An Evaluation of Commercial Fluorescent Bead-Based Luminex Cytokine Assays

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Abstract

The recent introduction of fluorescent bead-based technology, allowing the measurement of multiples analytes in a single 25–50 μl sample has revolutionized the study of cytokine responses. However, such multiplexes approaches may compromise the ability of these assays to accurately measure actual cytokine levels. This study evaluates the performance of three commercially available multiplex cytokine fluorescent bead-based immunoassays (Bio-Rad's Cytokine 17-plex kit; LINCO Inc's 29-plex kit; and RnD System's Fluorokine-Multi Analyte Profiling (MAP) kit) by RnD Systems and Biosource International and comparatively assessed multiplex kits from LINCO Research, Bio-Rad Laboratories, RnD System's IFN-γ Quantikine ELISA kits and a 29-plex and MAP kit by RnD Systems IFN-γ. A positive correlation was found in the levels of IFN-γ measured in antigen stimulated whole blood culture supernatants by the LINCO Inc 29-plex, RnD Fluorokine-(MAP) and RnD system IFN-γ Quantikine ELISA kits across a panel of controls and stimulated samples. Researchers should take the limitation of such multiplexed assays into account when planning experiments and the most appropriate use for these tests may currently be as screening tools for the selection of promising markers for analysis by more sensitive techniques.

Introduction

Cytokines are important modulators of immune response pathways [7,18,19]. Cytokine expression profiling (CEP) has become a popular and established method for the identification and characterisation of disease-associated immune responses [4,6,8,9]. Previously, CEP was a laborious process requiring substantial sample volumes when multiple cytokines were under investigation. However, CEP methodology has been revolutionised by the recent introduction of fluorescent bead-based luminex technology, a capture/detection sandwich type immunoassay allowing the measurement of up to 100 different analytes in a single 50 μl sample [16]. The reduced sample volume and time-saving advantages of the luminex system have made it an attractive method for large-scale cross-sectional, association or cohort studies which investigate the host immune response [1,13,15,16]. Khan et al. [11] have comparatively assessed multiplex kits from LINCO Research, Bio-Rad Laboratories, RnD Systems and Biosource International and compared them to an enzyme-linked immunosorbant assay (ELISA). The comparison was based on the measurement of a sample of five cytokines (serum samples from healthy individuals intravenously injected with endotoxin). They reported that the cytokine concentrations, as measured by the different kits, showed similar trends, although the absolute concentrations measured were different.

There are also a number of reports validating luminex systems. These studies often used kits with a narrow panel of cytokines. The present study not only has the advantage of combining a head-to-head comparison of different kits and assays on their ability to measure cytokine levels in blood samples, but is also the first independent study to comparatively assess the recovery of each cytokine by the commercially available Bio-Rad 17-plex, LINCO 29-plex and RnD Fluorokine-(MAP) kits. A positive correlation was found in the levels of IFN-γ measured in antigen stimulated whole blood culture supernatants by the LINCO Inc 29-plex, RnD Fluorokine-(MAP) and RnD system IFN-γ Quantikine ELISA kits across a panel of controls and stimulated samples. Researchers should take the limitation of such multiplexed assays into account when planning experiments and the most appropriate use for these tests may currently be as screening tools for the selection of promising markers for analysis by more sensitive techniques.

Methods

Study design

This study followed an integrated methodology, comparing 3 commercially available multi-plex luminex kits (Cytokine 17-plex kit by Bio-Rad; a 29-plex kit by LINCO Research; and Fluorokine-Multi Analyte Profiling (MAP) kit by RnD System as well as the RnD Systems IFN-γ Quantikine ELISA kit. We have used the following two approaches: 1) Measurement of recombinant cytokines in serum and in unstimulated diluted whole blood culture supernatant samples, each spiked with serial dilutions of the multiplex standard provided by the luminex kit manufacturer in order to calculate the recovery (accuracy) of the assay for each of the different cytokines; 2) Measurement of native, induced IFN-


\[ \text{\textit{\( \gamma \)}} \text{, in vitro, whole blood culture supernatants where, whole blood culture supernatants were stimulated with Mycobacterium tuberculosis (MtB) antigens or Bacille Calmette Guerin (BCG).} \]

**Definitions**

**Recovery.** Ratio of the observed amount of cytokine compared to the expected known amount of cytokine in a sample, expressed as a percentage. An acceptable recovery falls within the range of 70–150%.

**Curve build upon a five parameters logistic equation and that was kept to a minimum and never exceeded 10**

\[ \text{order to keep the matrix of the spiked samples as similar as possible, which produced in total six concentration replicates. In cytokine using the Bio-plex kit standard. The assays were run in concentrations (ranging from 7–5461 pg/ml) with recombinant supernatant samples SN1, SN2 and SN3 were each spiked at three test showed that this method was superior to dilution of standards resembled that of the samples as closely as possible as preliminary validation protocol.} \]

\[ \text{array reader, using a 5 PL regression curve to plot the standard curve. Samples and controls were read at both a low RP1 target setting (used to maximize assay sensitivity when the expected concentrations are below 3 200 pg/ml) and a high RP1 target setting (used for broad range concentrations) on the Bio-plex suspension array using a high throughput fluids (HTF) system (cat# 171000005). Data was subsequently analysed using the Bio-plex manager software, version 3.} \]

**Experiment 2.** The standard curve was generated and the assay conducted as described above in the section on the Bio-Rad human 17-plex assay experiment 1. Two sets of samples were used. The first set was generated using whole blood from a healthy laboratory donor diluted one in ten with RPMI-1640 with glutamax and stimulated with different MtB antigens, generously donated by Tom Ottenhoff, Leiden University, and a phytohaemagglutinin (PHA)-stimulated positive control. Unstimulated culture supernatant served as a negative control. The second set of samples was generated from unstimulated whole blood culture as described above. Supernatants were harvested on day seven and spiked at five different concentrations with recombinant cytokine from the Bio-Rad standard (lot # 5004060). The six concentrations at which samples were spiked were unique for each cytokine with the lowest spike ranging from 2–43 pg/ml and the highest from 1191–8062 pg/ml. The results of these tests were used to calculate recovery.

