



Research paper

Validation and comparison of two multiplex technologies, Luminex® and Mesoscale Discovery, for human cytokine profiling

Ferdousi Chowdhury, Anthony Williams*, Peter Johnson

Cancer Sciences Division, CRUK Clinical Centre, Somers Cancer Research Building, University of Southampton, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, England, United Kingdom

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ABSTRACT

Biomarker research has rapidly expanded over recent years aided by the progressive development of research tools, in particular the different multiplex technologies allowing simultaneous measurement of multiple analytes. It is foreseeable that such technology will have an integral role in clinical studies for establishing biomarker profiles of disease status, but validation of the tools is essential to confirm the reliability of their application. More comparable studies between multiplex platforms are required to enable users to determine which of these are best for a particular clinical study, as different platforms will have varying levels of performance for the validation parameters. Comparison of two multiplex platforms, the Luminex® and the Mesoscale Discovery, has been performed to determine their performance for the validation parameters of sensitivity, precision and accuracy for the cytokines IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α . When measuring high concentrations both platforms show good accuracy (within $\pm 25\%$ recovery) with all cytokines except IL-12 for the MSD. At low concentrations, $\pm 25\%$ recovery was seen with all cytokines except IL-2 and IL-8 for the Luminex and IL-2 and IL-12 for the MSD. Although quantitative differences are found, relative differences are comparable, and consequently both platforms have been shown to be suitable for analyzing trends in multiple cytokine profiles, with the Luminex having better precision and the Mesoscale Discovery having greater sensitivity.

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1. Introduction

The recognition of the importance of cytokines in cell-to-cell communication and their role in cellular signaling, activation, growth and differentiation, has resulted in a wealth of information on single cytokines in settings of both health and disease (Fahey, 1998; Renauld, 2001; Lauts, 2003). The understanding of such roles has led to the realization of their potential therapeutic use in clinical conditions, either by direct use, blocking or indirect modulation of pathways associated with the release of specific cytokines (Maher et al., 2007; Bermel and Rudick, 2007; Bell and Kamm, 2000;

Yamagata and Ichinose, 2006). However, cytokines form a complex network of proteins with both paracrine and autocrine functions (Goldsby et al., 2000), and advancements in this intricate field have been hampered by a lack of techniques for simultaneous analysis of multiple cytokines. Traditionally, methods such as the enzyme-linked immunosorbent assay (ELISA) and ELISpot have enabled the analysis of single cytokines, and although such techniques have been invaluable in our current understanding, this has limited research to focus on a few key targets. With the advent of multiplex technology, cytokine research is now rapidly expanding, enabling analysis of large panels from small volume samples. The challenge for the new technologies is to prove their performance is adequate to allow such cytokine profiles to be used in a clinical setting.

As with any biological assay, the potential of simultaneously analyzing multiple analytes poses problems in terms of validation and standardization, especially for use in clinical

* Corresponding author.

E-mail address: apw2@soton.ac.uk (A. Williams).

studies (Mire-Sluis et al., 1995; Kopp et al., 1999; Aziz et al., 1999; Cummings et al., 2008). Furthermore, there are no regulatory guidelines for the validation of multiplex biomarker assays and existing guidelines for traditional immunoassays such as the ELISA (Findlay et al., 2000; DeSilva et al., 2003; Mire-Sluis et al., 2004; Shah et al., 2000) are proving too rigid to be applied to the new emerging technologies. The FDA guidelines suggest 'further research is needed to establish the validity of available tests and determine whether improvements in biomarkers predict clinical benefit' (Guidance for Industry: Clinical Trial Endpoints for the Approval of Cancer Drugs and Biologics, FDA, May 2007). Several reports have now been published on the validation of multiplex systems, most of which have involved a comparison against the enzyme-linked immunosorbent assay (ELISA), (de Jager et al., 2003; Ray et al., 2005; Prabhakar et al., 2002; Khan et al., 2004; Pickering et al., 2002; DuPont et al., 2005). Quantitative comparisons between multiplex and ELISA assays using culture supernatants show good agreement (de Jager et al., 2003; Tam et al., 2002; Carson and Vignali, 1999), but poor quantitative agreement is seen in more complex biological matrices (Liu et al., 2005; Young et al., 2008; Elshal and McCoy, 2006). In addition, cross-comparison of different reagent suppliers for the same multiplex platform shows variability in absolute levels of cytokines measured in serum (Khan et al., 2004), which mirrors differences reported with the 'gold standard' immunoassay – the ELISA (Ledur et al., 1995; Aziz et al., 1999). This variability in reagent choice for different technology platforms makes cross technology comparisons even more difficult to standardize. We have compared one reagent source for the Luminex[®] technology (Biosource [Invitrogen] 10 plex kit) against another (TH1/TH2 10 plex kit) for the Mesoscale Discovery system.

We have performed a detailed validation of a 10-analyte multiplex assay from Biosource (Invitrogen) for the Luminex[®] (Luminex Corp. Texas) in comparison to a 10 analyte assay (MesoScale Discovery, Maryland) for the MesoScale Discovery technology. The two assays have 7-cytokines in common, IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α , and cross-comparison of these has been performed in this study. Such a comparison is inherently reflective of the kit source as well as the associated technology that is examined.

The Luminex[®] xMAP technology is based on polystyrene bead sets encoded with different intensities of red and infrared dyes and coated with a specific-capture antibody against one of the analytes of interest. Interrogation of the beads by two lasers identifies the spectral property of the bead and hence associated analyte, in addition to the R-phycoerythrin (RPE) labeled secondary antibody against the specific analyte. The second platform assessed was the Mesoscale Discovery (MSD) electrochemiluminescence detection system. This system uses plates fitted with up to 10 carbon electrodes per well, each electrode being coated with a different capture-antibody. The assay procedure follows that of a sandwich ELISA, with any analytes of interest captured on the electrode being detected with an analyte-specific ruthenium-conjugated secondary antibody. Upon electrochemical stimulation the ruthenium label emits light at the surface of the electrodes allowing the concentration of the analyte to be determined relative to the particular electrode.

