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Bead-based multiplex immuno-assays for cytokines, chemokines, growth factors and other analytes: Median fluorescence intensities versus their derived absolute concentration values for statistical analysis

Edmond J. Breen *, Veronika Polaskova, Alamgir Khan

Australian Proteome Analysis Facility (APAF), Level 4, Building F7B, Research Park Drive, Macquarie University, Sydney, NSW 2109, Australia

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ABSTRACT

Within the scientific literature, analyses of data from bead based multiplex immunoassays are based on either median fluorescence intensities (MFI) or derived absolute concentration values (ACV) but no consideration of which set of data is the most appropriate for analysis has been published. Here we look at the variance of MFI versus their ACV from the expression of 14 analytes in plasma, using 6 commercially available kits, across 177 patients, recorded at two time points and the associated analyte standards. In total 60 micro titre plates were used resulting in 4965 MFI. In doing so we develop a new background subtraction procedure that reduced by 50% the number of out-of-range values observed in our data set. Using a linear mixed-effect model, which normalizes for assay-to-assay variation, MFI produced similar significant differences than that observed using absolute concentration values. We show that subtracting analyte blanks produces 15% negative MFI resulting in uncertainty of the data being analysed. We argue for analysis of protein expression values MFI are generally a better choice than absolute concentration values. It is argued that analyte standards are not required on each plate, or not at all, in multi-plate experiments, but knowledge of the concentration curve and the range of MFI values that fall within the limits of this curve for each analyte is required. The significance of using MFI over concentration values for the life scientist means higher statistical power and lower costs.

1. Introduction

The reading of fluorescence emission, such as in bead-based multiplex immunoassays, is a standard method used in the life sciences for analysing biological molecules within biological tissue [1–3], for biomarker discovery and for screening targeted molecules in clinical trial studies [4–6]. The technology for multiplexed immunoassays have been reviewed recently [7,8] and therefore, is not dealt with here. The Luminex system, a commercially available multiplex immunoassay platform, is a magnetic or polystyrene bead-based application that uses microscopic beads that are dyed with different ratios of two fluorophores enabling quantification of up to 100 different analytes in a single sample. The Bio-Plex Manager software version 5 interprets these readings and outputs results in the form of a spread sheet that contains observed fluorescence, background corrected fluorescence and observed concentration values for each test-sample analyte, control, standard or blank being analysed and this output forms the basis of our comparative analysis.

Typically, relative median fluorescence intensities (MFI) obtained from each assay for each analyte are converted into absolute concentration values (ACV) by (a) creating a serial dilution set of standards of known concentrations for the analyte under investigation, (b) plotting the associated MFI obtained from these standards against their known concentration, and (c) by mapping MFI from the unknowns, test samples, through the graph to obtain the ACV. Background subtraction of associated blank MFI from the test samples and standards is also often performed [9]. However, as shown here subtracting background values often produce many negative readings and we develop and recommend an alternative approach, and with this procedure we are able to reduce the number of out-of-range values in our plasma data set by 50%.

While there are many issues with producing reliable robust standard concentration curves [6,10] a common problem is selecting the appropriate dilution series [11]. If the dilution series is too low (as in most cases) or too high then the unknowns (test samples) will be out of range (OOR) with respect to the standard curve, and from the data analysis point of view this is a problem as large unbalanced data sets are often generated. For life scientist it is even worst, as it can mean that entire analytes need to be ignored and

* Corresponding author. Tel.: +61 2 9850 9255; fax: +61 2 9850 6200.
E-mail address: ebreen@proteome.org.au (E.J. Breen).

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removed from the data set. Further, for large experiments and clinical trials where multiple micro titre plates are used the level of repeatability by the technician and instrument with respect to the production of controls, blanks, standards and test samples is a real issue [12] and considered by some to be the key to obtaining predictable outcomes [9]. While robotic liquid-handling platforms [7] can help alleviate this, not every lab will have access to such expensive equipment. The results presented here show that these problems are overcome greatly by using MFI rather than their associated concentration values.

This report addresses the question, for protein expression analysis obtained from quantitative multiplex immunoassays, should MFI or concentration values be used. While, reportedly, Luminex has determined that the MFI for analytes are best for analysis and reproducibility [13], bioinformaticians are often asked to do statistics on protein expression values from quantitative multiplex immunoassays using the Bio-Plex Manager observed concentration values. For a statistician this is perplexing because, as shown here, the MFI has greater statistical power, than these observed concentration values reported by the Bio-Plex Manager platform and therefore, greater discriminating ability and by association less likely to miss true positives than from an analysis based on the observed concentration values. Also, since the concentration values are derived from the use of standards it is interesting to note that for many multiplexed bead based systems the high variability and lack of agreement of protein expression levels across platforms and experiments is often attributed to the use of these standards and not to the MFI [7,10]. While there are numerous papers profiling protein expression for the comparisons of various multiplexed immunoassay platforms [6,10,14–18] and for protocol optimization [19] there is no published research done comparing the MFI to their derived observed concentration values that we could find. This may be in part because these two values, as many would realise, are highly correlated but as shown here it does not mean they have the same statistical power and that the standard concentration curve can have varied outcomes depending on how the curve is derived.