**Manufacturer 2 assay**

**Experiment 1.** A human 29-plex LINCO assay (cat no HCYTO -60-K-PMX29) was done according to manufacturer's instructions. Briefly, a standard curve ranging from 3.2 pg/ml to 10 000 pg/ml was generated by serial dilution of reconstituted standard. Two sets of samples were used, as described earlier, with the exception that for the second set of samples the LINCO Research standard (provided with the kit) was used to spike unstimulated whole blood culture at final concentrations of 5000, 1000, 500, 50 and 10 pg/ml. Additionally for the assessment of the LINCO kit reproducibility five aliquots of the same PHA-stimulated whole blood supernatant were produced and each aliquot was run in five different experiments on different days. Briefly the filter plates were blocked by pipetting 200 µl of assay buffer into each well. After 10 minutes the assay buffer was discarded by vacuum aspiration and 25 µl of assay diluent was added to the wells designated for standards, while 25 µl of RPMI-1640 with glutamax (GIBCO) was added to the wells designated for standards. According to the plate layout, 25 µl of either standard or sample was then added to the appropriate wells after which 25 µl of antibody coated fluorescent beads was added. Biotinylated secondary (detection) and Streptavidin-PE-labelled antibodies were then added to the plate respectively, with alternate incubation and washing steps. Finally 100 µl of sheath fluid was added to the wells and the plate read immediately on the Bio-plex array reader, at high and low RP1 targets, using a 5 PL regression curve.

**Experiment 2.** A repeat of the experiment 1 was done by measuring 21 cytokines in two sets of samples including 17 MtB antigens stimulated culture supernatant and spiked unstimulated whole blood culture (as previously described). Only this time samples were spiked at 2 different concentrations. The Plate was read at low RP1 targets as previously described.

**Manufacturer 3 assay**

**Experiment 1.** The assay was done according to the manufacturer’s instructions. Briefly, the standard curves for the RnD System fluorokine-[MAP] human base kits A (cat # LUH000) and B (cat # LUH001) were generated by reconstitution of standards in standard diluent provided with the
Table 1. Bio-Rad human 17-plex expected and observed cytokine concentrations and recovery (experiment 1).

| Cytokine | Expected (pg/ml) | High RP1 target | Low RP1 target | Recovery (%) |
|----------|-----------------|-----------------|----------------|--------------|
| IFN-gamma | 1797.5 | OOR> | NA | 931.6 | 518.3 |
| | 179.7 | 600.25 | 334 | 491.23 | 273.36 |
| TNF-alpha | 341.2 | 344.8 | 81.4 | 1660.55 | 160.4 |
| | 1035.1 | 2212 | 213.7 | 1582.7 | 152.9 |
| | 103.5 | 178.7 | 172.6 | 138.7 | 134.0 |
| IL-1beta | 3132.0 | 4082.4 | 130.3 | 10131.7 | 323.5 |
| | 313.2 | 1800.33 | 574.8 | 421.8 | 134.7 |
| IL-2 | 31.3 | 56.6 | 180.7 | 49.4 | 157.7 |
| | 1762.7 | 1882.8 | 106.8 | 2895.7 | 164.3 |
| | 176.3 | 363.8 | 206.4 | 302.2 | 171.4 |
| | 17.6 | 40.3 | 228.7 | 32.6 | 185.0 |
| IL-4 | 695.4 | 707.1 | 101.7 | OOR> | NA |
| | 69.5 | 202.8 | 291.6 | 164.7 | 236.8 |
| IL-5 | 5159.5 | 8582.8 | 166.3 | 13496 | 261.6 |
| | 516.0 | 2727.2 | 528.6 | 739.7 | 143.4 |
| IL-7 | 51.6 | 96.4 | 186.8 | 79.5 | 154.1 |
| | 3422.8 | 2817 | 82.3 | 8190.6 | 239.3 |
| IL-8 | 3423 | 893.2 | 261.0 | 550.5 | 160.9 |
| | 34.2 | 107.9 | 315.3 | 60.4 | 175.6 |
| IL-10 | 5460.6 | 9190 | 168.3 | 10819.1 | 198.1 |
| | 546.1 | 1392 | 254.9 | 1032 | 189.0 |
| | 54.6 | 145.1 | 265.8 | 98.6 | 180.6 |
| IL-12-p70 | 3672.0 | 3702.8 | 100.8 | 5661.5 | 154.2 |
| | 367.2 | 861.3 | 234.6 | 613 | 166.9 |
| IL-13 | 36.7 | 89 | 242.4 | 39.1 | 106.5 |
| IL-17 | 4810.6 | 4540.7 | 94.4 | 7128.86 | 148.2 |
| | 481.1 | 1348.9 | 280.4 | 732.23 | 152.2 |
| | 48.1 | 98.43 | 204.6 | 75.53 | 157.0 |
| IL-18 | 4798.2 | 4641.6 | 96.7 | OOR> | NA |
| | 479.8 | 1191.7 | 248.4 | 942 | 196.3 |
| IL-19 | 480.0 | 114 | 237.6 | 68.4 | 142.6 |
| IL-20 | 762.5 | 1116.1 | 146.4 | 1401 | 183.7 |
| | 76.3 | 169.7 | 222.6 | 140.1 | 183.7 |
| IL-21 | 7.6 | 15.7 | 205.8 | 14.1 | 184.8 |
| IL-22 | 2765.6 | 3074.8 | 111.2 | 9773.8 | 353.4 |
| | 276.6 | 940.6 | 340.1 | 791.9 | 286.3 |
| GM-CSF | 27.7 | 110.2 | 398.4 | 70.6 | 255.2 |
| | 2552.7 | 4100.5 | 160.6 | 4716.7 | 184.8 |
| G-CSF | 255.3 | 597.3 | 234.0 | 478.6 | 187.5 |
| | 25.5 | 55.1 | 215.8 | 42 | 164.5 |
| MCP-1 | 2536.7 | 21494.94 | 847.4 | 3861 | 152.2 |
| | 253.7 | 564.8 | 222.7 | 431.6 | 170.1 |
| | 25.4 | 50.1 | 197.5 | 40.2 | 158.5 |
| | 3412.6 | 5958.1 | 174.6 | 7755.5 | 227.3 |

