



## RESEARCH ARTICLE

# Validation of novel multiplex technologies

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**Abstract:** Cytokine and chemokine levels in body fluid provide information of altered conditions in patients. The parallel analysis of multiple factors, such as cytokines, from small sample sizes is an interesting approach for the assessment of in vivo activation signatures and functionality after *ex vivo* stimulation. One interesting application is for therapy monitoring—such as safety data, pharmacodynamics, evidences for mode-of-action and side effects—which is particularly useful for accompanying early phase clinical trials. There are different platforms for multiplex analyses of ligands available. An assay validation of three different platforms for simultaneous quantification of cytokines (Luminex Bio-Plex<sup>®</sup> 200, Meso Scale Discovery<sup>®</sup> and Ella<sup>®</sup>) was performed in this study. The comparison of multiplex platforms, which use different ways of achieving parallel measurements of biomarkers, reveal the performance strengths and weaknesses. We showed examples of in-house assessments of intra- and inter-assay variations, determination of the range and recovery of classical immunological serum markers and discussed advantages and disadvantages of these three platforms in relation to the question addressed. All the platforms show low intra-assay variances. The Luminex platform shows a high inter-assay variance for the majority of parameters, whereas the MSD and Ella platforms show low inter-assay variances.

**Keywords:** biomarker; multiplex; cytokine; chemokine; soluble factors

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## Introduction

Soluble factors circulating in the body, such as in peripheral blood, urine and liquor, are effective sources for evaluating cell/tissue-based activity useful for diagnostics and patient stratification<sup>[1,2]</sup>. Cytokines, chemokines and growth factors are important factors in cell communication, activation and differentiation. Alterations in their pattern could provide valuable information and allow the evaluation of health condition. In order to obtain a clinical evaluation, several factors need to be considered: levels of circulating soluble factors reflect, in most cases, an excess of locally produced mediators and are therefore an early indicator of changes in the homeostasis but might not always reflect locally restricted clinical situations. Furthermore, the measurement usually reflects only the state at the time point (snapshot) the sample was obtained and different pathological situations show a distinct secretion pattern. Most importantly, in the majority of clinical

evaluations, more than one biomarker needs to be available to support the interpretation of the activation state and clinical course<sup>[3]</sup>.

Profiling these soluble factors is an important part in routine diagnostics for intensive care units and as a safety and exploratory parameter in clinical studies to obtain the current stage of the immune response after infections, vaccination or therapeutic intervention.

The ELISA system is the most common method to determine the quantitative changes of cytokines/chemokines<sup>[4]</sup>. Due to the limitation of the classic method, only one parameter per run can be analysed. The only possibility to analyse more than one parameter with this conventional method is to measure several ELISAs one after the other. However, this might face another problem if the source of samples is limited in volume and has resource issues as well. Therefore, several multiplex technologies were developed to quantify simultaneously the concentration of multiple analytes.

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Multiplex technologies allow a wide-range screening with very small sample volumes.

In this study, we compared the validation of three different multiplex technologies based on different platforms: the Luminex Bio-Plex<sup>®</sup> 200 System, BioRad<sup>®</sup> (Luminex Corp., Texas, USA) technology, the Meso Scale Discovery<sup>®</sup> (Meso Scale Diagnostics, Maryland, USA) technology and the Ella<sup>®</sup> (ProteinSimple, California, USA) technology<sup>[5]</sup>. The performances of each of these technologies were evaluated in accordance with the international guidelines of ICH/GCP under “Validation of Analytical Procedures”<sup>[6]</sup>.

The Luminex Biorad Bio-plex<sup>®</sup> 200 platform can analyse theoretically up to 100 analytes in parallel and works on a bead-based sandwich ELISA principle. Instead of 96-well plates as a solid phase for the binding of capture antibodies as in the classic ELISA system, the Luminex concept is the simultaneous measurement of multiple analytes by using differentially color-coded beads. Each bead type is characterized by a unique emission wavelength when excited by the red laser (660 nm). Cytokine-specific capture antibodies are then bound to a particularly coded bead. Quantitation is accomplished by a sandwich assay using a fluorescently labelled detection antibody with an emission wavelength of 532 nm when excited by the red laser. Multiple readings on each bead set further validate the results<sup>[7]</sup>.

The principle of the Meso Scale Discovery (MSD) method is based on linking capture antibodies to the solid phase on ten specific carbon spots per well of a 96-well plate. The detection antibody is linked with a sulfo-tag and upon electrical stimulation, the sulfo-tag leads to light emission. This electrochemiluminescence is detected and measured with a CCD camera<sup>[8]</sup>.

The concept of the Ella system is rather an automated multi-analysis system based on a microfluidic cartridge than a real multiplex system. The cartridges used by the Ella system separate the sample into four nanocapillaries. While migrating through microfluidic channels, it subsequently passes specific capture antibodies, then passes the detection antibody and is finally scanned in the glass nano-reactor where the fluorescence intensities are measured. In contrast to the Luminex and MSD technology, the standard values of the Ella system are provided by the company for each lot of cartridge<sup>[9]</sup>.

