Low volume, highly sensitive immunoassays for detecting cytokines in animal fluids

Jean-Francois Michaud, Nathalie Rouleau and Francesco Lipari

1 Abstract

Cytokine levels are measured in many different experimental models, with samples obtained in a variety of biological matrices such as cell culture supernatant, serum, plasma, saliva, cerebrospinal fluid, and tissue homogenate. The most common method of cytokine analysis is enzyme-linked immunosorbent assay (ELISA). However, ELISA often requires high sample volume (at least 50 µL), which is difficult to obtain when working with small animals such as mice. Also, the ELISA method has a limited dynamic range and low throughput due to its numerous wash steps. Considering the limitations of ELISA technology, a faster approach was developed for cytokine analysis in complex biological samples. This novel analytical approach is a chemiluminescent homogeneous bead-based immunoassay that is performed without wash steps. The cytokine of interest is sandwiched between two highly specific antibodies bound to the beads. In presence of the analyte, the beads come into proximity resulting in an emission of light upon laser excitation. The amount of light emitted is directly proportional to the amount of analyte present. Sample volumes as low as 5 µL can be utilized in this sensitive detection platform. In addition, upper quantitation limits can extend to much higher concentrations than achievable using traditional ELISA assay. For example, mouse IL6 can be detected from 1 pg/mL to 100,000 pg/mL without dilution, whereas a comparable ELISA has a range of 8 pg/mL to 500 pg/mL. The application of this immunoassay to different types of biological samples was verified by performing spike recovery experiments, and also by comparison of the levels measured to the levels reported in the literature. Overall, this assay technology will aid researchers in evaluating cytokines from many different biological sources that, in some cases, have been problematic to measure in the past.

2 Materials and Methods

Mouse and rat IL6, TNFα and MCP-1 detection:
AlphaLISA™ kits available from Revvity: IL6 (mouse) (AL504), TNFα (mouse) (AL505) and CCL2/MCP-1 (mouse/rat) (AL509). Assay buffer is AlphaLISA Immunoassay Buffer (AL000C) for mouse IL6 (mIL6) and mouse TNFα (mTNFα), and NaCl Buffer (AL007C) for mouse MCP-1 (mMCP-1). All calibration curves were done using mouse analyte supplied in the kit. All 3 kits similarly detect mouse and rat cytokines.

Mouse/rat sample fluids:
All mouse/rat sample fluids were supplied as pools of individuals, non-medicated, non-immunized, from Bioreclamation LLC:

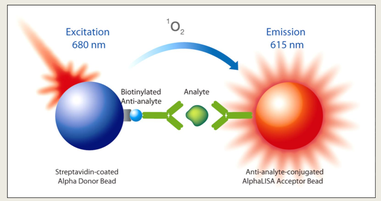
- Mouse bronchial lavage fluid (BALF): Strain CD-1, cat# MSE-BRONLEV
- Mouse serum: Strain BALB/C, cat# MSESRM-BALB
- Rat amniotic fluid (AF): Strain Sprague Dawley, cat# RATAMNFL
- Rat cerebrospinal fluid (CSF): Strain Sprague Dawley, cat# RATCSF

Diluents tested:
AlphaLISA HiBlock Buffer: Revvity, cat# AL004C.
Foetal bovine serum Premium (FBS): Wisent, cat# 080150.
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
- Beagle cerebrospinal fluid (CSF): cat# BGLCSF
- Rabbit amniotic fluid (AF): Strain New Zealand White, cat# RABAMNFL

Fluids pre-treatment and storage:
Prior to being used in AlphaLISA assays: FBS was triple filtered at 0.1µm by Wisent; mouse serum was filtered at 0.2µm by Bioreclamation LLC; BALF and AF fluids were centrifuged at 650xg for 15 min at 4°C to remove cells and cell debris, and supernatants were used in assays; CSF fluids were not pre-treated.
Storage: Mouse serum and FBS were kept at -20°C. All other fluids (BALF, CSF, AF) were kept at -80°C.

