

Short communication

Analytical performance of a multiplexed, bead-based cytokine detection system in small volume samples

Martin Bomert^{1,a}, Gabi Köllisch^{1,a}, Marjut Roponen², Roger Lauener^{3,4}, Harald Renz¹, Petra I. Pfefferle¹ and Nadia Al-Fakhri^{1,*}

¹ Institute of Laboratory Medicine and Pathobiochemistry, Molecular Diagnostics, Philipps University, Marburg, Germany

² Department of Environmental Health, National Institute for Health and Welfare, Kuopio, Finland

³ University of Zürich, Children's Hospital, and Christine Kühne-Center for Allergy Research and Education, Zürich, Switzerland

⁴ Children's Allergy and Asthma Hospital, Hochgebirgsklinik, and Christine Kühne-Center for Allergy Research and Education, Davos, Switzerland

Abstract

Background: Multiplexed cytokine measurement offers many advantages over the conventional enzyme-linked immunosorbent assay (ELISA) format when applied in large-scale epidemiological studies or clinical trials. In the present study we set out to define the reliability and consistency of a suspension multiplexed protein array, the cytometric bead array (CBA), in large-scale, longitudinal studies.

Methods: The cytokines interleukin (IL)-5, IL-10, tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) were measured in pediatric samples from childhood asthma and allergy studies. Analytical performance of CBA was determined in sample supernatants and CBA was compared to conventional ELISA.

Results: Within-run and total imprecision were between 5.2%–10.8% and 5.6%–13.2%, respectively, at three different concentrations for all cytokines. Slopes of dilution linearity were between 1.01 and 1.31 for the four cytokines. The recovery rate at two different concentrations of the cytokines was between 97% and 113%. Lower limits of detection and quantification as well as functional sensitivity were determined. Comparison of the multiplex array and solid phase method showed good correlation with r between 0.82 and 0.93. The

sample volume required for the multiplex format was 25% of the ELISA sample volume.

Conclusions: CBA analytical evaluation and comparison to an ELISA format demonstrated high reproducibility, sensitivity and good applicability for small volume samples.

Keywords: allergy; analytical performance; cytokine measurement; immunology; multiplex assay system.

Analysis of immune functions in bio-samples collected in population-based epidemiological studies or large-scale clinical trials is of increasing importance. An important aspect in this regard is the simultaneous assessment of a large panel of cytokines to obtain a “fingerprint” of innate and/or adaptive immune responses. Clinical trials employing pediatric samples add further requirements on careful utilization of the often limited sample volume. This may require multiple cytokine measurements under high throughput conditions in small sample volumes. Therefore, we have validated and implemented a multiplex immunoassay system allowing measurement of multiple analytes simultaneously in a single assay procedure, the cytometric bead array (CBA) (1), using a FACSArray bioanalyzer system (BD Biosciences, San Jose, CA, USA).

A recently published review by Ellington and colleagues provided an extended overview on the usage of multiplex assay techniques for protein determinations (2). Besides many advantages of this assay format, the reliability and consistency of suspension multiplexed protein arrays in large-scale studies still remains unknown and the ability to analyze many samples without affecting inter-assay imprecision has not yet been adequately documented (2). We therefore compiled the evaluation data on the CBA assay and our technical experience with this assay format in a concise report to document the applicability of this multiplex assay technique for the analysis of longitudinal series of small volume samples.

Our laboratory participates in several multicentered studies, including the birth-cohort PASTURE (Protection Against Allergies-Study in Rural Environments)/Efrain (Mechanisms of Early Protective Exposures on Allergy Development) studies, designed to assess prospectively the role of farm exposures in the development of childhood asthma and allergies in rural environments across Europe and to gain insights into cytokine networks (3). Comparability of measurements from different sampling time points and stimulations is of great importance for longitudinal studies like PASTURE or Efrain requiring high analytical quality of the employed methods. In all study samples, the signature cyto-

^a M. Bomert and G. Köllisch contributed equally to this work.

*Corresponding author: PD Dr. Nadia Al-Fakhri, Institute of Laboratory Medicine and Pathobiochemistry, Molecular Diagnostics, Philipps University Marburg, Baldingerstr., 35043 Marburg, Germany
Phone: +49-6421-5866265, Fax: +49-6421-5866189,
E-mail: alfakhri@med.uni-marburg.de

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Table 1 Validation of the multiplex array-based CBA system.

Cytokine	Analytical sensitivity (CI)		Functional sensitivity at 20%, ng/L	Within run-imprecision, %			Total imprecision, %			Recovery rate, %		Dilution linearity Slope $\pm$ SD, intercept $\pm$ SD
	LOD, ng/L	LOQ, ng/L		80 ng/L	1250 ng/L	5000 ng/L	80 ng/L	1250 ng/L	5000 ng/L	625 ng/L	1250 ng/L	
IL-5	1.3 (1.1–1.5)	3.1 (3.0–3.3)	1.0	5.25%	5.26%	7.02%	6.89%	9.88%	9.07%	97.6%	113.1%	1.31 $\pm$ 0.03 10.89 $\pm$ 42.10
IL-10	2.3 (2.0–2.6)	5.7 (5.4–6.0)	2.5	6.45%	8.80%	8.67%	5.67%	9.32%	9.57%	97.0%	98.1%	1.01 $\pm$ 0.01 22.08 $\pm$ 12.74
TNF- α	3.9 (3.3–4.4)	10.2 (9.6–10.7)	15.0	6.78%	8.51%	7.67%	6.84%	8.76%	8.50%	98.8%	106.1%	1.03 $\pm$ 0.02 70.06 $\pm$ 40.11
IFN- γ	5.9 (5.1–6.8)	15.2 (14.4–16.0)	15.0	5.72%	8.78%	10.85%	9.18%	12.80%	13.24%	100.0%	108.4%	1.11 $\pm$ 0.02 44.67 $\pm$ 40.04

