV Max	Counts Min	SD Min	CV Min
11607	1812	15.6%	1395	103	7.4%

2,500 cells + 30 µM staurosporine

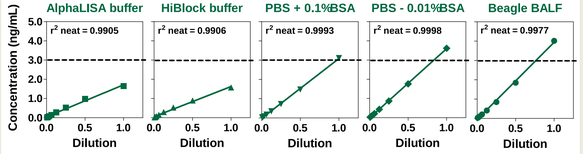
Assay	Z'	S/B	CV max	CV min
1	0.69	9	8.2%	7.4%
2	0.76	8	6.3%	7.0%

2,500 cells + 30 µM staurosporine

Counts Max	SD Max	CV Max	Counts Min	SD Min	CV Min
5407	573	10.6%	636	56	8.8%

7 Mouse TNFα in BALF

Step 1: Linearity of dilutions in 5 diluents for spiked mouse BALF (3 ng/mL mTNFα)



Dilution factor (DF)	AlphaLISA buffer Recovery from neat (%)	HiBlock buffer Recovery from neat (%)	PBS-0.1%BSA Recovery from neat (%)	PBS-0.01%BSA Recovery from neat (%)	Beagle BALF Recovery from neat (%)
1	100	100	**100**	**100**	**100**
2	119	113	**95**	**97**	**92**
4	128	135	**93**	**96**	**85**
8	137	150	**90**	**96**	**82**
16	139	146	**92**	**95**	**83**
32	149	145	**94**	**99**	**83**

Step 2:

Diluents not chosen:
Linearity not good from neat

Choose 2 good diluents (PBS-0.1%BSA and Beagle BALF):
• Good linearity from neat
• Observed concentration X DF close to spiked value (3 ng/mL) for all dilutions

Step 3: Chosen diluent showing the best spike and recovery results

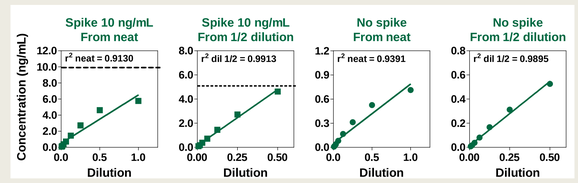
Diluent: PBS-0.1%BSA			
	Spiked diluent control	Spiked neat Mouse BALF	
Spike (pg/mL)	Concentration (pg/mL)*	Concentration (pg/mL)*	Recovery (%)**
No spike	0.0	17.1	**NA**
10	12.0	9.2	**76**
30	35.2	32.3	**92**
300	300.2	292.6	**97**
3000	3141.2	2952.1	**94**

Excellent linearity of dilutions from neat (Step 1) and excellent recovery for all 4 spikes tested. Measured TNFα physiological level in mouse BALF (No spike) = 17.1 pg/mL

* Concentration for 10 to 3000 pg/mL spikes = measured concentration – no spike value
** Recovery (%) = recovery compared to spiked diluent control

8 Mouse VEGF A in Lung Homogenates

Step 1: Linearity of dilutions in PBS-1%BSA for non-spiked or spiked mouse lung homogenates (10 ng/mL mVEGF A)



	PBS 1%BSA (spiked 10 ng/mL)		PBS 1%BSA (no spike)	
Dilution factor (DF)	Recovery from neat (%)	Recovery from 1/2 dil. (%)	Recovery from neat (%)	Recovery from 1/2 dil. (%)
1	100		100	
2	161	**100**	148	**100**
4	190	**118**	176	**119**
8	203	**126**	188	**127**
16	203	**126**	183	**124**
32	212	**132**	170	**115**

Step 2:

PBS-1%BSA is a good diluent:
- Good linearity starting at dilution 1/2 (so, 1/2 dilution required)
- Observed X DF close to spiked value (10 ng/mL) starting at dilution 1/2

Step 3: Spike and recovery results

Diluent: PBS-1%BSA			
	Spiked diluent control	Spiked 1/2 mouse lung homogenates	
Spike (ng/mL)	Concentration (ng/mL)*	Concentration (ng/mL)*	Recovery (%)**
No spike	0.00	0.63	**NA**
1	0.94	0.76	**81**
3	2.77	2.23	**81**
10	9.47	7.01	**74**

Excellent recovery for all 3 spikes tested in 1/2 mouse lung homogenates. Measured VEGF A physiological level in mouse lung homogenate (No spike) = 0.63 ng/mL X 2 = 1.26 ng/mL

* Concentration for 1 to 10 ng/mL spikes = measured concentration – no spike value
** Recovery (%) = recovery compared to spiked diluent control

Step 4: Test biological samples from individuals

Analyte	Biological sample	LDL (pg/mL)	Number of individuals tested	Average concentration	Range (AlphaLISA)	Expected range (literature)
VEGF A (mouse)	Mouse Lung Homogenates	1	10	0.96 ng/mL	0.5 - 1.3 ng/mL	350 - 400 pg/mg ptn*
				240 pg/mg	110 - 350 pg/mg ptn	

* Mori H et al. Am J Physiol Lung Cell Mol Physiol. 2008. Feb;294(2):L196-204.

9 Conclusion

AlphaLISA technology has been applied to a wide variety of biological matrices from different species. Different types of analytes such as small peptides, cytokines, or proteases can be detected in cell lysate, BALF, CSF, or lung homogenate. A caspase-3 assay was validated for both HeLa and Jurkat cells by showing comparable results to Western blotting. The assay was shown to be appropriate for HTS since good Z' values and inter-assay precision can be achieved. Quantitative assays for detecting mouse TNFα in BALF or mouse VEGFA in lung homogenate were optimized by choosing the correct matrix for the calibration curve and shown to detect endogenous level of cytokine.
These assays are fast and easy no-wash assays. Moreover, they are extremely sensitive, as we obtained good recoveries from 10 pg/mL spikes. Since these assays can use volumes as low as 2.5 µL, the AlphaLISA technology offers the possibility to accurately detect physiological levels of analytes even if very low volume of fluid is collected per animal.

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