

Technical note

Effect of serum content and diluent selection on assay sensitivity and signal intensity in multiplex bead-based immunoassays

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Abstract

Introduction: In the Luminex multiplexed immunoassay system, complex samples such as human serum are diluted to minimize disturbing matrix effects with a specific diluent. This diluent has to imitate the sample matrix to allow interpolation and has to provide optimal cytokine–antibody binding for all cytokines. Because diluents influence multiplex immunoassay results, this paper explores several methods to determine the quality of a chosen diluent.

Materials and methods: Two commercially available diluents, DY997 and RD6 from R&D Systems, were compared in a 19-plex immunoassay setup from Luminex.

Results: Using diluent DY997, multiplex signal intensity was reduced by 55% when spiked samples (chemokines and cytokines at 100 pg/mL) contained 50% v/v human serum, compared to samples containing 25% v/v. When using diluent RD6, signal intensity was reduced by 20% when samples contained 50% v/v human serum, compared to 25% v/v human serum. Diluent DY997 showed decreasing multiplex assay sensitivity with increasing protein concentrations, but not as low as in the presence of 50% v/v human serum.

Conclusions: In a 19-plex setup, this paper describes signal intensity, assay sensitivity and background signal levels in relation to the total volume-fraction of serum and protein concentration. For the determination of cytokines in serum samples with the multiplexed system Luminex the diluent RD6 seems more appropriate than the diluent DY997.

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1. Introduction

There is increasing demand for multiplexed immunoassay systems that allow multiple cytokine determination by using a small amount of samples (Kurkjian et al., 2006; Martins et al., 2006; Nolan and Mandy, 2006; Skogstrand et al., 2005).

The Luminex-100 bead-based system combines the principle of ELISA and fluorescent bead-based flow cytometry and is theoretically capable of measuring up to 100 analytes simultaneously.

An important factor in multiplex assay optimization is the sample diluent; a protein mixture that minimizes disturbing influences such as heterophilic antibodies and unspecific binding. In addition, the diluent optimizes cytokine detection by sample dilution and, at the same time, mimics the sample matrix in the calibration curve (i.e. human serum) to allow accurate interpolation of

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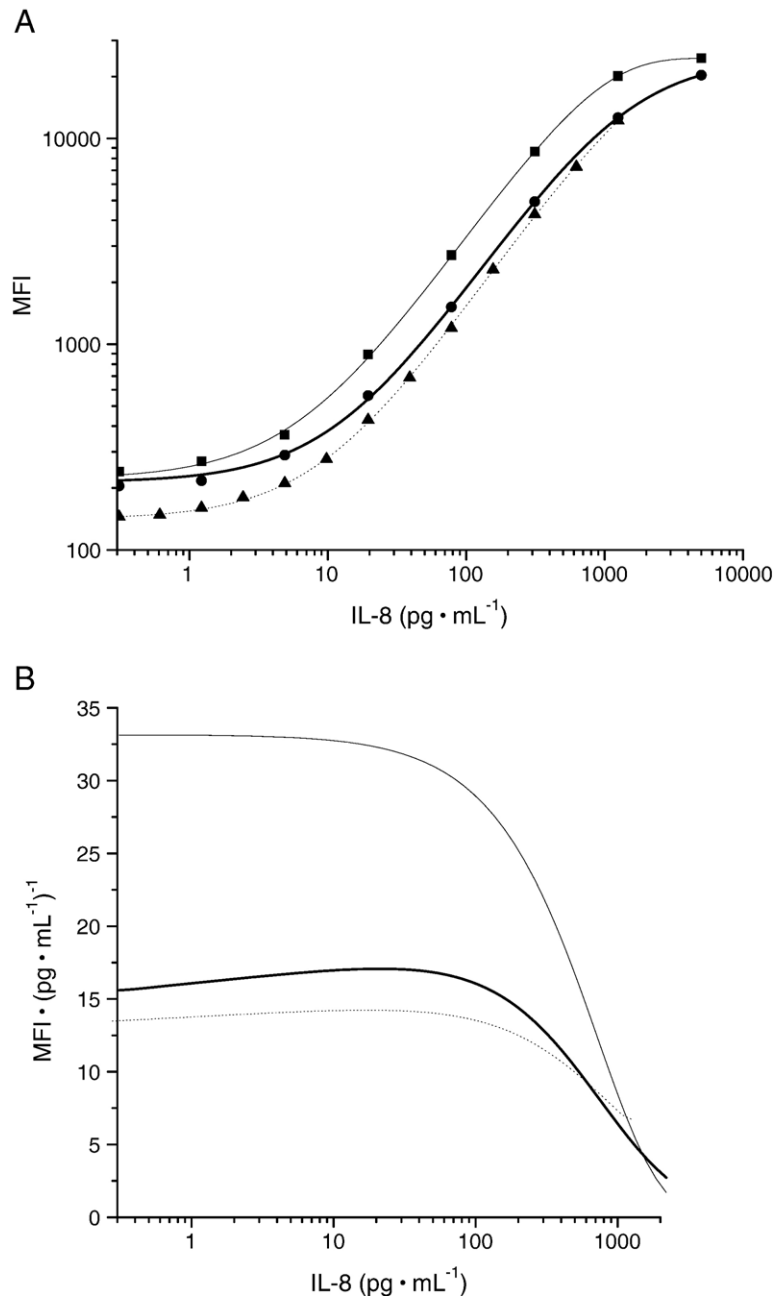