We performed a detailed validation of the novel multiplex platforms and compared the performance of the quality controls, the standard curve and the robustness of the method for *ex vivo*-stimulated whole blood samples. The multiplex platforms have different properties; therefore, the parameters for the validation were chosen for each platform with special regards to their applicability in clinical trials and diagnostics<sup>[10]</sup>.

## Material and Method

### Cell Stimulation

Whole Heparin blood from healthy volunteers was stimulated with 0,1 ng/mL LPS for 24 h at 37 °C. After centrifugation for 15 min at 1,000 × g at room temperature (RT), the supernatant (blood plasma) was collected and aliquoted. The whole Heparin blood was stimulated with 25 ug/ml Concavalin A (ConA) for 24 h at 37 °C, 1:10 diluted with serum matrix, and the concentration of IFN-g, IL-17A, IL-2 and IL-5 was measured on the Ella platform.

### Luminex Method

The human Luminex kit is custom-designed (for IFN-γ, IL-10, IL-1β, IL-6, IL-8, IP-10, MCP-1 and TNF-α) and was purchased from Millipore<sup>®</sup> (Merck Millipore, Darmstadt, Germany). The Luminex kits provide ready-to-use cocktails of the respective cytokine analytes as standard for the assay. The standard was reconstituted with 250 μL deionized water to obtain a concentration of 10000 pg/mL for all cytokines. The vials were inverted multiple times for mixing. After vortexing, the vials were kept for 5–10 min at RT and then on ice until use. Preparation of further serial 1:5 dilution of Cytokine Standard was done with expected final concentrations of 2000 pg/mL, 400 pg/mL, 80 pg/mL, 16 pg/mL and 3.2 pg/mL. Quality Controls (low and high) are components of the kits and the respective Quality Control ranges are provided by the manufacturer. The two Quality Controls (low and high) were reconstituted with 250 μL deionized water (low and high). The vials were inverted multiple times for mixing and, after vortexing, the vials were kept for 5–10 min at RT and then on ice until use. The samples were measured on a suspension array multiplex system (Bio-Plex<sup>®</sup> 200 System, BioRad<sup>®</sup>). The Bio-Plex Manager Software controls the instrument, acquires and analyses data without the need to import files. Analysed data can then be exported as Excel files.

### ELLA Method

The human inflammatory kit (for IL-10, IL-12p40, IL-1β and IL-6) and the T cell activation kit (for IFN-γ, IL-17A, IL-2 and IL-5) for the Ella system were purchased from ProteinSimple. The Ella system runs a maximum of four parameters in triplicates on a 16-well cartridge. The standard is generated by the factory and is provided lot-wise. The 5PL curve is an average of five replicates of each standard value. The information of the factory-standard curve is encoded in the barcode on each cartridge. The cartridges contain the capture antibodies, biotinylated detection antibodies and the streptavidin-dye conjugate in the appropriate area of the cartridges.

Quality Controls (low and high) are components of the kits and respective Quality Control ranges are provided by the manufacturer. Quality Controls (low and high) were reconstituted with the respective volume of deionized water. The vials were inverted multiple times for mixing. After vortexing, the vials were kept for 5–10 min at RT and then on ice until use.

### MSD Method

The human Proinflammatory Panel 1 kit (for IFN- $\gamma$ , IL-10, IL-2, IL-4 and TNF- $\alpha$ ) was purchased from MSD. The kit provides all reagents, together with a 96-well plate with specific pre-coated spots, the detection antibodies and assay diluent. The standard was reconstituted with assay diluent to obtain a lot-specific concentration which differs for all cytokines. The vials were inverted multiple times for mixing and, after vortexing, the vials were kept for 5–10 min at RT and then on ice until use. Preparation of further serial 1:5 dilution of Cytokine Standard was performed. Quality Controls (low and high) are components of the kits and respective Quality Control ranges are provided by the manufacturer. The Quality Controls (low and high) were reconstituted with 250  $\mu$ l of deionized water. The vials were inverted multiple times for mixing. After vortexing, the vials were kept for 5–10 min at RT and then on ice until use.

### Accuracy

The accuracy of a measurement system is the degree of closeness of measurements of a quantity to its actual (true) value. Accuracy is the combination of precision and repeatability.

### Precision

Repeatability (intra-assay variation) is defined as the degree of precision of multiple measurements using the same test conditions (*e.g.* identical sample material, and analysis conducted by the same analyst on the same day using identical equipment). Intermediate precision (inter-assay variation) is defined as the degree of precision of multiple measurements using varying conditions within the same lab (*e.g.* same material tested on multiple days by multiple analysts). Intra-assay and inter-assay variations were assessed as described below. For inter-assay variation, plasma samples from three stimulated donors were analysed on five independent days. For intra-assay variation, plasma from three stimulated donors in ten replicates were analysed on the same day.