A direct comparison of the two systems in determining plasma cytokine levels in ex-vivo stimulated whole blood is reported.

2. Materials and methods

2.1. Cytokine standards and controls

Lyophilized cytokine standards were provided as a cocktail mix with both the Biosource (Invitrogen) Luminex[®] and Mesoscale Discovery (MSD) multiplex kits. The Biosource (Invitrogen) Luminex[®] cytokine standard concentrations vary between the kits with each cytokine having a different pre-defined stock concentration. MSD standards were always supplied at the same stock concentration of 10,000 pg/ml for each cytokine. Negative and spiked sample controls were prepared from the pooled plasma samples of 6 healthy individuals. Plasma was separated from whole blood within 4 h of collection and immediately frozen as 50 μ l aliquots at -70°C . Unspiked pooled plasma from 6 healthy individuals was used as the negative control. Spiked controls were prepared from the pooled plasma following spiking with high or low concentrations of the cytokine standards provided in the multiplex kits. Two sets of spiked controls were prepared, one using the standard cocktail from the Biosource (Invitrogen) Luminex[®] kit, and the other using the standards from the MSD kit. All spiked samples were gently vortexed and stored immediately as 50 μ l aliquots at -70°C .

2.2. Luminex[®] cytokine detection

Human cytokine Luminex custom 10-plex kits (for IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IFN γ , TNF α , MIP-1 β and MIP-1 α) were purchased from Invitrogen (Biosource). All reagents were provided with the Biosource (Invitrogen) kit (LCP0010), and all reagents were prepared according to the manufacturers' protocol booklet provided. The assays were performed in 96-well filter bottom plates according to the protocol provided. Antibody conjugated beads are used at a concentration of 5000 beads per marker, per well requiring a 30 s vortex followed by a 30 s sonication to prevent bead aggregation. The volume of concentrated beads required for each assay was determined using the protocol chart and diluted to the correct volume using wash solution. The beads were protected from light throughout the course of the assay. The lyophilized standard was reconstituted in 1 ml of assay diluent and 1/3 serial dilutions undertaken to generate a 7 standard concentration set. Diluent alone was used as a blank. The wells to be used were pre-wet with 200 μ l of wash solution for 30 s, and then aspirated using a vacuum manifold with the bottom of the plate blotted on paper towels to remove excess fluid. The bead solution was vortexed and sonicated immediately prior to adding 25 μ l into each well. The plate was washed twice by adding 200 μ l of wash solution, soaking for 30 s and then aspirating using a vacuum manifold with the bottom of the plate once again blotted to remove excess fluid. Incubation buffer (50 μ l) was added to each well and each standard was added in duplicate (100 μ l per well). A further 50 μ l of assay diluent was added to each sample well followed by the addition of the sample (50 μ l). The plate was covered in foil and incubated for 2 h at room temperature on an orbital plate shaker (600 rpm). At the end of this incubation, the

fluid from the wells was removed using a vacuum manifold, and then the plate was washed twice as before. Biotinylated detection antibody (100 μ l) was added to each well, covered and incubated on the orbital shaker for a further 1 h at room temperature. The plate was then washed twice prior to addition of Streptavidin-RPE (100 μ l). The plate was covered and incubated for a further 30 min at room temperature on an orbital shaker. Finally the plate was washed three times as before. Wash solution (100 μ l) was added to each well, and the plate covered and placed on an orbital shaker for 2–3 min at room temperature prior to analysis. The plate was uncovered and measured on the Luminex xMAP 100 system (Luminex Corp.), and analysed using StarStation software version 2.0 (Applied Cytometry Systems). The analysis software was set to acquire data using 50 μ l of sample per well, to collect a total of 1000 beads (average of 100 events per single bead set). The raw data was measured as mean fluorescence intensity (MFI) and the concentration of each analyte for each sample was calculated using a 4 or 5-parameter logistic fit curve generated for each analyte from the 7 standards. The lower limit of quantification (LLOQ) was determined using the lowest standard that was at least 3 times above background. The calculation of the LLOQ was performed by subtracting the MFI of the background (diluent) from the MFI of the lowest standard concentration and back-calculating the concentration from the standard curve.

2.3. MesoScale Discovery cytokine detection

TH1/TH2 human 10-plex kits (for IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IFN γ , TNF α , IL-1 β and IL-13) were purchased from MSD (K110101A-4). All reagents were provided with the MSD kit. Each 96-well plate had 10-carbon electrodes in the bottom of each well, each pre-coated with one of the 10-anti-cytokine antibodies of interest. The standards were reconstituted in the assay diluent provided. Assay diluent (25 μ l) was added to all wells and the plate sealed and incubated for 30 s at room temperature on an orbital shaker (600 rpm). Samples, standards and controls were added at 25 μ l per well. The plate was sealed and incubated for 2 h at room temperature on an orbital shaker (600 rpm). At the end of the incubation the wells were washed three times using 200 μ l PBS + 0.05% Tween 20, soaking for 30 s and then discarding. Detection antibody was added at 25 μ l per well, and the plate sealed and incubated for 1 h at room temperature on an orbital shaker (600 rpm). At the end of the incubation the plate was washed three times as before. 150 μ l of the MSD Read Buffer was added to each well and the MSD plates were measured on the MSD Sector Imager 2400 plate reader. The raw data was measured as electrochemiluminescence signal (light) detected by photodetectors and analysed using the Discovery Workbench 3.0 software (MSD). A 4-parameter logistic fit curve was generated for each analyte using the standards and the concentration of each sample calculated.