Fig. 1. A) Calibration curves of IL-8 prepared in RD6 (dotted line with triangles), DY997 (thin line with squares) and DY997 with 50% v/v serum (thick line with circles). MFI = Mean Fluorescence Intensity. B) Derivative of calibration curves of IL-8 prepared in RD6 (dotted line), DY997 (thin line) and DY997 with 50% v/v serum (thick line) ($n=2$). Sensitivity = $\text{MFI}/(\text{pg}/\text{mL})$.

unknown sample response in an external calibration curve. Diluents, of course, lack potentially disturbing factors such as endogenous cytokines and heterophilic antibodies (de Jager et al., 2005; Muller et al., 2002).

In a 19-plex setup, this paper describes signal intensity, assay sensitivity and background signal levels in relation

to the total volume-fraction of serum and protein concentration. We present several methods to determine the quality of a diluent. As an example we used two widely used diluents from R&D Systems; DY997 and RD6. We demonstrate the importance of using a diluent that adequately imitates the sample matrix.

2. Material and methods

2.1. Luminex multiplex assay

Proteins and antibodies used were purchased from R&D Systems (Wiesbaden, Germany) with the exception of recombinant proteins IL-1 β , IL-2, IL-5, IL-6, IL-8, IL-10, IL-13, IFN- γ which were from Strathmann (Hamburg, Germany), capture antibody IL-4 was from Bioscience (Cambridge, United Kingdom), IL-6, IL-10, IL-13 were from Pelikine (Amsterdam, The Netherlands) and detection antibodies IL-4 and IL-10 were from Bioscience and Pelikine, respectively. All cytokines and proteins were reconstituted as recommended by the manufacturers.

Samples were prepared containing 25% or 50% v/v serum of a serum pool that was prepared by combining serum of 10 healthy blood donors after informed consent, in diluents DY997 and RD6 with spiking of 100 pg/mL for each cytokine. Experiments were performed two times with 4 independent runs in duplicate. Two times, calibration curves were prepared in two-fold dilution steps using the same diluent and recombinant protein standards. All assays were performed as described previously (de Jager et al., 2003). Calibration microspheres were purchased from Bio-Rad (Munich, Germany), sheath fluid from Qiagen (Hilden, Germany).

Protein concentrations were quantified using a BCA protein assay kit from Pierce (Bonn, Germany) according to the specifications of the manufacturer.

2.2. Data and statistical analysis

Data were analyzed by the five parameter curve fitting in Origin 6.0 (Microcal Software Inc.,

Northampton, MA, U.S.A.) and BioPlex Manager 4.1 software (Bio-Rad).