### Calculation

For each sample, respective OD values were obtained by subtracting the blank. A respective calibration curve was used to determine the corresponding

analyte concentration of the OD values. The mean concentrations and standard deviations of the samples were calculated. As a measure for the intra-assay and inter-assay precision, the percentage of coefficient of variation (CV) was calculated for each analyte:

$$\% \text{ CV} = \text{standard deviation (SD)} / \text{mean concentration } \bar{c} \times 100$$

### Statistics

The analysed data was exported from the platform-specific software as Excel file and further statistical data analysis was performed by using Prism Software (GraphPad<sup>®</sup> version 6.0).

### Ethics

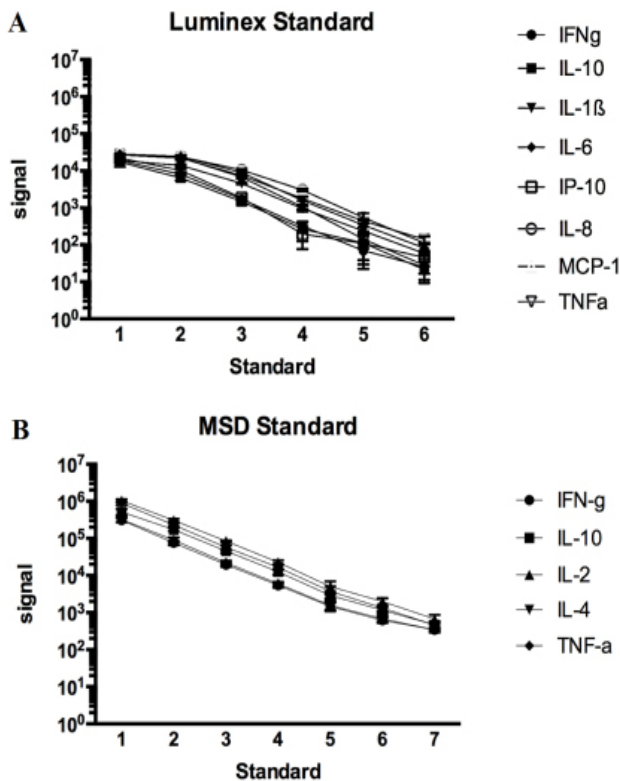
The study was approved by the local ethics committees (application number: EA2/152/16). All healthy volunteers in the study provided written informed consent before blood donation.

## Results

### Quantification Limits of the Standards

The Luminex standards from Milliplex<sup>®</sup> (Merck Millipore) were 1:5 diluted to generate a six-point standard curve. All analytes started with the same stock concentration of 10000 pg/mL. According to the manufacturer, the detection range of TNF- $\alpha$ , MCP-1, IP-10, IL-8, IL-6, IL-1 $\beta$ , IL-10 and IFN- $\gamma$  is from 10000 pg/mL – 3,2 pg/mL (Figure 1A).

The MSD standards (Meso Scale Diagnostics) were 1:4 diluted to generate a seven-point standard. The stock concentration for the analytes differed between the analytes. The detection ranges were as follows: TNF- $\alpha$  313 pg/mL – 0,08 pg/mL, IL-4 240 pg/mL – 0,06 pg/mL, IL-2 1220 pg/mL – 0,30 pg/mL, IL-10 307 pg/mL – 0,07 pg/mL and IFG-g 1270 pg/mL – 0,31 pg/mL (Figure 1B). Compared to the Luminex system, the MSD starts with a lower stock concentration and therefore detects values inside a lower concentration range. However, the detection ranges of the MSD span an overall larger range compared to the Luminex platform since it reaches a far lower sensitivity. The Luminex platform shows a signal range over four logs, whereas the MSD platform detects a range over five logs. The Ella platform provides a standard detection range over six logs, according to manufacturer, and thereby covers the high concentration and the lower concentration range. The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) of Luminex and MSD were calculated by using the mean of five standard curves from individual experiments of the same kit. Since the standard performance for the ELLA platform is assessed



**Figure 1.** Standard curves demonstrating the range of detection of the signal and the concentration of the Luminox platform (A) and the MSD platform (B). Standard curve for the ELIA platform was not performed during the daily measurement; data were provided by factory-generated standard. The standard curves were measured on five different days and the error bars show the standard deviation (SD) of the mean.

by the company, we generated the LLOQ by diluting the controls. The ULOQ could not be assessed with the standard. Table 1 shows the lower and upper limits of quantification.