2.4. Whole blood collection and stimulation

Blood samples were collected from healthy individuals in lithium heparin-containing vacutainer tubes. Whole blood stimulation assays were set up within 4 h of blood collection. One ml of whole blood was added to 15 ml polypropylene

Falcon tubes, with or without 0.1 μ g/ml LPS. The tubes, with the lids loosened and the tubes held slanted in a rack, were placed in a 5%CO₂ incubator at 37 °C for 4 h. At the end of the incubation, 500 μ l of each sample was removed and centrifuged at 300 g for 15 min. The plasma was removed and stored as 50 μ l aliquots at –70 °C. All cytokine analyses were performed on frozen plasma samples.

3. Results

3.1. Comparison of the limits of quantification

The Biosource (Invitrogen) Luminex cytokine standards were diluted 1/3 to produce 7-standard concentrations; each cytokine standard was used to produce a 7-point standard curve. The starting stock concentrations for the highest standards for the Biosource (Invitrogen) kit were variable, resulting in standard curves being generated with different detection ranges of 6150–8.4, 6530–8.9, 5850–8.1, 7940–10.9, 14290–19.6, 12580–17.3 and 2900–3.98 pg/ml for IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α respectively, (Fig. 1A). The MSD cytokine standards were diluted 1/4 to produce 8-standard concentrations, each generating an 8-point standard curve with a similar detection range of 10,000–0.61 pg/ml due to all stock concentrations starting at 10,000 pg/ml (Fig. 1B). In general, the MSD standards produced a greater detection range than the Biosource (Invitrogen) Luminex standards, due to 1/4 dilutions being recommended and the highest standard concentration being greater (except for IL-12 and IFN γ). However, the high concentration range is unlikely to be physiologically relevant, and if required, samples can be diluted to allow reliable quantification whereas the lower end of the standard curve is likely to be of more physiological relevance. The lower limits of quantification calculated using the mean of two identical kits and standards for the Biosource (Invitrogen) Luminex kit were 8.7, 8.6, 7.7, 11.6, 21.3, 14.4 and 5.9 pg/ml for IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α respectively (Table 1). This compared with performance characteristics of this kit of 6, 5, 3, 5, 6, 5 and 10 pg/ml. The LLOQ calculated using the mean of two identical kits and standards for the MSD platform were 2.5, 0.7, 0.7, 3.6, 2.3, 0.7 and 3.2 pg/ml for IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α respectively (Table 1) with kit performance limits of <1 pg/ml for all cytokines. Comparison of the two platforms and associated kits for the analytes IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α , shows that the MSD kit has a lower level of quantification and is therefore more sensitive than the Biosource (Invitrogen) Luminex kit.

3.2. Comparison of inter and intra-assay precision

Samples spiked with known concentrations of standard taken from the Luminex kit were run on the Luminex platform and samples spiked with known concentrations of standard taken from the MSD kit were run on the MSD platform to establish intra-assay and inter-assay variability. High concentration spiked samples for MSD were all at 2000 pg/ml, and for Biosource (Invitrogen) were at 3075, 3265, 2925, 3970, 7145, 6290 and 1450 pg/ml for IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α respectively. Low concentration spiked samples for MSD were all at 20 pg/ml and for