Table 1. Cont.

| Cytokine | Expected (pg/ml) | High RP1 target | Low RP1 target | Recovery (%) |
|----------|-----------------|-----------------|----------------|--------------|
| IFN-gamma | 341.3 | 700.5 | 205.3 | 595.1 | 174.4 |
| | 34.1 | 16.7 | 48.9 | 7.5 | 22.0 |
| MIP-beta | 2544.7 | 1962.6 | 77.1 | 6157.1 | 242.0 |
| | 254.5 | 636.56 | 250.2 | 382.1 | 150.2 |
| IL-10 | 25.4 | 47.5 | 186.7 | 44.9 | 176.4 |

Unstimulated whole blood culture supernatant samples were spiked at three different concentrations of recombinant cytokines. Samples were analysed on the Bio-plex system instrument and the recovery of each cytokine in the panel assessed. The expected concentrations, after subtraction of the endogenous levels of cytokines, are represented. DOI:10.1371/journal.pone.0002535.t001

kit. Samples included the same set of antigen-stimulated whole blood culture supernatants used for the Bio-plex experiment 2 and LINCO 29-plex assays described earlier, as well as serum (diluted one in four) and whole blood supernatant spiked at six different concentrations with recombinant cytokine from the RnD System's standard [Part # 895531, lot # 238222 and Part # 895546, lot # 238223 [base kit A] and Part # 892794, lot # 233020 [base kit B]]. The six concentrations at which samples were spiked were unique for each cytokine with the lowest spike ranging from 14–600 pg/ml and the highest from 950–19 000 pg/ml. An eight-point standard curve, with each cytokine spanning its own unique specific range, was generated and 50 μl of each standard and sample were added to a 96-well plate containing fluorescent antibody coated beads. After alternate incubation and washing steps, detection and PE-labelled secondary antibodies were added and the plate read on the Bio-plex array reader, at a low RP1 target, using a 5 PL regression curve.

**Experiment 2.** The assay was done according to the manufacturer’s instructions. Serum (diluted one in four) and whole blood culture supernatant were spiked with seven different concentrations (ranging from 5–1700 pg/ml) of recombinant cytokine from the RnD System’s standard and measured as previously described.

**RnD-system Quantikine ELISA**

The same Mtb antigen- and PHA-stimulated samples used for the Bio-Rad human 17-plex assay experiment 2, LINCO human 29-plex assay and RnD Systems fluorokine-(MAP) experiment 1 assay were also assessed by ELISA. The ELISA was done using the RnD Systems IFN-γ Quantikine ELISA kit (cat# DIF50) according to the manufacturer’s instructions. Briefly, lyophilised Quantikine standard was reconstituted in distilled water and serially diluted one in two in kit standard diluent to produce a seven-point standard curve ranging from 15.6 pg/ml to 1000 pg/ml. Thereafter, 100 μl of assay diluent was added to the designated wells in a 96-well polystyrene microplate (provided with the kit) coated with polyclonal antibody against IFN-γ, followed immediately by 100 μl of standard, sample or control. The standard curve, samples and controls were run in duplicate. The plate was incubated for two hours at room temperature, washed and thereafter 200 μl of horseradish peroxidase (HRP)-conjugated IFN-γ antibody followed by 200 μl of substrate solution was added to the wells, followed by another incubation period and washing step between the two additions. After
30 minutes of incubation, 50 μl of stop solution was added to the wells and the plate read at 450 nm, with the wavelength correction set at 570 nm, on a multi-detection microplate reader (Bio-Tek instruments Inc, part # 7081000). Sample concentrations were determined using the KC4 microplate data analysis software, version 3.34, revision 12.

Statistics: Manufacturer 1d, 2, 3 assays and ELISA comparison (Study 2)

The correlation between the concentrations of cytokines as measured by the different Immunoassays for the same sample was assessed by the mean of intra-class correlation coefficients and the Pearson product-moment correlation coefficient. The analysis was done using STATISTICA (version 7).

Results

Manufacturer 1 assay

Experiment 1. The recovery of the Bio-Rad human 17-plex was assessed using spiked whole blood culture supernatants from three healthy individuals and each of the supernatants was spiked at three different concentrations. Generally, a lack of accuracy was observed as illustrated in Table 1. At a high RP1 target, 21% of positive readings were in the recovery range of 70 to 130%, whereas only 12.4% were within that range when samples were read at a low RP1 target.

Experiment 2. In this study, recovery of the Bio-Rad human 17-plex was assessed for five different concentrations of individual cytokines. Fluorescence was read both at high and low RP1 targets, with 85 readings made for each RP1 target. A total of 65 readings out of 85 were positive for the low RP1 target, with 54% of these positive readings (41.2% of the total readings) falling within the acceptable recovery range of 70 to 130%. There were 62 positive readings out of 85 at the high RP1 target, with 61% of these (44.7% of the total readings) falling within the acceptable recovery range of 70 to 130%. The cytokines IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-12p70, IL-13, IL-17, GM-CSF and MCP-1 measured most accurately when samples were read at the high RP1 target, whereas measurements of IL-5, IL-10, G-CSF and MIP-1β showed better recoveries when samples were read at a low RP1 target. Interfering interactions in the samples presumably led to falsely increased and signal inhibition for IL-8 detection, which resulted in out-of-range readings for four of the five assessed concentrations.