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 Immunoassays – quick and simple

Assay protocol with 3 incubation steps (No wash steps)

5 µL Standard or animal fluid

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Add 10 µL of AlphaLISA Anti-Analyte Acceptor beads

↓ Incubate 30 minutes

Add 10 µL of Biotinylated Anti-Analyte Antibody

↓ Incubate 60 minutes

Add 25 µL of Streptavidin Alpha Donor beads

↓ Incubate 30 minutes

Read at 615 nm using Envision® or EnSpire™ Reader and analyze data

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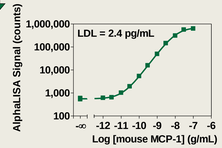
Calibration curve for mouse MCP-1 (LDL = Lower Detection Limit)



5 Fluids and cytokines chosen

Cytokines and mouse/rat fluids were selected according to availability of mouse/rat Revvity AlphaLISA kits, as well as availability and cost of fluids from suppliers.

Expected physiological range:

	Physiological range found in literature (pg/mL)			
Cytokine	Mouse serum	Mouse BALF	Rat CSF	Rat AF
IL6	5 - 70	20	Not found	10 - 60
TNFα	5 - 100	30-90	Not found	10 - 60
MCP-1	30 - 1500	40	Not found	1500

6 Experimental approach

Step 1: Perform linearity experiment with the following diluents for all 3 cytokines:

	Spiked sample fluid (3ng/mL or 10ng/mL mouse cytokine)			
Tested diluent	Mouse serum	Mouse BALF	Rat CSF	Rat AF
Assay buffer from kit		X	X	X
HiBlock buffer		X	X	X
PBS-0.1%BSA		X	X	X
PBS-0.01%BSA		X	X	X
Same fluid as sample fluid, but other species		X	X	X
FBS*	X			

*For serum samples, FBS was the only diluent tested, as FBS has previously been shown to be the best diluent for all serum samples (data not shown).

↓

Step 2: Diluent selection:
With step 1 results, select the two best diluents based on:

- Observed concentration X dilution factor (DF) as close as possible to spiked value, for all dilution points
- Recoveries between 70-130% for all dilution points

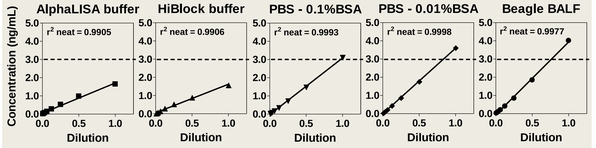
↓

Step 3: Evaluate spike and recovery value:

- Spike neat and ½ diluted mouse/rat fluid with mouse cytokine
- Use spiked diluent as spike control
- Select 3-4 spikes and include a "no spike" control (to measure physiological level)
- Recoveries between 70-130% are targeted
- Evaluate other diluents if good recovery is not obtained

7 Example (no dilution required)

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



	AlphaLISA buffer	HiBlock buffer	PBS-0.1%BSA	PBS-0.01%BSA	Beagle BALF
Dilution factor (DF)	Recovery from neat (%)	Recovery from neat (%)	Recovery from neat (%)	Recovery from neat (%)	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 (3ng/mL) for all dilutions

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

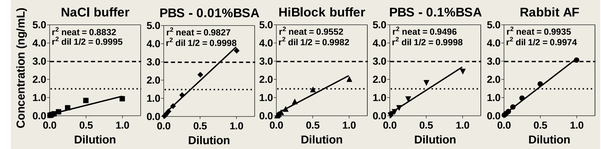
	Diluent: PBS-0.1%BSA		
	Spiked diluent control	Spiked neat Mouse BALF	
Spike (pg/mL)	Concentration (pg/mL)*	Concentration (pg/mL)*	Recovery (%)**
No spike	0.0	17.1	**NA**
10	12.0	9.2	**76**
30	35.2	32.3	**92**
300	300.2	292.6	**97**
3000	3141.2	2952.1	**94**

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

8 Example (½ dilution required)

Step 1: Linearity of dilutions in 5 diluents for spiked rat AF (3ng/mL mMCP-1)