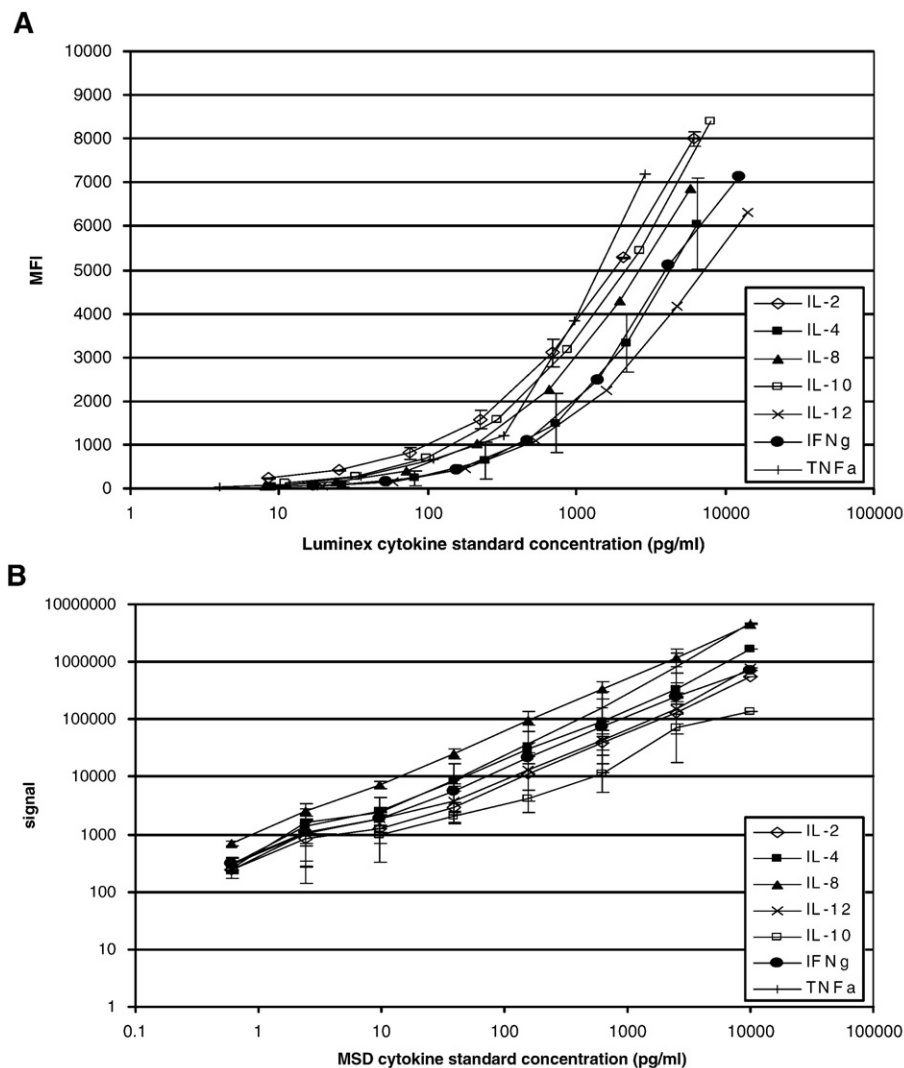


Fig. 1. Standard curves generated showing the range of detection for common analytes in the two platforms. The error bars show the standard deviation of the mean of two separate assays using (A) two identical Biosource (Invitrogen) Luminex kits and standards, (B) two identical MSD kits and standards. The Y axis for (A) is linear and for (B) is logarithmic, reflecting the different primary readouts of the technologies.

Biosource (Invitrogen) were at 62, 65, 59, 79, 143, 126 and 29 pg/ml for IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α respectively. These were all well within the LLOQ determined for each technology. Inter-assay variation was determined as the percentage coefficient of variation (%CV) from three

assays performed on three different occasions for each platform, using the same batch of spiked samples stored as single use aliquots. Results are shown in Fig. 2. The inter-assay %CV for samples spiked with high concentrations of Biosource (Invitrogen) kit standard was 12.3, 14.9, 37.9, 3.1, 83.9, 14.0 and 11.7%, and with MSD kit standard was 7.8, 18.1, 27.2, 75.4, 37.8, 19.2 and 14.9% for the cytokines IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α respectively (Fig. 2A). The inter-assay %CV for samples spiked with low concentrations of Biosource (Invitrogen) kit standard was 13.5, 25.4, 3.0, 31.7, 79.7, 15.9 and 41.8% and with the MSD kit standard was 71.1, 22.1, 47.8, 125.9, 87.0, 21.5 and 54.8% for the cytokines IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α respectively (Fig. 2B). Measurements of standards at high concentration show the inter-assay %CV was above the 25% acceptance criteria for IL-8 and IL-12 for the Biosource (Invitrogen) kit and IL-8, IL-10 and IL-12 for the MSD kit. At low concentrations the inter-assay

Table 1
Limits of quantification

Cytokine	LLOQ (pg/ml $\pm$ S.D.)	
	Luminex (Biosource kit)	MSD (MSD kit)
IL-2	8.7 ($\pm$ 2.2)	2.5 ($\pm$ 3.0)
IL-4	8.6 ($\pm$ 2.9)	0.7 ($\pm$ 0.1)
IL-8	7.7 ($\pm$ 1.5)	0.7 ($\pm$ 0.1)
IL-10	11.6 ($\pm$ 0.8)	3.6 ($\pm$ 5.0)
IL-12	21.3 ($\pm$ 6.1)	2.3 ($\pm$ 2.8)
IFN γ	14.4 ($\pm$ 3.0)	0.7 ($\pm$ 0.03)
TNF α	5.9 ($\pm$ 4.9)	3.2 ($\pm$ 4.3)