The recoveries of cytokines included in the Bio-Rad human 17-plex panel are shown in Figure 1 (recoveries from two independent experiments). Detailed statistics on Bio-Rad human 17-plex recovery and variations are shown in Table 2.

Manufacturer 2 assay

Experiment 1. The recovery of the cytokines forming part of the LINCO-Inc 29-plex panel were assessed at five different concentrations (5 000, 1 000, 500, 50 and 10 pg/ml), and read at high and low RP1 targets. The test showed an acceptable performance. A total of 145 readings were made at each RP1 target and 123 of these fell within the detection range when read at high RP1, compared to 136 out of 145 when read at the low RP1 target. Of the positive readings, 78.4% (66.2% of the total readings) were within the recovery range of 70 to 130%.

Figure 1. Recoveries of the Bio-Rad 17-plex assay. Un-stimulated whole blood culture supernatant samples from healthy donors were spiked at three (experiment 1) and five (experiment 2) different concentrations (2–8062 pg/ml) with the standard from the Bio-Rad 17-plex kit. Samples were assayed in duplicate and read at high and low RP1 targets on the Bio-plex system instrument. Recoveries were calculated for each of the cytokines in the panels for each of the spiked concentrations. The figure shows the recoveries obtained for each individual cytokine after two independent experiments.

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Figure 2. Recoveries of the Linco 29-plex assay. Un-stimulated whole blood culture supernatant samples from healthy donors were spiked at five (experiment 1) and two (experiment 2) different concentrations ranging from 10–5000 pg/ml with the standards from the LINCO 29-plex kit. Samples were assayed in duplicate and read at high and low RP1 targets on the Bio-plex system instrument. Recoveries were calculated for each of the cytokines in the panels for each of the spiked concentrations. The figure shows the recoveries obtained for each individual cytokine after two independent experiments.

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Table 2. Detailed statistics of Bio-Rad 17-plex assay recoveries (two independent experiments).

| Cytokines | IFN-γ | TNF-α | IL-1β | IL-2 | IL-4 | IL-5 | IL-6 | IL-7 | IL-8 |
|-----------|-------|-------|-------|------|------|------|------|------|------|
| Number of positive reads | 14    | 13    | 13    | 13   | 13   | 13   | 13   | 13   | 10   |
| Minimum | 20.3  | 51.47 | 23.77 | 88.89| 43.07| 14.7 | 19.63| 67.52| 100.2|
| Median | 98.37 | 91.43 | 114.5 | 106.3| 101.7| 115.7| 92.05| 168.3| 104  |
| Maximum | 518.3 | 152.9 | 323.5 | 692.5| 319.2| 261.6| 176.5| 824  | 166.9|
| Mean | 165.2 | 100.2 | 138.4 | 203.8| 147  | 126.4| 101.1| 225  | 118.8|
| Std. Deviation | 160.8 | 36.1  | 91.58 | 219.2| 110.6| 77.82| 54.27| 269.7| 32.21|
| Std. Error | 56.84 | 13.64 | 34.61 | 82.85| 41.8 | 27.51| 20.51| 101.9| 16.11|
| Coefficient of variation | 97.32%| 36.03%| 66.15%| 107.55%| 75.22%| 61.56%| 53.67%| 119.86%| 27.13%|

| Cytokines | IL-10 | IL-12p70 | IL-13 | IL-17 | MCP1 | MIP-1β | G-CSF | GM-CSF |
|-----------|-------|----------|-------|-------|------|--------|-------|--------|
| Number of positive reads | 14    | 14       | 13    | 13    | 12   | 12     | 13    | 13     |
| Minimum | 17.69 | 17.47    | 83.66 | 63.5  | 48.9 | 61.9   | 26.49 | 83.32  |
| Median | 86.81 | 96.84    | 146.4 | 111.2 | 69.05| 93.07  | 105.5 | 160.6  |
| Maximum | 194.4 | 230.3    | 259.1 | 554.4 | 174.6| 176.4  | 170.1 | 850.1  |
| Mean | 102.1 | 117.3    | 150.5 | 204.3 | 99.58| 108.6  | 114.3 | 232.1  |
| Std. Deviation | 60.77 | 69.12    | 63.35 | 178.7 | 58.53| 44.71  | 50.41 | 275.8  |
| Std. Error | 21.49 | 24.44    | 23.95 | 67.52 | 23.89| 18.25  | 19.05 | 104.3  |
| Coefficient of variation | 59.50%| 58.91%   | 42.09%| 87.46%| 58.77%| 41.16% | 44.11%| 118.82%|

Unstimulated whole blood culture supernatant samples were spiked at different concentrations with recombinant cytokines. Samples were analysed on the Bio-plex system instrument and the recovery of each cytokine was assessed after subtraction of the endogenous levels of cytokines.

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readings) read at the high RP1 target had recoveries falling within
the acceptable range of 70 to 130%, whereas approximately 70%
of positive readings (65.7% of the total readings) made at a low
RP1 target achieved this acceptable recovery. Measurements of
IFN-γ, IL-1β, IL-4, IL-6, IL-7, IP-10, MCP-1 and G-CSF were
found to be most accurate when the plate was read at a high RP1
target, with recoveries falling between 70 to 130%, whereas those
to TNF-α, IL-1α, IL-1α, IL-2, IL-5, IL-10, IL-12p40, IL-12p70,
IL-13, Fractalkine, MIP-1α, MIP-1β, GM-CSF, TGF-α, sCD40L,
VEGF, Eotaxine and EGF were most accurate when read at a low
RP1 target. IL-15 and IL-17 showed similar recoveries at both
high and low RP1 targets. The level of background signal was very
high for IL-8; this was most probably due to the fact that whole
blood supernatant used for the spiking experiment was not pre-
diluted. The coefficients of variation between the measurement at
high RP1 and low RP1 targets were less than 5% when applicable
(when both high and low RP1 showed positive readings).