### Comparison of Control Variability of the Different Platforms

To receive information about the accuracy of the measurement, all three platforms offer quality controls with different concentrations. The MSD platform provides high, medium and low quality controls covering a broader range compared to both of the other platforms with only two controls of high and low. The concentrations for the MSD high and medium controls are however comparable to the high and low controls of the competitors, whereas the MSD low control is significantly lower. We calculated the percentage of variation coefficient (% CV) by using the mean of five independent experiments of the same platform-specific control lot for all three platforms (see Figure 2A–C).

The % CV of the Luminox platform for the high control was below our acceptance criteria of 25% for

TNF- $\alpha$ , MCP-1, IP-10, IL-6, IL-1 $\beta$ , IL-10 and IFN- $\gamma$ . However, the % CV of IL-8 showed rates above our acceptance criteria of 25%. For the low concentration control, the variation coefficient was lower than our acceptance criteria of 25% for TNF- $\alpha$ , MCP-1, IP-10, IL-6, IL-1 $\beta$  and IL-8, but not for IFN- $\gamma$  and IL-10 (Figure 2A).

The variation coefficient of the MSD platform for the high and medium control was below our acceptance criteria of 25% for all analysed cytokines (TNF- $\alpha$ , IL-4, IL-2, IL-10 and IFN- $\gamma$ ). In contrast, for the low controls, all cytokines failed to satisfy our acceptance criteria of a % CV below 25% (Figure 2B).

The % CVs of the Ella platform for high and low control were lower than the acceptance criteria of 25% for the inflammatory kit (IL-10, IL-12p70, IL-1 $\beta$  and IL-6) and for the T cell activation kit (IFN- $\gamma$ , IL-17A, IL-2 and IL-5) (Figure 2C).

### Comparison of Spike Recovery of the Controls

To compare the recovery rate of the three platforms, the control samples with an expected control range were measured. To determine the control recovery rate of the detected spiked concentration, the percentage of the recovered concentration related to the expected 100% concentration was calculated. Our acceptance criteria for accuracy of recovery were fulfilled if the measured concentration differed only  $\pm 25\%$  from the expected concentration.

The Luminox high control was below our acceptance criteria of 25% for all analysed cytokines (TNF- $\alpha$ , MCP-1, IP-10, IL-8, IL-6, IL-1 $\beta$ , IL-10 and IFN- $\gamma$ ). The low control was below our acceptance criteria of 25% for most of the analysed cytokines (TNF- $\alpha$ , MCP-1, IP-10, IL-8, IL-6, IL-1 $\beta$  and IFN- $\gamma$ ), apart from IL-10 (Figure 3A). The MSD high, middle and low control fulfilled the acceptance criteria for IFN- $\gamma$ , IL-10 and IL-4. However, the recovery rate for TNF- $\alpha$  and IL-2 did not meet the acceptance criteria for high, middle and low control (Figure 3B). The Ella platform fulfilled the acceptance criteria for high and low control (Figure 3C) for the inflammatory kit (IL-10, IL-12p70, IL-1 $\beta$  and IL-6) as much as for the T cell activation kit (IFN- $\gamma$ , IL-17A, IL-2 and IL-5).

### The Intra-Assay Precision of Cytokines from Stimulated Whole Blood

So far, we have only used the controls and standards provided by the manufacturer to evaluate the assay performance. In order to simulate patient samples better, we stimulated the whole blood with either 0.1 pg/mL LPS or with 25 ug/mL ConA. The stimulated whole blood is a relevant test to simulate the physiological immune response with medium to high levels of cytokine