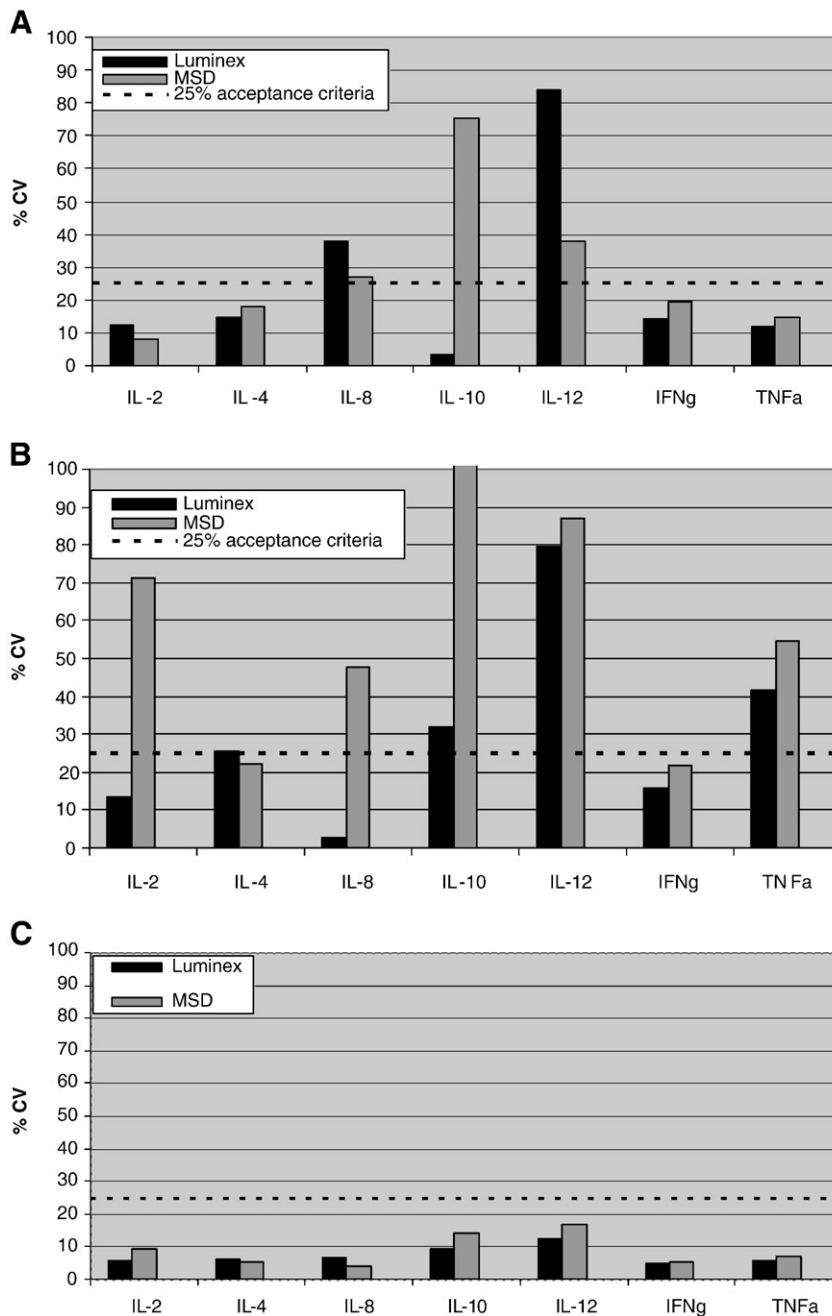


Fig. 2. Inter assay variability for spiked plasma samples from three replicate assays performed on three different days using samples stored as single-use aliquots, are shown as the percentage coefficient of variation (%CV) for the MSD and Luminex platforms. The solid bars show the %CV at (A) high concentration (MSD cytokines at 2000 pg/ml and Luminex at 3075, 3265, 2925, 3970, 7145, 6290 and 1450 pg/ml for IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α respectively) and (B) low concentration (MSD cytokines at 20 pg/ml and Luminex at 62, 65, 59, 79, 143, 126 and 29 pg/ml for IL-2, IL-4, IL-8, IL-10, IL-12, IFN γ and TNF α respectively). Intra assay variability was calculated as the %CV for 10 replicate samples run on a single plate (C). The dotted line on each plot shows the 25%CV acceptance criteria.

variation was above the 25% acceptance criteria for IL-10, IL-12 and TNF α with the Biosource (Invitrogen) kit and for IL-2, IL-8, IL-10, IL-12 and TNF α with the MSD kit. This gives a mean %CV of 25% and 29% for the Biosource (Invitrogen) Luminex kit and the MSD kit respectively when measuring cytokines at high concentration; and 30% and 61% for the Biosource (Invitrogen) Luminex kit and the MSD kit respec-

tively, when measuring cytokines at low concentration. The product characteristics report an inter-assay CV of standard curves of <10% for both the Biosource (Invitrogen) Luminex and MSD kits. Intra-assay precision was determined as the %CV from 10 replicate samples on a single plate for each platform. Excellent intra-assay precision was seen with both platforms, % CV < 25% (Fig. 2C) consistent with kit characteristics. Therefore,

the results indicate overall inter assay precision was better for the Biosource (Invitrogen) Luminex kit than the MSD kit but both platforms have good intra assay precision.

3.3. Comparison of accuracy

Samples spiked with either a high or low concentration of the MSD kit standard or Biosource (Invitrogen) Luminex kit standard were run in duplicate using the MSD and Luminex platform respectively. The concentrations of the samples were determined from the standard curves generated and accuracy was determined as the concentration recovered expressed as a percentage of the actual spiked concentration. The acceptance criterion for accuracy was set at $\pm 25\%$ recovery of actual spiked concentration. Results are shown in

Fig. 3. The % recovery for high concentrations of cytokines all fell within $\pm 25\%$ of actual spiked concentration except for high concentration of IL-12 with the MSD platform which fell just below acceptance at 73% (Fig. 3A). For low concentration cytokines (Fig. 3B) using the Luminex, only IL-2 and IL-8 fell within the $\pm 25\%$ acceptance criteria, the remaining cytokines falling outside the acceptance range, (IL-4 (38%), IL-10 (53%), IL-12 (48%), IFN γ (53%) and TNF α (41%)); using the MSD, better accuracy was seen with IL-4, IL-8, IL-10, IFN γ and TNF α all falling within the $\pm$ acceptance criteria, and only IL-2 (512%) and IL-12 (0% as below level of detection) falling below the acceptance range. The results show both platforms to have good accuracy when measuring high concentrations of cytokines, but accuracy is variable at lower concentrations. For the panel tested at low concentra-