Experiment 2. The assessment of LINCO-plex kit was
repeated only this time 21 cytokine were assessed. The repeat
experiment was done using a healthy donor whole blood
supernatant spiked at two different concentrations. The
recoveries of all the cytokines in panel fell within the acceptable

| Cytokines | IL-1α | IL-1β | IL-1α (−) | IL-2 | IL-4 | IL-5 | IL-6 | IL-7 |
|-----------|-------|-------|-----------|------|------|------|------|------|
| Number of positive reads | 6     | 7     | 4         | 7    | 7    | 7    | 5    | 7    |
| Minimum   | 61.44 | 70.7  | 35.02     | 77.09| 70.92| 75.23| 55.12| 64.04|
| Median    | 87.88 | 93.63 | 82.98     | 87.9 | 92.48| 112.8| 78.96| 77.5 |
| Maximum   | 99.01 | 123.2 | 180.5     | 102.9| 314.4| 177  | 110.2| 120.9|
| Mean      | 85.43 | 94.49 | 95.37     | 87.58| 121.1| 118.6| 81.48| 84.32|
| Std. Deviation | 12.92   | 18.51 | 61.23     | 8.522| 34.65| 22.5 | 25.2 |
| Std. Error | 5.275  | 6.997 | 30.62     | 3.221| 10.06| 7.433|
| Coefficient of variation | 15.13% | 19.59% | 64.21%    | 9.73%| 29.22%| 27.62%| 23.32%|

| Cytokines | IL-8 | IL-10 | IL-12p40 (−) | IL-12p70 | IL-13 | IL-15 | IL-17 | IFN-γ |
|-----------|------|-------|--------------|-----------|-------|-------|-------|-------|
| Number of positive reads | 4    | 7     | 4            | 7         | 7     | 7     | 7     | 7     |
| Minimum   | 76.67| 52.7  | 67.87        | 91         | 30.2  | 77.64 | 76.84 | 80.07 |
| Median    | 108.1| 77.15 | 68.75        | 97.1       | 78.02 | 85.7  | 90.58 | 90.29 |
| Maximum   | 233  | 86.8  | 71           | 304.3      | 95.84 | 128.3 | 134.9 | 128.2 |
| Mean      | 131.5| 74.81 | 69.09        | 131.8      | 74.22 | 96.48 | 94.31 | 95.23 |
| Std. Deviation | 69.34   | 10.69 | 1.371        | 77.84      | 20.59 | 20.33 | 19.22 | 17.9  |
| Std. Error | 34.67 | 4.039 | 0.6857       | 29.42      | 7.781 | 7.685 | 7.264 | 6.767 |
| Coefficient of variation | 52.74% | 14.28% | 1.98%       | 27.74%     | 21.07%| 20.38%| 18.80%|

| Cytokines | TNF-α | MCP1 | G-CSF | GM-CSF | MIP-1α | MIP-1β (−) | IL-10 | TGF-α (−) |
|-----------|-------|------|-------|--------|--------|-------------|------|-----------|
| Number of positive reads | 7     | 6    | 5     | 7      | 5      | 4           | 7    | 5         |
| Minimum   | 61.6  | 70.88| 46.55 | 81.44  | 53.06  | 59.3        | 22.3 | 90.15     |
| Median    | 76.02 | 135.3| 74.83 | 87.1   | 69.19  | 78.55       | 95.25| 99.96     |
| Maximum   | 139.2 | 251.5| 92.58 | 100.1  | 109.5  | 93.94       | 218.3| 121.4     |
| Mean      | 84.19 | 148.7| 71.77 | 87.84  | 76.99  | 77.58       | 96.86| 105.3     |
| Std. Deviation | 25.56   | 60.57 | 17.57 | 6.146  | 21.91  | 14.22       | 61.94| 12.94     |
| Std. Error | 9.659 | 24.73| 7.856 | 2.323  | 9.8    | 7.109       | 23.41| 5.785     |
| Coefficient of variation | 30.35% | 40.73% | 24.47%  | 7.00%  | 28.46% | 18.33%      | 63.94| 12.28%    |

| Cytokines | Fractalkine (−) | sCD40L (−) | VEGF (−) | EGF (−) | Eotaxin |
|-----------|-----------------|-------------|---------|---------|--------|
| Number of positive reads | 5     | 5    | 4     | 5      | 7     |
| Minimum   | 66.46 | 76.8 | 65.1  | 81.6   | 17.3  |
| Median    | 76.12 | 79.4 | 102.4 | 86.58  | 80.17 |
| Maximum   | 128.2 | 90.38| 108.3 | 97.24  | 86.1  |
| Mean      | 90.79 | 81   | 94.53 | 87.18  | 69.37 |
| Std. Deviation | 26.22   | 5.407 | 19.82 | 6.149  | 23.74 |
| Std. Error | 11.72 | 2.418| 9.911 | 2.75   | 8.973 |
| Coefficient of variation | 28.88% | 6.67% | 20.97% | 7.05%  | 34.23% |

| Cytokines not included in the second test. |

Unstimulated whole blood culture supernatant samples were spiked at different concentrations with recombinant cytokines. Samples were analysed on the Bio-plex system instrument and the recovery of each cytokine assessed after subtraction of the endogenous levels of cytokines.
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range of 70 to 130% except for MCP-1 and IL-6 for which the recoveries were 66 and 251% respectively. Figure 2 shows the recoveries of the LINCO-plex assay after the 2 independent experiments. Detail statistics are shown in Table 3.

The reproducibility of the LINCO kits was done by measuring cytokine concentrations of the same whole blood supernatants across five LINCO 29-plex kits. Data analysis showed that the coefficient of variation, standard deviation and error were within acceptable ranges for most of the cytokines (see Table 4 for more details).