**Table 1.** Limits of LLOQ and ULOQ provided by the company in comparison to in-house generated data

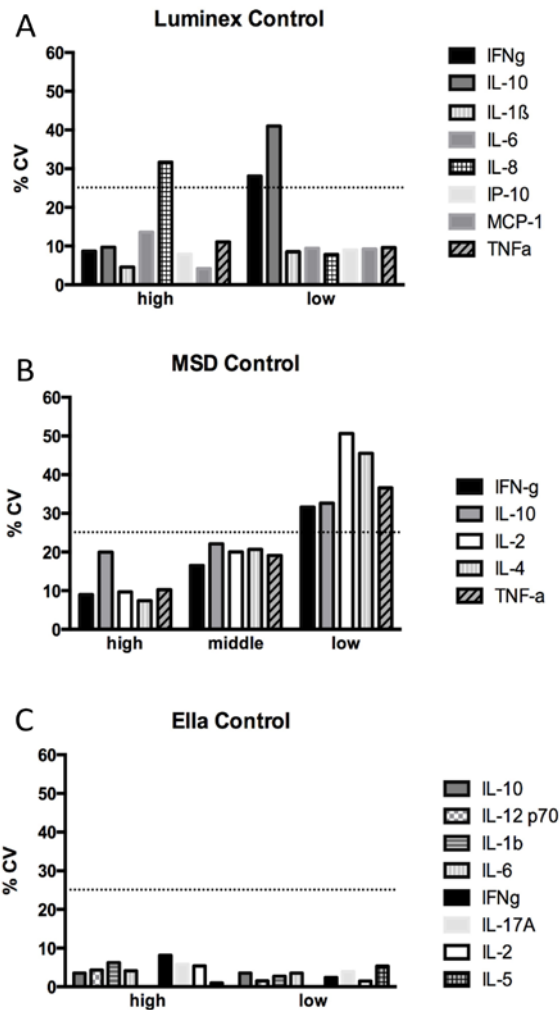
Luminex				
Analyte	LLOQ (pg/mL)		ULOQ (pg/mL)	
	Company	In-house	Company	In-house
IFN- $\gamma$	N/A	3.20	N/A	9997.63
IL-10	N/A	3.18	N/A	10010.73
IL-1 $\beta$	N/A	3.21	N/A	9798.76
IL-6	N/A	3.20	N/A	4292.48
IP-10	N/A	7.16	N/A	10008.52
IL-8	N/A	3.22	N/A	2046.70
MCP-1	N/A	3.25	N/A	3439.38
TNF- $\alpha$	N/A	3.22	N/A	6766.02
MSD				
Analyte	LLOQ (pg/mL)		ULOQ (pg/mL)	
	Company	In-house	Company	In-house
IFN- $\gamma$	7.47	1.48	938.0	1312.2
IL-10	0.68	1.10	233.0	80.2
IL-2	0.89	4.34	938.0	1253.4
IL-4	0.45	0.94	488.0	244.6
TNF- $\alpha$	0.69	1.15	248.0	326.3
Ella				
Analyte	LLOQ (pg/mL)		ULOQ (pg/mL)	
	Company	In-house	Company	In-house
IL-10	0.46	2.88	5530.0	N/A
IL-12p70	0.46	2.95	2570.0	N/A
IL-1 $\beta$	0.41	4.12	3850.0	N/A
IL-6	0.41	3.24	3850.0	N/A
IFN- $\gamma$	1.16	20.24	6050.0	N/A
IL-2	0.64	141.85	990.0	N/A
IL-5	0.07	1.80	3120.0	N/A
IL-17A	0.82	27.63	8490.0	N/A

responses. Additionally, the intra-assay variability of all platforms of Luminex (Figure 4A), MSD (Figure 4B) and the inflammatory and T cell activation kit from Ella were assessed (Figure 4C). The intra-assay variability was determined as the percentage of variability (% CV) from ten replicates of the stimulated samples. The % CV of all platforms remained below the acceptance criterion of <25% CV and showed a good intra-assay precision.

### The Inter-Assay Precision of Stimulated Whole Blood

The inter-assay variability was determined by measuring samples of stimulated whole blood on five consecutive days. The percentage of variation coefficient (% CV) was calculated to establish the inter-assay precision

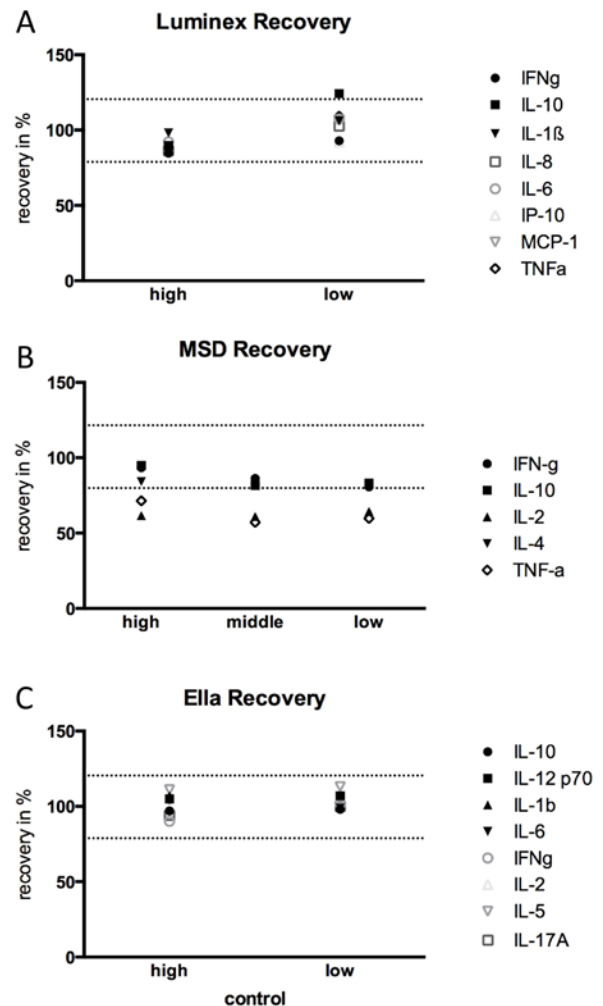
with an acceptance criterion of <25% CV. The Luminex platform showed a variation coefficient below 25% for IL-8 and TNF- $\alpha$  and fulfilled our acceptance criterion. However, none of the other parameters, *i.e.* MCP-1, IP-10, IL-6, IL-1 $\beta$ , IL-10 and IFN- $\gamma$  matched our inter-assay precision criterion (Figure 5A). The MSD platform fulfilled the inter-assay acceptance criterion for TNF- $\alpha$ , IL-2, IL-10 and IFN- $\gamma$ . The high % CV for the IL-4 measured on the MSD platform was due to the absence of higher IL-4 concentrations after LPS stimulation (Figure 5B). The Ella platform showed an excellent inter-assay precision for the inflammatory kit (IL-10, IL-12p70, IL-1 $\beta$  and IL-6) as much as for the T cell activation kit (IFN- $\gamma$ , IL-17A, IL-2 and IL-5) with an inter-assay variance even below 20% CV (Figure 5C).