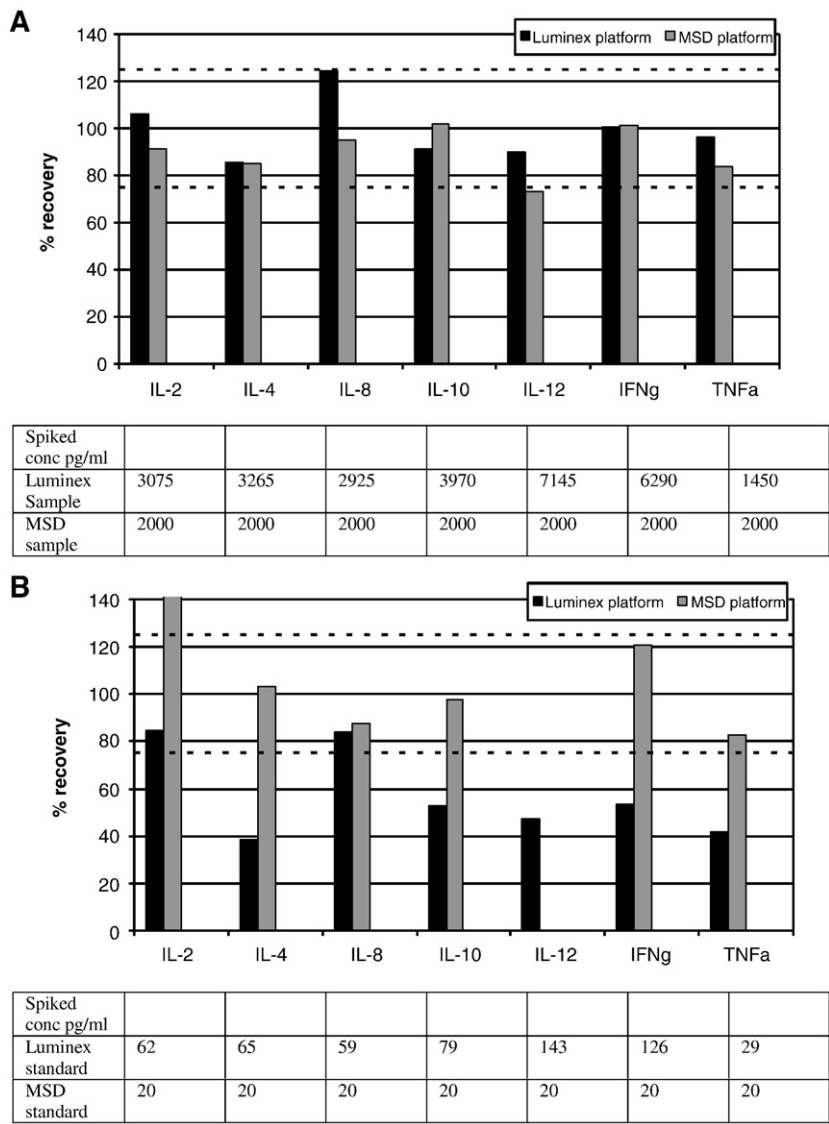


Fig. 3. Plasma samples spiked with either a high (A) or low (B) concentration of the MSD kit standard or Biosource (Invitrogen) Luminex kit standard were analyzed in duplicate using the MSD and Luminex platform respectively. The table below each of the graphs show the actual concentration of each cytokine spiked into the samples. The bars show the percentage recovery of each cytokine determined as the concentration recovered expressed as a percentage of the actual spiked concentration. The dotted lines on each graph show the acceptance range of $\pm 25\%$ of actual spiked concentration.

tion, MSD has shown better accuracy for most cytokines in comparison to the Luminex, except for IL-2 where Luminex has better accuracy.

In addition to determining accuracy using the kits own standard, spike recovery assays were performed with the alternate kit suppliers' standard. Thus, plasma samples spiked with the MSD kit standard were assayed using the Biosource (Invitrogen) Luminex kit and plasma samples spiked with the Biosource (Invitrogen) Luminex kit standard were assayed using the MSD kit. Accuracy was determined as the percentage recovery calculated as the recovered concentration expressed as a percentage of the actual spiked concentration, and the acceptance criterion was set as $\pm 25\%$ of the actual

concentration. The % recovery for measuring high concentration cytokine standards using the alternate platform was poor (Fig. 4A), with all cytokine recoveries falling well below the $\pm 25\%$ acceptance criteria for both the MSD and Luminex® platforms. The % recovery for low concentration Biosource (Invitrogen) Luminex cytokine standards analyzed on the MSD platform also fell below the 25% acceptance criteria except for IL-12 (91%). Four of the low concentration MSD cytokine standards (IL-4, IL-10, IL-12 and TNF α) when measured with the Biosource (Invitrogen) Luminex kit fell within the $\pm 25\%$ acceptance criteria. These results show that both platforms have variable accuracy when measuring standards designed to be used for a different platform, with