### Manufacturer 3 assay

**Experiment 1.** The recoveries of 13 cytokines measured in whole blood culture supernatant and serum samples were assessed for six different concentrations using the RnD Systems Fluorokine-MAP assay. A total of 78 readings were made using whole blood culture supernatant and 67 from serum samples. All whole blood supernatant and serum sample readings were positive and within the standard curve range. A total of 67% of whole blood supernatant samples achieved recoveries within 70 to 130%, compared to approximately 56% of the serum samples.

**Experiment 2.** The recoveries of IFN-γ, TNF-α and IL-4 were assessed for seven different concentrations in whole blood culture supernatant and serum samples. In this experiment all whole blood supernatant readings were positive, whereas the detection limits in spiked serum samples were 44 pg/ml. About 30% of spiked whole blood supernatant achieved acceptable recovery (70 to 130%).

### Table 4. Linco 29-plex assay reproducibility.

| Cytokines       | IL-1B | IL-2 | IL-1ra | IL-4 | IL-5 | EGF  | IL-6 | IL-7 | TGF-α |
|-----------------|-------|------|--------|------|------|------|------|------|-------|
| Number of experiments | 5     | 5    | 5      | 5    | 5    | 5    | 5    | 5    | 5     |
| Minimum concentration | 6.46  | 1.6  | 52.92  | 5.8  | 6.05 | 1.6  | 238.7| 3.53 | 1.6   |
| Median concentration | 7.27  | 1.6  | 59.7   | 9.36 | 6.87 | 1.6  | 258.8| 3.99 | 1.6   |
| Maximum concentration | 9.17  | 4.33 | 79.18  | 14.09| 8.55 | 1.6  | 304.9| 8.69 | 1.6   |
| Mean concentration | 7.378 | 2.538| 65.58  | 10.29| 7.038| 1.6  | 261.6| 5.182| 1.6   |
| Std. Deviation   | 1.099 | 1.313| 12.37  | 3.294| 0.9504| 0    | 26.13| 2.19 | 0     |
| Std. Error       | 0.4914| 0.5872| 5.533  | 1.473| 0.425| 0    | 11.69| 0.9794| 0   |
| Coefficient of variation | 14.89%| 51.73%| 18.87% | 32.01%| 13.50%| 0.00%| 9.99%| 42.26%| 0.00% |

| Cytokines       | Fractalkine | IL-8 | IL-10 | IL-12p70 | IL-13 | IL-15 | IL-17 | IL-1α | IFN-γ |
|-----------------|-------------|------|-------|----------|-------|-------|-------|-------|-------|
| Number of experiments | 5     | 5    | 5     | 5        | 5     | 5     | 5     | 5     | 5     |
| Minimum concentration | 1.6   | 828.4| 143.1 | 1.6      | 18.9  | 1.6   | 12.34 | 22.73 | 687.4 |
| Median concentration | 1.6   | 960.2| 161.8 | 1.6      | 20.15 | 1.6   | 13.86 | 24.16 | 801.5 |
| Maximum concentration | 43.48 | 1153 | 200.4 | 4.71     | 26.7  | 1.6   | 20.83 | 32.38 | 993.7 |
| Mean concentration | 11.31 | 969.8| 167.9 | 2.222    | 21.17 | 1.6   | 14.96 | 25.65 | 807.1 |
| Std. Deviation   | 18.21 | 116.9| 21.57 | 1.391    | 3.238 | 0     | 3.351 | 3.851 | 127.1 |
| Std. Error       | 8.145 | 52.29| 9.648 | 0.622    | 1.448 | 0     | 1.499 | 1.722 | 56.85 |
| Coefficient of variation | 161.01%| 12.06%| 12.85% | 62.59%   | 15.29%| 0.00%| 22.41%| 15.01%| 15.75% |

| Cytokines       | G-CSF | GM-CSF | TNF-α | Eotaxin | MCP-1 | sCD40L | IL-12p40 | MIP-1α | MIP-1β |
|-----------------|-------|--------|-------|---------|-------|--------|----------|--------|-------|
| Number of experiments | 5     | 5      | 5      | 4       | 5     | 5      | 5        | 5      | 5     |
| Minimum concentration | 22.19 | 52.82  | 33.06  | 2.52    | 430.2 | 9      | 10.2     | 604.5  | 466.3 |
| Median concentration | 30.91 | 58.65  | 36.34  | 7.965   | 520.4 | 10.05  | 22.11    | 665.6  | 516.1 |
| Maximum concentration | 34.29 | 70.71  | 49.75  | 18.78   | 559   | 18.53  | 38.45    | 777.7  | 623.4 |
| Mean concentration | 29.65 | 59.71  | 38.3   | 9.308   | 502.6 | 12.85  | 21.96    | 690.1  | 519.2 |
| Std. Deviation   | 4.656 | 6.728  | 6.618  | 7.101   | 50.63 | 4.788  | 10.45    | 75.02  | 64.1  |
| Std. Error       | 2.082 | 3.009  | 2.96   | 3.55    | 22.64 | 2.141  | 4.674    | 33.55  | 28.67 |
| Coefficient of variation | 15.70%| 11.27%| 17.28% | 76.29%  | 10.07%| 37.26% | 47.58%   | 10.87%| 12.35% |

| Cytokine       | IP-10 | VEGF  |
|----------------|-------|-------|
| Number of experiments | 5     | 5     |
| Minimum concentration | 140   | 30.58 |
| Median concentration | 159.1 | 52.07 |
| Maximum concentration | 287.8 | 64.79 |
| Mean concentration | 182.1 | 50.43 |
| Std. Deviation   | 60.02 | 13.84 |
| Std. Error       | 26.84 | 6.189 |
| Coefficient of variation | 32.96%| 27.44% |

Five aliquots of the same PHA-stimulated whole blood supernatant were produced and each aliquot was run on different plates and on different days. doi:10.1371/journal.pone.0002535.t004
compared to 75% for serum samples that were within detection range. Details on cytokine recoveries after the two independent experiments are shown in Figure 3 detailed statistics in Table 5.