3. Results

3.1. Influence of different diluents on sensitivity of calibration curves

We compared calibration curves prepared in 100% v/v DY997 and in 50% v/v DY997+50% v/v human serum, representative of sample volume fraction used in Luminex assays (Herder et al., 2006). Calibration curves for IL-8, as an example for the effects observed in all investigated cytokines, were calculated using a 5-parameter logistic (PL) function (Fig. 1A). Interestingly, the IL-8 background mean fluorescence intensity (MFI) signal of 50% v/v DY997+50% v/v human serum was actually lower than DY997 alone, although serum itself contains a certain amount of IL-8. To better visualize assay sensitivity,

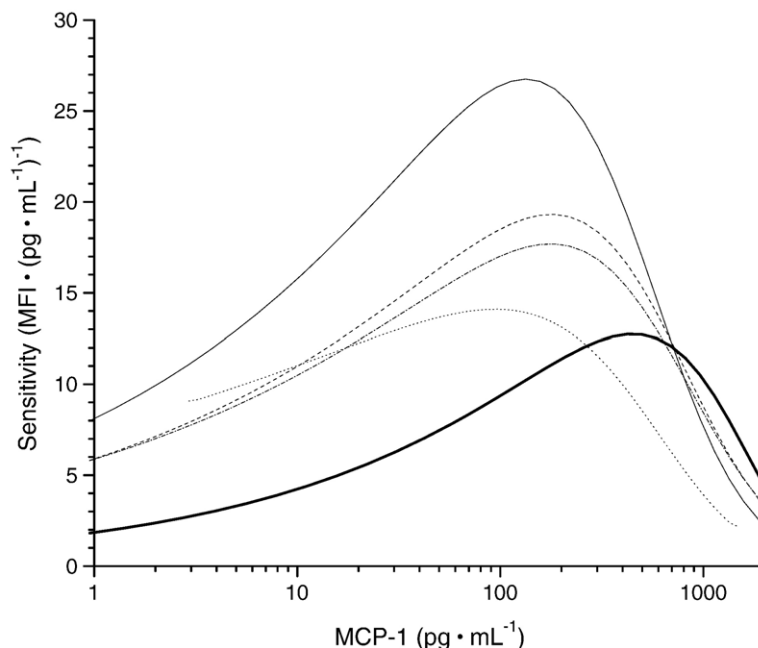


Fig. 2. Derivative of calibration curves of MCP-1 prepared in DY997 in the presence of 50% v/v serum (thick line), 50% v/v BSA solution at 56 (thin line), 85 (dashed line) or 128 mg/mL (dot-dashed line) and prepared in RD6 (dotted line) ($n=2$).