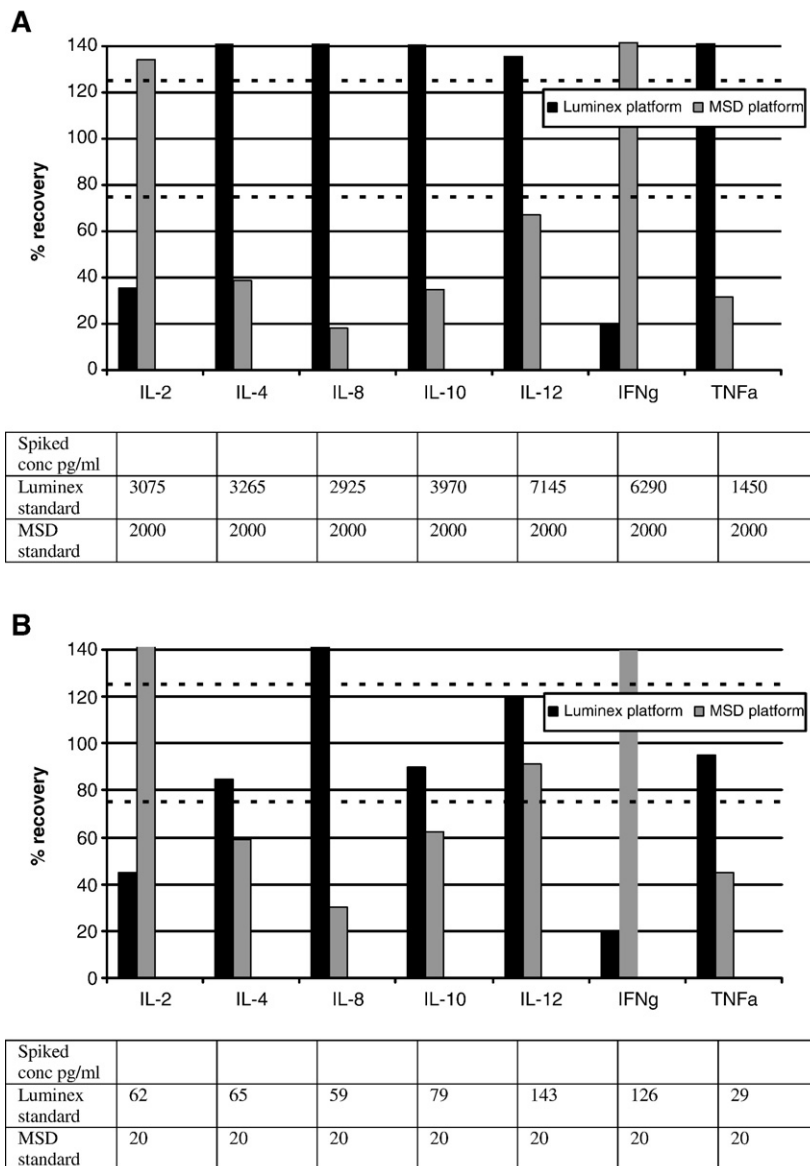


Fig. 4. Plasma samples spiked with either a high (A) or low (B) concentration of the MSD kit standard or Biosource (Invitrogen) Luminex kit standard were analysed in duplicate using the Luminex and MSD platform respectively. The table below each of the graphs show the actual concentration of each cytokine spiked into the samples. The bars show the percentage recovery of each cytokine determined as the concentration recovered expressed as a percentage of the actual spiked concentration. The dotted lines on each graph show the acceptance range of $\pm 25\%$ of actual spiked concentration.

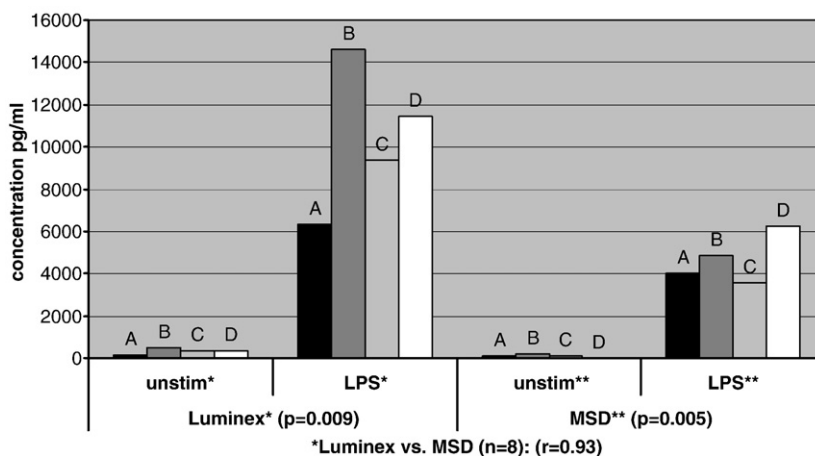


Fig. 5. Plasma from 4 sets of whole blood samples (A, B, C, and D) stimulated with and without 0.1 µg/ml LPS were analyzed using the Biosource (Invitrogen) Luminex kit and the MSD kit. The bars show the IL-8 levels measured using each platform.

poor accuracy for all cytokines tested at high concentrations falling below the $\pm 25\%$ acceptance range, and some cytokines falling within the acceptance range at lower concentrations.

In conclusion, accuracy for measurement of high levels of cytokines is within the 25% acceptance criteria and is comparable for both the Luminex and MSD platforms, but at lower levels the MSD has better accuracy but this is cytokine dependent. However, there is no compatibility across platforms for what is actually being measured. This relates to the standards used within each manufacturer's kits, typically recombinant cytokines which can vary between kit suppliers. As these kits are then linked to particular technology platforms, it is clear that different platforms must be individually assessed for the accuracy of the cytokines and cross compared to other integrated systems i.e. Luminex® and MesoScale Discovery.

3.4. Comparison of Luminex® with MSD for the analysis of plasma samples from whole blood assays

Whole blood sample assays require analysis of plasma, as opposed to serum which is extracted from clotted samples. Plasma extracted from whole blood assays provide a better clinical comparison than supernatant extracted from a cell culture assay. The validation results showed good accuracy when measuring high and low concentrations of IL-8 with both the Luminex and the MSD platform (when using their own kit standard), therefore, it was a good candidate for comparative analysis of the two platforms using the same set of stimulated samples. Validation also showed good intra assay precision, but to reduce the effect of poor inter-assay precision, samples were run simultaneously using a single Biosource (Invitrogen) Luminex kit and MSD kit. Plasma was removed from 4 sets of whole blood samples stimulated with/without 0.1 µg/ml LPS and analyzed for IL-8 levels using the Biosource (Invitrogen) Luminex kit and MSD kit (Fig. 5). The results show that low levels of IL-8 are detectable with both the MSD platform (93, 205, 88 and 23 pg/ml for samples A, B, C, and D respectively) and the Luminex platform (172,

514, 341 and 336 pg/ml for samples A, B, C and D respectively) for unstimulated samples. Significant increases in IL-8 levels are seen with both the MSD ($p=0.005$) and Luminex ($p=0.009$) platforms when samples are stimulated with LPS. However, as with the unstimulated samples, the absolute values for the stimulated samples are different between the MSD platform (4036, 4853, 3582 and 6256 pg/ml for samples A, B, C and D respectively) and the Luminex platform (6356, 14603, 9340 and 11420 pg/ml for samples A, B, C and D respectively). Although, quantitative differences are seen, there is good correlation between the two platforms ($r=0.93$). The results show that quantitative comparison of samples between the two platforms is not feasible, but relative changes and trends are comparable between the two platforms.

4. Discussion

Validation of the Luminex and MSD has shown both platforms to have potential use in analysis of clinical samples. In terms of practicality, both platforms effectively allow multiple analytes to be analysed simultaneously from small volume samples (25–100 µl/test), reducing time, cost and effort, making them an attractive alternative to the ELISA. The Luminex platform is a fluidics system which requires relatively more maintenance than the MSD platform, but has the advantage of being able to measure a larger panel of analytes.