**Figure 2.** Variability of quality controls of the different platforms. To evaluate the quality of a run, all platforms offer quality controls, which have to meet a specific range. The inter-assay variance of the controls of the Luminex platform (A), the MSD platform (B) and the Ella platform (C) are shown as the percentage coefficient of variance (% CV). The control concentrations of the Luminex and Ella platforms were comparable for the high and low control. The MSD high control was comparable to the other platforms, the MSD medium control was comparable to the low control of the other platforms. The MSD low control was clearly lower compared to the competitors.

## Discussion

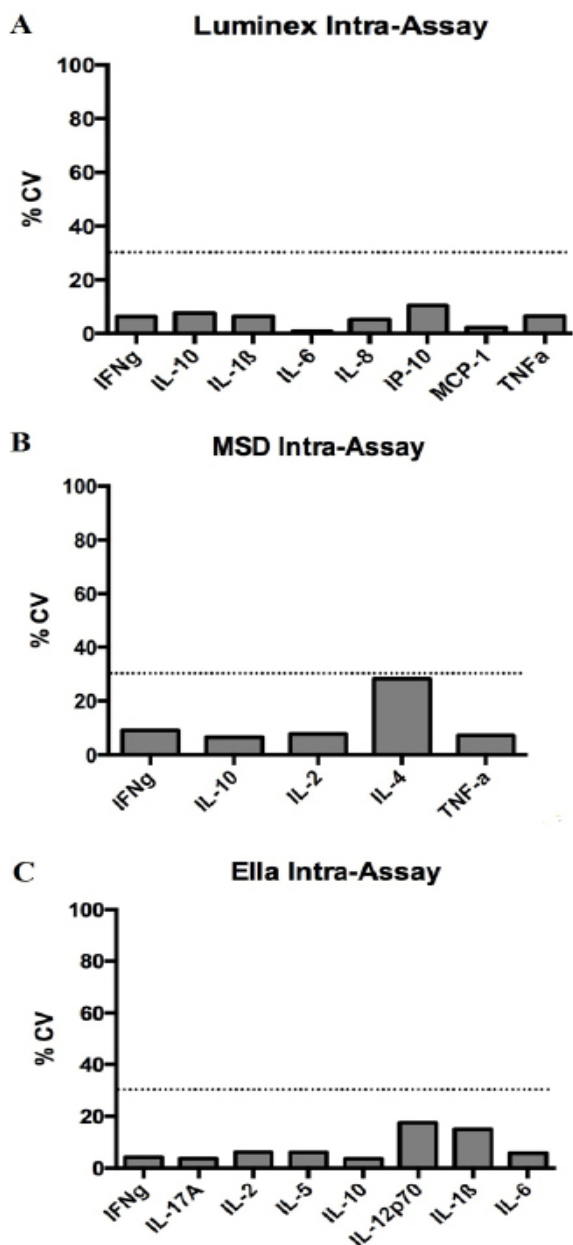
Immunological monitoring of patients during clinical studies with, for instance, new biologicals which target the immune system, is of high relevance for obtaining sufficient data about the immune status of these patients. Many studies in the past have tried to inhibit the immune system for treating chronic inflammatory diseases such as rheumatoid arthritis<sup>[11]</sup> or psoriasis<sup>[12]</sup>, or to treat cancer by using checkpoint inhibitors<sup>[13]</sup>. The changes in levels of immune mediators provide insights into the pharmacodynamics of drugs, and this might help to