	NACL buffer		PBS-0.01%BSA		HiBlock buffer		PBS-0.1%BSA		Rabbit AF	
Dilution factor (DF)	Recovery from neat (%)	Recovery from 1/2 dil. (%)	Recovery from neat (%)	Recovery from 1/2 dil. (%)	Recovery from neat (%)	Recovery from 1/2 dil. (%)	Recovery from neat (%)	Recovery from 1/2 dil. (%)	Recovery from neat (%)	Recovery from 1/2 dil. (%)
1	100		100		**100**		**100**		**100**	
2	179	100	127	100	**144**	**100**	**149**	**100**	**114**	**100**
4	187	104	131	103	**157**	**109**	**152**	**102**	**126**	**110**
8	180	100	129	101	**154**	**107**	**145**	**98**	**123**	**108**
16	170	95	129	101	**163**	**113**	**150**	**100**	**116**	**102**
32	192	107	129	101	**169**	**117**	**148**	**100**	**119**	**104**

Step 2:

Diluents not chosen: observed concentration X DF not close to spiked value (3ng/mL) (too low or too high).

Choose 2 good diluents: PBS-0.1%BSA and rabbit AF
- Excellent linearity from ½ dilution
- Observed concentration X DF close to spiked value (3ng/mL) for all dilutions.

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

	Diluent: PBS-0.1%BSA				
	Spiked diluent	Spiked neat Rat AF		Spiked 1/2 Rat AF	
Spike (pg/mL)	Concentration (pg/mL)*	Concentration (pg/mL)*	Recovery (%)**	Concentration (pg/mL)*	Recovery (%)**
No spike	0.0	910.5	**NA**	575.6	**NA**
300	267.3	195.2	**73**	317.2	**119**
1000	890.5	442.6	**50**	750.4	**84**
3000	2627.6	1542.0	**59**	2227.9	**85**

½ dilution in diluent required: excellent recovery for all 3 spikes tested in ½ rat AF, but not in neat rat AF. Measured MCP-1 physiological level in rat AF (No spike) = 575.6 pg/mL X 2 = 1151pg/mL

* Concentration for 300-3000 pg/mL spikes = measured concentration – no spike value.
** Recovery (%) = recovery compared to spiked diluent control

9 Summary table

Cytokine	Sample fluid	Best diluent	LDL in diluent (pg/mL)	Dilution of sample fluid required in diluent	Physiological level measured (pg/mL)	Spike range in sample fluid giving good recovery (pg/mL)
IL6	Mouse serum	FBS	2.1	1/2	33	30 - 10000
	Mouse BALF	PBS-0.1%BSA	2.4	Neat	22	10 - 3000
	Rat CSF	Beagle CSF	3.4	Neat	ND	10 - 3000
	Rat AF	PBS-0.01%BSA	2.9	1/2	ND	10 - 3000
TNFα	Mouse serum	FBS	1.6	1/2	55	30 - 3000
	Mouse BALF	PBS-0.1%BSA	1.6	Neat	17	10 - 3000
	Rat CSF	PBS-0.1%BSA	1.4	Neat	ND	3 - 3000
	Rat AF	PBS-0.1%BSA	1.4	1/2	5	3 - 3000
MCP-1	Mouse serum	FBS	5.4	1/2	444	300 - 3000
	Mouse BALF	Beagle BALF	5.7	Neat	22	10 - 3000
	Rat CSF	PBS-0.1%BSA	2.9	Neat	501	100 - 3000
	Rat AF	PBS-0.1%BSA	2.4	1/2	1151	300 - 3000

10 Conclusion

- Sensitive bead-based immunoassays were developed to detect IL6, TNFα and MCP-1 from rodent serum, BALF, CSF and AF. Good recoveries were obtained from fluids spiked as low as 3 pg/mL.
- This study highlights that the appropriate diluent is not easy to predict. In some cases where a perfect match of diluent and fluid is not found, sample dilution is required.
- These no wash rapid assays require only 5µL sample volumes. This represents a substantial improvement over ELISA-type assays in terms of speed of execution and sample consumption.
- Moreover, results presented demonstrate that measured physiological levels are also in agreement with the literature.

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