Quantifying Murine Anti-Viral Immune Responses: Development of a Bead-Based Multiplex Immunoassay for Standard Flow Cytometers

Amy Zhao, Binggang Sun, Jason Lehmann, Shaoquan Ji, and Weiping Jiang
BioLegend, San Diego, CA

Abstract

In response to pathogens, especially viruses, cells release interferons and other cytokines to combat infection. Interferons are typically divided into three types: 1 (*e.g.*, IFN-α, IFN-β), 2 (*e.g.*, IFN-γ), and 3 (*e.g.*, IFN-λ1, IFN-λ 2). All interferons are important for fighting viral infections and regulating the immune system. Beyond infections, interferons are also critically involved in cancer and autoimmune diseases such as psoriasis, systemic lupus erythematosus, and multiple sclerosis. Studying the expression profile of interferons and other related cytokines is essential to understanding the immune response against viral pathogens and other related disease processes.

We have developed a multiplex panel, using fluorescence-encoded beads, which is suitable for use on commonly available flow cytometers. The panel simultaneously quantifies 13 mouse anti-viral immune proteins, including IFN-α, IFN-β, IFN-γ, CXCL1 (KC), TNF-α, CCL2 (MCP-1), IL-12p70, CCL5 (RANTES), IL-1β, CXCL10 (IP-10), GM-CSF, IL-10, and IL-6. The assay was validated for specificity, accuracy, linearity of sample dilutions, cross-reactivity, and inter and intra-assay precision. The assay was further validated using biological samples to quantify analyte concentration changes in response to LPS and poly (I:C) stimulation compared to untreated controls.

This multiplex panel is a robust tool for measuring the concentration of anti-viral inflammatory mediators in murine biological samples, and offers greater efficiency and broader dynamic ranges compared to conventional

Materials and Methods

1. Instrument and Settings

Flow Cytometer	Laser to use	Reporter Channel	Reporter Channel Emission	Beads Classification Channel	Classification Channel Emission	Compensation needed?
BD FACS Calibur™	blue & red	FL2	575 nm	FL4	660 nm	No
BD Accuri™ C6	blue & red	FL2	585 nm	FL4	675 nm	No
BD FACSCanto™, BD FACSCanto™ II	blue & red	PE	575 nm	APC	660 nm	No
BD LSR, BD LSRII, BD LSRFortessa™	blue & red	PE	575 nm	APC	660 nm	No
Gallios	blue & red	PE	575 nm	APC	660 nm	No
CytoFLEX	blue & red	PE	585 nm	APC	660 nm	No
NovoCyte	blue & red	PE	572 nm	APC	660 nm	No
Attune™ NxT	blue & red	PE	574 nm	APC	670 nm	No

2. 96-well microtiter filter plates, V- or U-bottom plates, vacuum pump, filtration manifold and FACS tubes.
3. Capture antibody immobilized beads, biotinylated detection antibody cocktail, streptavidin-phycoerythrin (PE) conjugate and buffers.
4. Data analysis software and software dongle (provided free of charge).
5. **Biological Sample Preparation:**

   Mouse splenocytes and bone marrow cells were isolated from C57BL/6 mice (female, 8-12 weeks old) and cultured in T25 flasks under various stimulation conditions as indicated.

Assay Protocol

25 µL Matrix or Assay Buffer
25 µL Standard or samples
25 µL beads

↓ Shaking for 2h, RT
Vacuum and wash twice

25 µL Detection Antibody

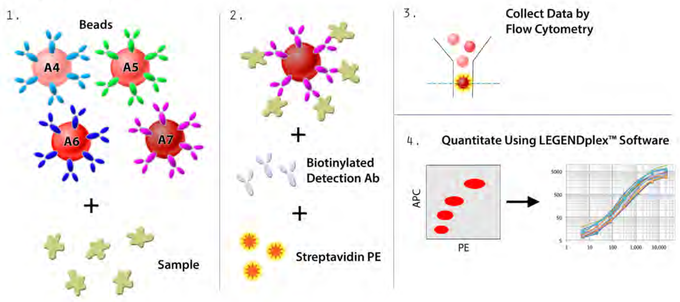
↓ Shaking for 1h, RT
No vacuum, no wash

25 µL Streptavidin-Phycoerythrin

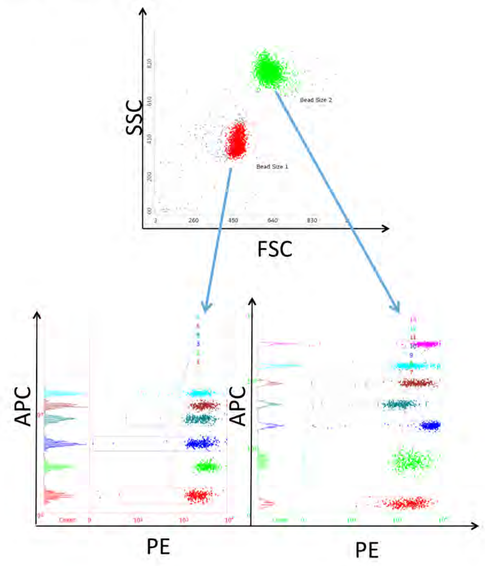
↓ Shaking for 30 min, RT
Vacuum and wash twice