LOD and LOQ were calculated from a series of repeated blank measurements ($n = 10$). Confidence intervals (CI) are given in parentheses. The functional sensitivity is given as absolute concentration obtained from the measurement of $n = 12$ concentrations in triplicate; the measurement range was 5–10,000 ng/L according to the manufacturer's standards. The within-run and total imprecisions ($n = 16$ or $n = 17$ measurements, respectively) are given as percent variation coefficient from the respective means. The recovery rate ($n = 6$ measurements) was calculated as follows: (final concentration–initial concentration)/added concentration. The dilution linearity is shown as slopes and intercepts for each cytokine; SD, standard deviation.

kines interleukin (IL)-5, IL-10, tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) were measured as key components of the adaptive immune balance. According to the study protocol, blood samples were drawn at birth (cord blood), 1, 4.5 and 6 years of age and stimulated as described previously (4), supernatants were stored at -80°C . The study was approved by the responsible university Ethics Committees of all participating study centers, in accordance with institutional guidelines and the Declaration of Helsinki (2008 version). Informed consent was obtained from all parents of the index children.

For validation and comparison studies, supernatants from blood samples of 4.5 years collection of the PASTURE/Efrain cohort were measured with the multiplex bead array CBA (Human CBA flex sets, BD Biosciences) according to the manufacturer's instructions, paralleled by analysis with solid phase technique using Opt-EIA ELISAs (BD Biosciences). For the CBA assay, standards from 5 to 10,000 ng/L were reconstituted and serially diluted in assay diluent, standards and samples were measured in a 96-well format by means of specific antibody-coated 7.5 μm capture beads and phycoerythrin-conjugated detection antibodies on the FACSArray system (BD Biosciences). The standard curve and cytokine concentrations were calculated with FCAP Array software (version 2.0, Soft Flow, Burnsville, MN, USA, <http://www.softflow.com>). CBA measurements were compared to conventional ELISA as the standard procedure by means of Opt-EIA kits for the individual cytokines performed according to the manufacturer's instructions, as described in detail previously (4).

Our results indicated a good analytical performance in small volume samples using the CBA system (see Table 1). The dilution linearity of the system was high. Within-run and total imprecision were below 15% at low, normal and above-normal concentrations tested in the biological matrix of study samples. Analytical specificity determined by measuring the recovery rate, as well as the lower limits of detection and quantification describing analytical sensitivity, and lastly the functional sensitivity are also given in Table 1.

Comparison of the multiplex array CBA to the solid phase OptEIA method (ELISA) by linear regression analysis according to Deming showed acceptable slopes and intercepts. There were 556 samples included in the study. Data of IL-5, IL-10, IFN- γ , and TNF- α were normally distributed

Table 2 Comparison of multiplex array CBA and OptEIA method.

Cytokine	Slope $\pm$ SD	Intercept $\pm$ SD	r-Value
IL-5	1.69 $\pm$ 0.07	16.03 $\pm$ 9.69	0.903
IL-10	1.66 $\pm$ 0.03	−44.29 $\pm$ 9.00	0.930
IFN- γ	0.88 $\pm$ 0.03	223.3 $\pm$ 113.0	0.917
TNF- α	0.45 $\pm$ 0.02	100.3 $\pm$ 22.39	0.815

Comparison of the multiplex array CBA to the solid phase OptEIA method (ELISA) by linear regression analysis according to Deming (slope, intercept) and correlation coefficient according to Pearson (r), the data were normally distributed (Shapiro-Wilk test); SD, standard deviation. Measurement range: 5–10,000 ng/L, $n = 556$ measured study samples.

according to Shapiro-Wilk and both methods showed good correlation coefficients according to Pearson (Table 2). The measurement range was 5–10,000 ng/L. Correlation between the two methods was high; differing slopes and intercepts were mainly due to different antibody reactivity of both methods.

In addition, our study revealed that the cumulative sample volume for measurements of all respective cytokines required for the multiplex array method was reduced to 25% (55 μ L vs. 221 μ L) compared to the total sample volumes needed in the solid phase test system.

Multiplexed cytokine measurement therefore offers several advantages over the conventional ELISA format when applied in large-scale longitudinal studies: analytical performance of the multiplexed system CBA described in this work was demonstrated to show high reproducibility, sensitivity and good applicability for small volume samples.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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References

1. Morgan E, Varro R, Sepulveda H, Ember JA, Apgar J, Wilson J, et al. Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clin Immunol* 2004; 110:252–66.
2. Ellington AA, Kullo IJ, Bailey KR, Klee GG. Antibody-based protein multiplex platforms: technical and operational challenges. *Clin Chem* 2010;56:186–93.
3. Von Mutius E, Schmid S. The PASTURE project: EU support for the improvement of knowledge about risk factors and preventive factors for atopy in Europe. *Allergy* 2006;61:407–13.
4. Pfefferle PI, Buchele G, Blumer N, Roponen M, Ege MJ, Krauss-Etschmann S, et al. Cord blood cytokines are modulated by maternal farming activities and consumption of farm dairy products during pregnancy: the PASTURE Study. *J Allergy Clin Immunol* 2010;125:108–15.