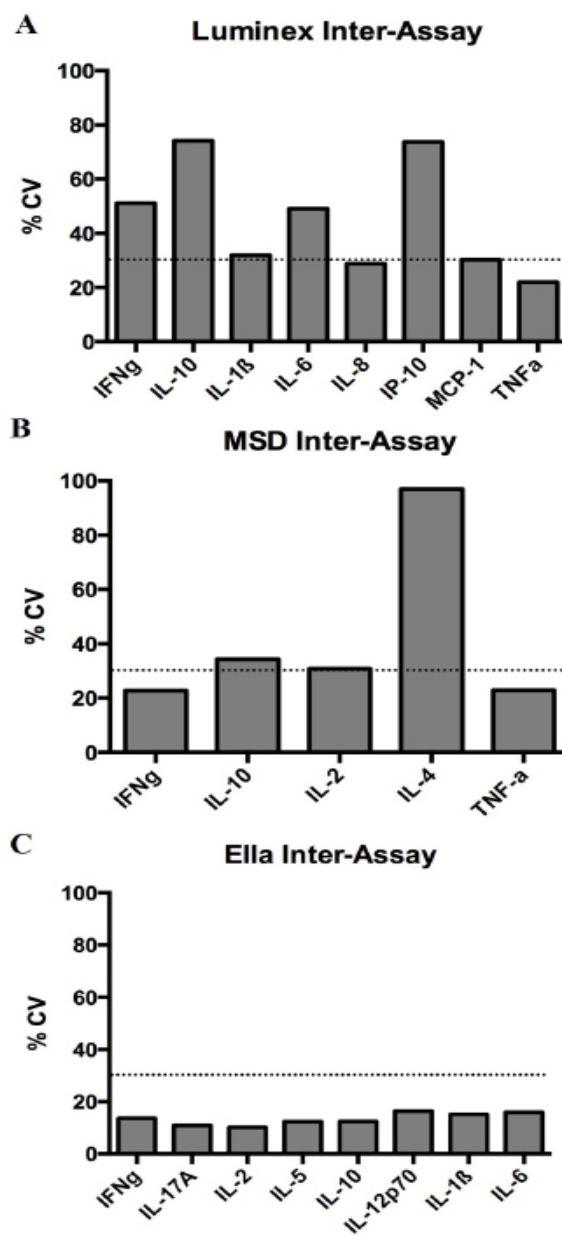
**Figure 3.** Recovery rates in different platforms with different concentrations of the Luminex (A), MSD (B) and Ella (C) kits. The mean of the measured control was analysed five times for all platforms with an expected concentration of  $\pm 25\%$  of the actual concentration. The acceptance range is displayed with dotted lines on each graph.

understand why drugs have failed to meet their clinical target. This allows us to learn from the potential failure of drugs and to re-adapt attempts aiming to modulate the immune system more precisely<sup>[14]</sup>. Furthermore, monitoring of immunological mediators is important also for safety reasons and for the understanding/interpretation of side effects<sup>[15]</sup>.

In order to achieve a more complete picture in order to allow an adequate interpretation of data, several multiplex analysis platforms have been developed, which allow the parallel analysis of multiple analytes from small amounts of material. We have compared three commonly used systems, which differ in their principles of achieving this goal, as much as in the number of different analytes they can handle in parallel.



**Figure 4.** The intra-assay variability of the different platforms of ten replicates was measured on the same day. Whole blood was stimulated with 0,1 ng/mL LPS, 1:10 diluted with serum matrix and measured on the Luminex platform to assess the concentration of IFN- $\gamma$ , IL-10, IL-1 $\beta$ , IL-6, IL-8, IP-10, MCP-1 and TNF- $\alpha$  (A). Whole blood was stimulated with 0,1 ng/mL LPS, 1:10 diluted with serum matrix and measured on the MSD platform to determine the concentration of IFN- $\gamma$ , IL-10, IL-2, IL-4 and TNF- $\alpha$  (B). Whole blood was stimulated with 200 ng/mL LPS, 1:10 diluted with serum matrix and run on the Ella platform to measure the concentration of IL-10, IL-12p70, IL-1 and IL-6; whole blood was stimulated with 25  $\mu$ g/mL ConA, 1:10 diluted with serum matrix and the concentration of IFN- $\gamma$ , IL-17A, IL-2 and IL-5 was measured on the Ella platform (C). Intra-assay variability was calculated with % CV for ten replicates and the dotted line shows the acceptance criteria.



**Figure 5.** The inter-assay variability of the different platforms of duplicates measured on five consecutive days. Whole blood was stimulated with 0,1 ng/mL LPS, 1:10 diluted with serum matrix and measured on the Luminex platform to assess the concentration of IFN- $\gamma$ , IL-10, IL-1 $\beta$ , IL-6, IL-8, IP-10, MCP-1 and TNF- $\alpha$  (A). Whole blood was stimulated with 0,1 ng/mL LPS, 1:10 diluted with serum matrix and measured on the MSD platform to determine the concentration of IFN- $\gamma$ , IL-10, IL-2, IL-4 and TNF- $\alpha$  (B). Whole blood was stimulated with 0,1 ng/mL LPS, 1:10 diluted with serum matrix and the concentration of IL-10, IL-12p70, IL-1 $\beta$  and IL-6 was measured on the Ella platform; whole blood was stimulated with 25  $\mu$ g/mL ConA, 1:10 diluted with serum matrix and the concentration of IFN- $\gamma$ , IL-17A, IL-2 and IL-5 was measured on the Ella platform (C). Intra-assay variability was calculated with % CV for 10 replicates and the dotted line shows the acceptance criteria.