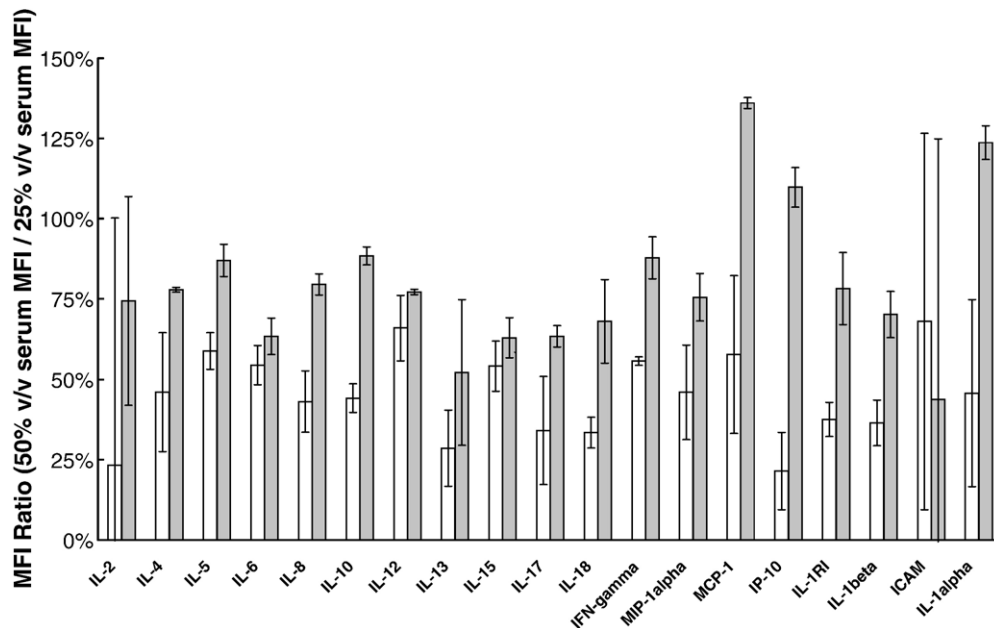


Fig. 3. Comparison of MFI values ratios obtained in either DY997 (white bars) or RD6 (grey bars) for various cytokines in the presence of 50% and 25% v/v serum. DY997 and RD6 with 25% or 50% v/v serum was spiked with 100 pg/mL of cytokine ($n=2$). Shown are means with standard deviation bars.

the derivative of the 5-PL function was plotted (Fig. 1B). In 100% DY997 average Luminex assay sensitivity was approximately 30 MFI/(pg/mL) from 1 to 100 pg/mL IL-8. However, adding 50% v/v human serum Luminex assay sensitivity decreased approximately twofold to 15 MFI/(pg/mL). When comparing calibration curves prepared in RD6 and 50% v/v DY997+50% v/v human serum, Luminex assay sensitivities were similar (13 and 16 MFI/(pg/mL), respectively). Thus, RD6 seems to be a diluent that more adequately imitates a human serum matrix.

3.2. Protein content influences sensitivity

To test whether serum protein concentration was the main reason for lowering sensitivity, we determined the protein concentration of DY997 and human serum. Protein content was 1.5 and 85.2 mg/mL for DY997 and human serum, respectively. To further investigate the effect of protein content on Luminex assay sensitivity we measured MCP-1 calibration curves (representative of all investigated cytokines and chemokines) in the presence 50% v/v bovine serum albumin (BSA) solution, at a BSA protein concentration of 67, 100 and 133% of human serum (56 mg/mL, 85 mg/mL or 128 mg/mL, respectively) (Fig. 2). At 100 pg/mL MCP-1, 56, 85 and 128 mg/mL BSA Luminex assay sensitivity was 25, 18 and 17 MFI/(pg/mL), respectively. In the same experiment 50% v/v human serum lowered

assay sensitivity to 9 MFI/(pg/mL). Using RD6 as diluent gave higher MFI/(pg/mL) values at MCP-1 concentrations below 100 pg/mL, but comparable results as DY997+50% v/v human serum, although RD6 had a protein concentration of 34.4 mg/mL. These results demonstrate that lowering of assay sensitivity cannot solely be attributed to protein concentration.

3.3. Comparison of signal intensity in DY997 and RD6 with respect to serum content

To investigate the effect of diluents DY997 and RD6 on Luminex assay sensitivity on samples, we spiked 19 cytokines and chemokines in diluent DY997 or diluent RD6 and human serum at a volume fraction of 25% and 50%. In DY997, samples containing 50% v/v human serum displayed 45% of MFI obtained with 25% v/v human serum (Fig. 3). In RD6, samples containing 50% v/v human serum showed 80% of MFI values obtained with 25% v/v human serum. This shows that serum matrix effects influenced the antibody-antigen equilibrium in both diluents, resulting in decreased Luminex signal intensity. However, this effect was less pronounced when using the RD6 diluent.

4. Conclusions

As shown in this paper for human serum, a diluent needs to mimic the sample matrix when establishing an

external calibration curve to allow accurate interpolation of unknown samples. With respect to our specific assay requirements, diluent RD6 from R&D Systems appears to be a more appropriate candidate for cytokine determination using the Luminex system. Our recommendation would be that one should compare several diluents in order to optimize a multiplex setup. More and more companies are offering high quality diluents, making it easier to find an appropriate diluent.

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