In this report we use the inter-assay coefficient of variance (CV) and a linear mixed-effect model to compare the expression levels of proteins in plasma across 60 micro titre plates from 177 patients, using both MFI and ACV and it is seen that MFI have statistically lower inter-assay CVs than the ACV and will produce protein expression differences that are the same to that identified using standard methods for determining ACV but are missed when considering the observed concentration obtained from the Bio-Plex Manager platform.

2. Material and methods

2.1. Samples and assay kits

Plasma samples were collected from 177 human subjects from a clinical trial study, from 4 collection sites. These involved 3 Australian ethics committees: Eastern Health Research and Ethics Ref:E20/0809, Baker IDI Heart and Diabetes Institute Human Research Ethics Committee Ref:7/2006, and the HARBOUR Human Research Ethics Committee of Northern Sydney Central Coast Health (NSCCH) Ref:Protocol 0611-217M (this covered two sites). The patients were randomly assigned to two groups (GroupA, GroupB), which represented diabetic and healthy subjects respectively. At 2 time points (baseline T1, 6 month endpoint T2) samples from each patient was collected. GroupA contained 93 patients, while GroupB 84, resulting in a total of 354 samples. Each sample was analysed for 14 analytes using 6 different types of customised kits (Milliplex kits, from Merck-Millipore, MA, USA) such as kit type 1 contained Interleukin-6 (IL.6), Interleukin -8 (IL.8), Leptin, Tumor Necrosis Factor-alpha (TNF.a) and Vascular Endothelial Growth Factor (VEGF) analytes (catalogue number HCCBP1MAG-58K), kit type 2 contained Interleukin-10 (IL.10) and Monocyte Chemotactic Protein-1 (MCP.1) analytes (catalogue number HCYTOMAG-60K), kit type 3 contained Adiponectin and Resistin analytes (catalogue number HADK1MAG-61K), kit type 4 contained Plasminogen Activator Inhibitor 1 (PAI) analyte (catalogue number HCVD1-67AK), kit type 5 contained C-Reactive Protein (CRP), Serum Amyloid A (SAA) and Serum Amyloid P (SAP) analytes (catalogue number HCVD2-67BK) and kit type 6 contained Brain Natriuretic Peptide (BNP) analyte only (catalogue number HCVD1MAG-67K). To acquire data for all 14 analytes, the same samples were analysed six times. Ten kits (same batch) of each kit type were used to cover all 354 samples as the maximum of 39 samples in duplicate were assayed using one kit (one assay plate). Kit types 4 and 5 were polystyrene bead based and all other kit types were magnetic bead-based.

2.2. Immunoassay procedures

Samples were defrosted on ice, vortexed for a few seconds and spun at 21,000 g for 10 min at 4 °C in order to separate plasma from lipid (flowing at the top) and any solid material such as cells (pelleted at the bottom of the tubes). The plasma was carefully pipetted out and filtered through 0.22 μm centrifugal filters at 10,000 g at 4 °C. The samples were then aliquoted and stored at −80 °C until analysed.

All reagents and samples were prepared, and assays performed according to the manufacturer's instructions provided with each kit. The standards and samples were assayed on a robotic liquid handling workstation (epMotion 5075, Eppendorf, Germany) for liquid delivery; plates were washed with Bio-Plex Pro II wash station (Bio-Rad, CA, USA) for magnetic beads and using an in-built vacuum manifold in epMotion 5075 for polystyrene bead-based kits. Assay plates were read with the Bio-Plex Systems 100 (Bio-Rad, CA, USA).

2.3. Plate layout design

In order to reduce plate to plate variation effects on group differences, an effort was made to distribute the samples, groups and time points as evenly as possible across all 10 plates of each analyte kit. For individual patients, time point T1 samples were on one plate while time point T2 samples were on another plate. Supplement Table S1 summarizes the distribution of these groups with respect to plate, by showing the number of analyte readings per plate with respect to Group and Time factors.

3. Results

It is common in a multiplex assay system to include 6–8 standards, 2 blanks and 2 quality controls (C1 low value, C2 high value) along with the test (unknowns) samples. The concentrations of test samples are often calculated after background subtraction of the test samples by subtracting the blank MFI from the test samples and by plotting them against the known concentrations of the back ground corrected standard curves. Here we start by considering the role of the blank in the analysis of multiplexed immunoassays.

3.1. Blanks and background correction

For the analyst, wanting to do a differential analysis using multiplexed immunoassay data or even to determine or report the concentration of various analytes within their test samples,