Our data show that each platform is useful and has its own advantages as well as limitations. Therefore, it is very important to select the right platform depending on the questions being addressed.

The Luminex platform is based on colour-coded beads, which provide the basis for up to 100 different parallel sandwich ELISAs from the same sample<sup>[7]</sup>. The MSD platform links up to ten different capture antibodies on defined spots in a 96-well format and then uses a highly sensitive electrochemiluminescence which exploits the company's patented sulfo-tag detection system<sup>[8]</sup>. The most recently developed ELLA system by ProteinSimple exploits a microfluidic system, which in fact distributes the sample to different classical single-plex ELISAs based on the established ELISA detection of R&D Systems<sup>[9]</sup>. So far, the system is restricted to measure only four analytes in parallel as triplicates.

In order to obtain highly reliable data from these kinds of analyses, several aspects have to be validated in-house by the laboratory according to the guidelines of the regulatory agencies<sup>[6]</sup>. Currently, none of the companies provide *In-Vitro* Diagnostic (IVD)-certified assays. Furthermore, one disadvantage of using multiplex analyses is that each individual combination of analytes needs validation, since the combination used can have an impact on specificity and performance. In this study, we focused mainly on aspects of precision: the variation of test results with the same sample was analysed in parallel on the same day (intra-assay variance), and the variation of test results with the same sample was analysed on different days (inter-assay variance). Particularly, the inter-assay variance is of high importance for clinical studies in order to obtain comparable results from the patient in the follow-up during the observation period. The companies provide some information about their own experimental variation between day-to-day inter-assay variances. However, our own data showed some differences. The larger variation we observed might be caused mainly by two reasons: 1) We had used not only the companies' control samples (mostly recombinant proteins, which also in our experience showed a relatively small variation in most cases) but also supernatants from *ex vivo* stimulated whole blood. These samples reflect patient samples better and also incorporate the intrinsic problem that the concentration of a particular cytokine is not always in the optimal range where the variation of the assay would be smaller. This was also reflected by the fact that the variation for the low concentration control by MSD was higher than the one for the middle and high controls. 2) We had used manual pipetting only, without any automated support. Variation of the assays might be smaller if pipetting automatization were used. This might be one of the reasons why the ELLA system

had the lowest inter-assay variance, because right after sample application, the entire further processing is fully automatized on this platform. Another aspect of ELLA is the multiplexing in separate circuits for each parameter, preventing undesired ligand/antibody interactions.

## Conclusion

The acceptance criteria of assay variation below 25% CV is rather arbitrary, although suggested by the regulatory agencies<sup>[6]</sup>. Whether this value is of real biological or even clinical relevance is a matter of debate. Many cytokines would impact on the physiological outcome only if they are elevated by a factor of ten or more, and therefore also assay variations of more than 25% CV would still allow an interpretation of data and could still be used at least for exploratory studies. However, this must be taken into account in the interpretation of changes. Nevertheless, it is still very much appreciated if the assay variation is as low as possible, and therefore the ELLA system by ProteinSimple seems to be the most reliable platform.

However, the disadvantage of the ELLA system is that it allows only the lowest number of analytes (four parameters) to be measured in parallel. Here, the Luminex system outperforms the other two providers clearly. Therefore, it might be the best way to select suitable biomarkers in a pre-study with the platform, which allows the highest number of mediators to be analysed in parallel and then to reduce the number of analytes to a number which can be handled by platforms with a particularly low inter-assay variance for the clinical study itself.

Another important aspect of choosing an appropriate platform is the question of what kind of concentration range can be analysed by the system. If the range is relatively low, it could cause a repeated measurement of a sample at another dilution, which would drive up costs and necessary manpower. This is particularly an issue if the concentration of cytokines inside the same sample is very different. Thus, IFN $\gamma$  for instance can be quite strongly induced in human samples (up to ng range) whereas IL-4 is hardly detectable. If one wants to analyse both cytokines inside the same sample, then the sensitivity of the cytokine assays has to be adapted to each other or the range has to cover very low and very high concentrations. None of the assays tested here were designed to adapt different cytokine sensitivities, and therefore the range is the decisive parameter. The Luminex platform shows a signal range over four logs, whereas the MSD platform detects a range over five logs. The ELLA platform provides a standard detection range over six logs, according to the manufacturer's information, and thereby covers the high concentration and the lower concentration range best.



## Authors' Contributions

L Akyüz: performing experiments, data analysis and manuscript writing; A Wilhelm: performing experiments and data analysis; F Butke: performing experiments and data analysis; SJ Park: performing experiments and data analysis; HD Volk: conceptual contribution and manuscript writing; G Grütz: design of validation, conceptual contribution and manuscript writing.

## Conflict of Interest

No conflict of interest is reported by all authors.

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