**RnD System’s ELISA**

The RnD System’s IFN-γ ELISA was used as a comparative test against which the different luminex kits were compared. Samples tested included antigen-stimulated samples with their negative and positive controls. As expected, the negative control showed a very low level of IFN-γ, whereas the positive control and antigen-stimulated samples showed higher levels of IFN-γ (Table 6).

**Manufacturer 1, 2, 3 luminex assays and RnD Systems ELISA: an IFN-γ based comparison**

Poor correlations were observed between the Bio-Rad luminex assay and the other assays. The correlation between the Bio-Rad luminex kit and the RnD Systems ELISA measurement gave an ICC of agreement of −0.01 and a Pearson correlation coefficient (r) of −0.09. The intra-class correlation coefficients (ICC) of agreement and the Pearson product-moment correlation coefficient (r) between LINCO luminex kits and RnD Systems ELISA kits were for the first test 0.64 (ICC) and 0.75 (r) and for the second test 0.75 and (ICC) and 0.84 (r) suggesting a positive

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**Figure 3. Recoveries of RnD System’s Fluorokine-MAP 13-plex base kits A and B.**

(A) Un-stimulated whole blood culture supernatant samples from a healthy donor were spiked at different concentrations (14–19000 pg/ml) with the standards from RnD-System Fluorokine-MAP base kits A and B. Samples were assayed in duplicate, read at a low RP1 target on the Bio-plex system instrument and recoveries calculated for each of the cytokines in the panel. The figure shows the individual cytokine’s best recovery obtained. (B) Serum samples from a healthy donor were spiked at concentrations (43.8–19000 pg/ml) with the standards from RnD-System’s Fluorokine-MAP base kits A and B. Samples were assayed in duplicate, read at a low RP1 target on the Bio-plex system instrument and recoveries calculated for each of the cytokines in the panel. The figure shows the individual cytokine’s recoveries obtained. The experiment was repeated for IFN-γ, TNF-α and IL-4 only.

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correlation between LINCO luminex kits and ELISA. The correlation analysis between RnD Systems Fluorokine-MAP and RnD Systems ELISA was also shown to be positive with an ICC of agreement of 0.1 and a Pearson correlation coefficient (r) of 0.99. The correlation between the different luminex kits measurements for the cytokines present in the three kit panels is shown in Table 7.

### Discussion

This validation study evaluated three commercially available cytokine multiplex bead immunoassays from Bio-Rad, LINCO-Inc and RnD Systems. The results suggest that, for the particular samples tested in this study, the LINCO Inc human 29-plex and the RnD Systems Fluorokine-MAP assays were the most accurate...
for the measurement of cytokine concentrations in whole blood culture supernatant and achieved good recovery ranges and reproducibility for most cytokines whereas the performance of the Bio-Rad human 17-plex assay was suboptimal.

The comparative study, including the Bio-Rad human 17-plex assay, LINCO 29-plex assay, RnD Systems Fluorokine-MAP assay and RnD Systems ELISA, was made based on IFN-γ responses in antigen-stimulated whole blood culture supernatant. It was found that all assays were capable of differentiating the positive and negative controls. Moreover, they were able to efficiently pick up the antigen-specific IFN-γ responses when applicable, with the exception of the Bio-Rad human 17-plex assay, where IFN-γ levels in two of the antigen-stimulated samples (ESAT-CFP-10 and Rv1115) went undetected.

Concentrations of IFN-γ measured by the LINCO 29-plex assay, RnD Systems Fluorokine-MAP assay and ELISA correlated, but results obtained using the Bio-plex assay correlated poorly with values obtained using the other three assays. Very similar to the findings by DuPont et al. [5], a very strong correlation between the level of IFN-γ measured by ELISA and the LINCO-plex kit in whole blood culture supernatant was found in the present study. Furthermore LINCO-plex assay, RnD Systems Fluorokine-MAP assay and the Bio-Rad 17-plex correlation of 13 cytokines showed a positive correlation between LINCO 29-plex and the RnD Systems Fluorokine-MAP assay for most of the cytokines, whereas the Bio-Rad 17-plex assay correlations to LINCO 29-plex assay and the Bio-Rad Systems Fluorokine-MAP assay for most of the cytokines, whereas the performance of the Bio-Rad human 17-plex assay was suboptimal.

Table 6. IFN-γ-based comparison of ELISA, LINCO 29-plex, Bio-Rad 17-plex and RnD Systems Fluorokine-MAP-13-plex assays.

| Whole blood supernatant | RnD Systems Fluorokine-MAP (pg/ml) | LINCO 29-plex (pg/ml) | Bio-Rad 17-plex (pg/ml) | RnD Systems ELISA (pg/ml) |
|--------------------------|----------------------------------|-----------------------|------------------------|-------------------------|
| PHA-stimulated           | 1731.38                          | 451.56                | 64.7                   | 86.83                   |
| Negative Control         | 0                                | 1.39                  | 0                      | NA                      |
| Mtb derived antigen 1 (supernatant A) | 1181.6                            | 969.12                | 0                      | NA                      |
| Mtb derived antigen 1 (supernatant B) | 908.83                            | 950.62                | 2.14                   | NA                      |
| Mtb derived antigen 2 (supernatant B) | 839.42                            | 536.92                | 193.53                 | NA                      |
| Mtb derived antigen 3 (supernatant D) | 489.1                             | 247                   | 49.08                  | 30.26                   |
| Mtb derived antigen 2 (supernatant C) | 235.67                            | 125.97                | 104.94                 | 17.65                   |
| Mtb derived antigen 4 (supernatant E) | 118.9                             | 133.82                | 172.19                 | NA                      |
| Mtb derived antigen 3 (supernatant C) | 57.5                              | 55.63                 | 0                      | 1.56                    |
| Mtb derived antigen 5 (supernatant E) | 23.78                             | 0.44                  | 99.2                   | 0.28                    |

NA: Not applicable because the sample was not measured with the RnD Systems Fluorokine-MAP base kit A. Supernatant was generated using whole blood from a healthy donor (A-E) in six day assays stimulated with 5 differents Mtb derived antigens and with phytohaemagglutinin (PHA). The LINCO 29-plex measurement most closely resembled that of the RnD Systems ELISA in absolute value, and followed a similar trend, but absolute values for all three luminex assay fell below that of the ELISA.