Read on a flow cytometer

Assay Principle



Beads Classification

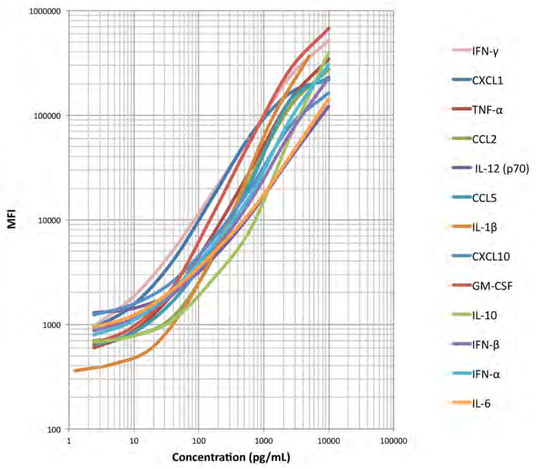


Panel Targets and Bead IDs

Targets included in the Mouse Anti-Virus Response Panel are listed below:

Mouse Anti-Virus Response Panel (Cat. No. 740621 or 740622)	
Bead ID	Target
A4	**IFN-γ**
A5	**CXCL1**
A6	**TNF-α**
A7	**CCL2**
A8	**IL-12 (p70)**
A10	**CCL5**
B2	**IL-1β**
B3	**CXCL10 (IP-10)**
B4	**GM-CSF**
B5	**IL-10**
B6	**IFN-β**
B7	**IFN-α**
B9	**IL-6**

Representative Standard Curves



Assay Sensitivity

Analyte	MDC* in Culture Media (pg/mL) (n=8)	MDC* in Serum (pg/mL) (n=5)
IFN-γ	1.44	0.73
CXCL1	1.54	0.66
TNF-α	1.18	0.92
CCL2	2.17	6.80
IL-12 (p70)	2.48	2.47
CCL5	2.38	1.85
IL-1β	1.63	1.55
CXCL10 (IP-10)	3.39	1.63
GM-CSF	2.66	1.31
IL-10	3.25	2.81
IFN-β	2.90	1.70
IFN-α	4.00	1.86
IL-6	3.16	1.67

*MDC = Minimum Detectable Concentration

Cross-Reactivity

No or negligible cross-reactivity was found with the following recombinant proteins (at 50 ng/mL)

Mouse	CCL20, CXCL2, CXCL9, CXCL12, IL-1α, IL-2, IL-4, IL-5, IL-7, IL-12 (p40), IL-13, IL-15, IL-17A, IL-21, IL-22, IL-23, IL-25, IL-27, IL-33
Rat	GM-CSF
Human	CXCL1, CXCL10, IFN-α1, IFN-α2, IFN-β, IFN-γ, IFN-λ1, IFN-λ2/3, IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-11, IL-12 (p40), IL-12 (p70), IL-15, IL-17A, IL-18, IL-23, IL-27, IL-33, GM-CSF, TNF-α

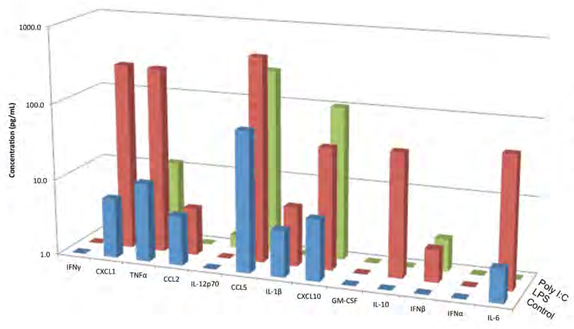
Spike Recovery and Linearity

Analyte	Spike Recovery	Spike Linearity
IFN-γ	92%	99%
CXCL1	94%	80%
TNF-α	90%	100%
CCL2	87%	98%
IL-12 (p70)	81%	83%
CCL5	90%	98%
IL-1β	95%	99%
CXCL10 (IP-10)	86%	112%
GM-CSF	87%	104%
IL-10	86%	103%
IFN-β	89%	114%
IFN-α	78%	103%
IL-6	81%	85%

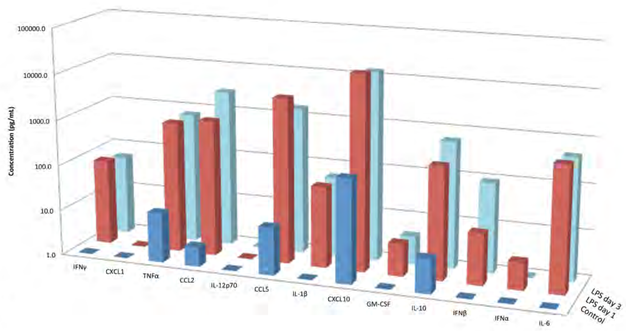
Spike Recovery: Natural samples were spiked with three different levels of recombinant proteins and assayed. Measured concentrations were compared with expected values for spike recovery analysis (%).

Linearity: Natural samples were spiked with known concentrations of recombinant proteins, then diluted 2, 4 and 8 fold. Measured concentrations of serially diluted samples were then compared to concentrations of the lowest dilution to calculate the linearity of sample dilution (%).

Biological Validation



Mouse splenocytes (1x10^6 cells/mL) were stimulated under various conditions (1 µg/mL LPS; 43 µg/mL Poly I:C), using unstimulated cells as a control. Supernatants were collected 1 day after stimulation and assayed.



Mouse bone marrow cells (1 x 10^6 cells/mL) were stimulated with 100 ng/mL LPS, using unstimulated cells as a control. Cell culture supernatants were collected 1 and 3 days after stimulation and assayed.

Conclusions

We have developed a bead-based multiplex assay for quantification of important mouse cytokines, interferons, and chemokines involved in innate and adaptive anti-viral immune responses.

The easy-to-use assay panel offers high performance, low cost, and user-friendly analysis tools without the need for dedicated assay-specific instrumentation.

The multiplex assay has been biologically validated using relevant sample types that include serum, plasma, and cell culture supernatants; and it should be a useful tool for biomedical and drug discovery research.

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