In terms of performance, the MSD was found to be more sensitive than the Luminex, with the lowest level of quantification (LLOQ) being lower than the Biosource (Invitrogen) Luminex kit for all the cytokines tested in our panel. This may be related to the ultrasensitive TH1/TH2 kit which is provided for MSD 10 plex evaluations compared to the custom 10 plex kit from Biosource for Luminex. Both platforms are capable of detecting a concentration below the LLOQ for each of the 10 cytokines. However, an absolute value for the lower level of detection (LLOD) could not be determined as this signal was prone to variation and falls below the standard curve making quantification unreliable.

This same problem has prevented other validation studies determining LLOD for serum samples (Prabhakar et al., 2004). The intra-assay precision (or repeatability and reproducibility) was shown to be high for both platforms (<25%CV). Inter-assay precision was comparable for the two platforms when measuring high concentration cytokines, but for measuring low concentrations, inter-assay variation was variable and cytokine dependent with the overall mean inter-assay precision being improved using the Biosource (Invitrogen) Luminex kit than the MSD kit. Results also showed both platforms to have comparable accuracy when measuring high concentration cytokines but again at low concentration accuracy is variable and cytokine dependent, with the overall mean accuracy being improved using the MSD. Consequently, the Biosource (Invitrogen) Luminex kit is less sensitive, and loses accuracy when measuring low concentrations, but has more precision whilst the MSD has lower precision but is more sensitive with better accuracy. These results demonstrate that different technologies need to be assessed for the different parameters of analytical performance, and the user has to decide which aspect of the assay is more important for their purpose.

Regardless of the multiplex platform used, to assess results for possible analytical trends emerging in a cytokine profile, the assay must be monitored for consistency and repeatability. Controls must be established to enable multiple assay results from a single platform to be reliably compared. The negative control for this study was pooled plasma samples from healthy individuals having negligible levels of each analyte being analysed so that any analytes detected in test samples can be considered as 'real'. Establishing quality controls is more problematic and this is an area of constant discussion for biological assays (Stenman, 2001; Miller et al., 2001). For most biomarkers, no certified standards are available, and researchers have to rely on the standards provided with the commercial kits for quantification, and although many of these are calibrated to WHO/NIBSC standards, differences still remain when samples are analysed (Khan et al., 2004; Ledur et al., 1995). Our own results show analysing samples spiked with analytes from the opposing platform shows poor accuracy, each platform giving a quantifiably different value to the quantity spiked. In addition, the commercial multiplex kits use 'diluent' as a surrogate matrix to generate calibration curves, and do not reflect the complex biological matrices raising the need for in-house controls to be established with the test matrix, in this case plasma. To minimise these problems in this study, quality controls were established using the same pooled plasma samples as the negative control and spiking at high and low concentrations with the same source for analytes as the standard used in the associated kit. For our purposes, these controls cannot be used for assessing analytical accuracy, but only to monitor analytical precision of an assay. The results show good inter-assay precision for the Luminex for both high and low concentration analytes; however, the MSD results show poor inter-assay variation at low concentrations. This could be a potential problem if low concentration analytes need to be monitored, although good intra-assay precision would allow for all samples from an individual to be combined on a single plate and batch-run to produce a cytokine profile.

Comparison of analysing the same stimulated and unstimulated test samples on each platform shows that absolute values of quantification differ depending on which platform is used, but interestingly there is good correlation between datasets, suggesting that the relative changes or 'trends' are comparable. As with all antibody based assays, the quality of the antibody pairs is crucial to the performance of the assay and is especially important in multiplex assays where minimal cross reactivity between all antibodies is essential. The antibody pairs for each platform's kit will have been optimised for their own cytokine standards and combination of antibodies, so it is unsurprising that variation in absolute values of the same sample is seen. Variation in absolute values for cytokine analysis has been reported between different ELISA kits, in which only a single analyte is analysed (Ledur et al., 1995), as well as between different Luminex kit suppliers (Khan et al., 2004). The most common explanation for the problem is differences between the antibody pairs used by different suppliers, each being manufactured, tested and optimised differently, with no regulatory guidelines for cross comparison of methods and no well-established reference standards for confirming accuracy. However, this should not invalidate the results of multiplex assays, as it is the trends between normal and disease or pre-, post- and during treatment that are central to clinical research, and the trends have been shown to be comparable between the Luminex and MSD platforms. Multiplex technology has rapidly expanded over recent years and several platforms are now available and competing for use in biological research. However, the technology needs to be evaluated for clinical research if they are to have any potential application in the analysis of clinical samples.

In conclusion, both the Luminex and MSD are valid platforms for measuring multiple analytes from small volume samples, to investigate possible trends in cytokine profiles in a clinical setting. Each platform has its own merits with the Luminex having better precision and the MSD better sensitivity and accuracy. However at high cytokine concentrations both perform comparably apart from IL10 measurements by MSD. Without any direct regulatory guidelines for validating such complex biological assays, and with no clear reference standards or controls, validation of such assays has to be attempted using existing guidelines for well-established 'gold standard' methods such as the ELISA. These guidelines are often too stringent to apply to complex biological assays and a less constrained approach is required. The issues associated with biomarker assays are recognised and a different approach in biomarker method development and validation has been suggested in the context of 'fit-for-purpose' (Lee et al., 2006). Despite the problems encountered with validation, the new multiplex technology offers many advantages in terms of time, cost and labour. Indeed, it is the approach many are now taking to help elucidate not just the complex cytokine network but to measure many new biomarkers, and continued validation and comparison of these platforms is essential in this rapidly expanding field.

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