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In conclusion, the most appropriate use for multiplex cytokine assays based on luminex technology currently is as a screening tool, for example for the selection of candidate markers characteristic of disease-associated immune responses. Promising candidates can then be validated using a method with higher accuracy and proven reliability, such as ELISA.

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Author Contributions

Conceived and designed the experiments: GW JD CB GB. Performed the experiments: JD TR NB CB KS HG. Analyzed the data: JD HG. Contributed reagents/materials/analysis tools: Pv GW SP JD EH. Wrote the paper: GW JD TR GB.
**Table 7.** Correlation between ELISA, LINCO 29-plex, Bio-Rad 17-plex and RnD Systems Fluorokine-MAP 13-plex assays.

| Rater 1                  | Rater 2                  | ICC agreement | ICC consistency | r    |
|-------------------------|-------------------------|---------------|-----------------|------|
| IFN-γ                   | LINCO 29-plex            | 0.638         | 0.67            | 0.75 |
| RnD System ELISA        | Bio-Rad 17-plex          | -0.014        | -0.02           | -0.1 |
| RnD System ELISA        | RnD-System MAP           | 0.1           | 0.1             | 0.99 |
| LINCO 29-plex            | Bio-Rad 17-plex          | -0.06         | -0.085          | -0.2 |
| LINCO 29-plex            | RnD-System MAP           | 0.26          | 0.36            | 0.97 |
| Bio-Rad 17-plex          | RnD-System MAP           | 0.18          | 0.21            | 0.22 |
| IL-2                    | LINCO 29-plex            | 0.56          | 0.65            | 0.71 |
| IL-4                    | Bio-Rad 17-plex          | 0.098         | 0.17            | 0.23 |
| LINCO 29-plex            | RnD-System MAP           | -0.005        | -0.01           | -0.3 |
| Bio-Rad 17-plex          | RnD-System MAP           | 0.002         | 0.0064          | 0.23 |
| IL-6                    | LINCO 29-plex            | -0.05         | -0.07           | -0.1 |
| LINCO 29-plex            | RnD-System MAP           | 0.77          | 0.74            | 0.88 |
| Bio-Rad 17-plex          | RnD-System MAP           | -0.08         | -0.075          | -0.1 |
| IL-8                    | LINCO 29-plex            | 0.017         | 0.03            | 0.04 |
| LINCO 29-plex            | RnD-System MAP           | 0.3           | 0.56            | 0.73 |
| Bio-Rad 17-plex          | RnD-System MAP           | -0.046        | -0.042          | -0.04 |
| IL-10                   | LINCO 29-plex            | -0.02         | -0.02           | -0.04 |
| LINCO 29-plex            | RnD-System MAP           | 0.007         | 0.014           | 0.68 |
| Bio-Rad 17-plex          | RnD-System MAP           | 0.004         | 0.007           | 0.45 |
| GM-CFS                  | LINCO 29-plex            | -0.014        | -0.021          | -0.2 |
| TNF-α                   | Bio-Rad 17-plex          | -0.06         | -0.067          | -0.1 |
| LINCO 29-plex            | RnD-System MAP           | 0.9           | 0.9             | 0.91 |
| Bio-Rad 17-plex          | RnD-System MAP           | 0.42          | 0.38            | 0.38 |
| IL-1βi                  | LINCO 29-plex            | -0.27         | -0.27           | -0.3 |
| IL-5                    | Bio-Rad 17-plex          | -0.1          | -0.093          | -0.1 |
| LINCO 29-plex            | RnD-System MAP           | -0.046        | -0.038          | -0.1 |
| Bio-Rad 17-plex          | RnD-System MAP           | -0.18         | -0.15           | -0.5 |
| IL-13                   | LINCO 29-plex            | -0.14         | -0.138          | -0.2 |
| IL-17                   | Bio-Rad 17-plex          | -0.1          | -0.11           | -0.2 |
| MCP-1 (MCAF)            | LINCO 29-plex            | 0.5           | 0.48            | 0.48 |
| LINCO 29-plex            | Bio-Rad 17-plex          | 0.02          | 0.043           | 0.62 |
| Bio-Rad 17-plex          | RnD-System MAP           | -0.008        | -0.018          | -0.2 |
| MIP-1βi                  | LINCO 29-plex            | 0.04          | 0.037           | 0.05 |
| LINCO 29-plex            | RnD-System MAP           | 0.25          | 0.25            | 0.73 |
| Bio-Rad 17-plex          | RnD-System MAP           | 0.27          | 0.26            | 0.43 |
| MIP-1α                   | LINCO 29-plex            | 0.72          | 0.78            | 0.95 |
| IL-7                    | Bio-Rad 17-plex          | 0.11          | 0.25            | 0.36 |
| IL-12                   | LINCO 29-plex            | -0.16         | -0.14           | -0.1 |
| G-CSF                   | Bio-Rad 17-plex          | 0.004         | 0.005           | 0.01 |
| IL-1αi                  | LINCO 29-plex            | 0.33          | 0.49            | 0.97 |
| IL-1rru                 | LINCO 29-plex            | 0.23          | 0.39            | 0.85 |

10 Whole blood supernatant from a healthy donor were stimulated with antigens in six day assays. The table shows the intra-class correlation coefficients (ICC) of agreement and consistency as well as Pearson correlation coefficients (r) between measurements obtained with the different luminex kits and the ELISA.

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