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OVERVIEW OF LUMINEX ASSAY PERFORMANCE

Luminex Assay Design and Methods. Luminex assays use antibody-conjugated bead sets to detect analytes in a multiplexed sandwich immunoassay format. Each bead in the set is identified by a unique content of two addressing dyes, with a third dye used to read out binding of the analyte via a biotin-conjugated antibody and streptavidin-conjugated second step detector. Data is acquired on a dedicated flow cytometry-based platform (Luminex MAP 200).

We have validated a 50-plex Luminex bead kit, provided by Affimetrix, for the analysis of 50 human cytokines and chemokines (Table 1), and have an optimized SOP for the assay (available on request). Briefly, 96-well filter-bottom plates are wetted with buffer, and beads conjugated with capture antibodies for each cytokine are added. This is followed by addition of serum, plasma, or other samples (in duplicate wells), and serial dilutions of cytokine standards in designated wells. Plates are incubated for two hours at room temperature, with shaking, then for another 18 hours at 4C. The plates are washed on a vacuum manifold, then incubated with a biotin-labeled detector antibody cocktail for two hours at room temperature with shaking. Plates are washed again and incubated with streptavidin-PE for 40 minutes, then a final set of washes are performed and the wells resuspended in reading buffer. Samples are acquired on the Luminex MAP200 instrument, with collection criteria set for 100 beads per analyte (2000 beads total). Data is analyzed using MasterPlex software (Hitachi Software Engineering America Ltd., MiraiBio Group), and both median fluorescence intensity (MFI) and calculated concentration values are reported for each analyte.

Table 1. Analytes in the human 50-plex Luminex kit (with bead address)

ENA-78 (17)	IL-1alpha (62)	IL-12p40 (33)	MCP-1 (51)	Resistin (86)
EOTAXIN (52)	IL-1beta (18)	IL-12p70 (34)	MCP-3 (13)	SCF (7)
FGF-basic (55)	IL-1RA (63)	IL-13 (35)	M-CSF (90)	sFas Ligand (16)
G-CSF (46)	IL-2 (19)	IL15 (65)	MIG (11)	sICAM-1 (73)
GM-CSF (44)	IL-4 (20)	IL-17A (36)	MIP-1alpha (12)	sVCAM-1 (88)
GRO-alpha (61)	IL-5 (21)	IL-17F (39)	MIP-1beta (47)	TGFa (23)
HGF (79)	IL-6 (25)	IL-18 (57)	NGF (38)	TGF-beta (29)
IFN-alpha (48)	IL-7 (26)	IP-10 (22)	PAI-1 (14)	TNFalpha (45)
IFN-beta (30)	IL-8 (27)	LEPTIN (6)	PDGF-BB (37)	TNF-beta (32)
IFN-gamma (43)	IL-10 (28)	LIF (49)	RANTES (42)	VEGF (56)

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Quality control procedures. We have established a set of quality control criteria, which include the following:

1. AssayChex beads: A set of four QC beads with unique fluorescence addresses (AssayChex, Radix Biosolutions) are added to each well to provide quality assurance for: (a) addition of biotinylated detector antibodies; (b) addition of streptavidin-PE; (c) instrument performance; and (d) non-specific background fluorescence. Acceptance criteria for the AssayChex beads have been established and are applied to all samples. Wells that do not meet the acceptance criteria are excluded and may be re-run if necessary.
2. Bead counts: Both total bead count (>2000) and individual bead counts (>40) are verified and wells below threshold are flagged for possible exclusion.
3. Standard curves: Best-fit curves for each analyte are established by the analysis software (MasterPlex) with log-log transformation and weighting applied to the low end of the curve (for maximum low-end precision). All curves are manually inspected, and any outlier points are excluded.
4. Duplicate well CV: The coefficient of variation of duplicate wells is checked, and a plate is deemed to pass if the mean CV<15%, and if not more than 20% of duplicates have CV>25%. Wells with CV>20% are flagged for possible exclusion.
5. Control serum: An aliquot of a known control serum is included on each plate, and the MFI and concentration of each analyte in the control is plotted against historical data. While we have not yet established strict acceptance criteria for these values, this allows us to flag an outlier plate for possible re-run.

Assay Reproducibility. The mean inter-assay C.V. of multiplexed bead-based assays for cytokine detection has been shown to be 10-14% in published studies [1, 2]. We have obtained a similar average C.V. (10.5%) across the 50 analytes in our Luminex assay, using a healthy control serum (Figure 1). This relatively low C.V. was obtained despite the fact that most of the 50 analytes in the control serum were in very low abundance, often near the limit of detection of the assay.

We have also compared our standard curves over a large number of assays (Figure 2). These show that raw MFI values for each point in the standard curve are highly reproducible from run to run.

Finally, intra-assay precision of duplicate wells averages <10% C.V. in the 50-plex assay (data not shown).

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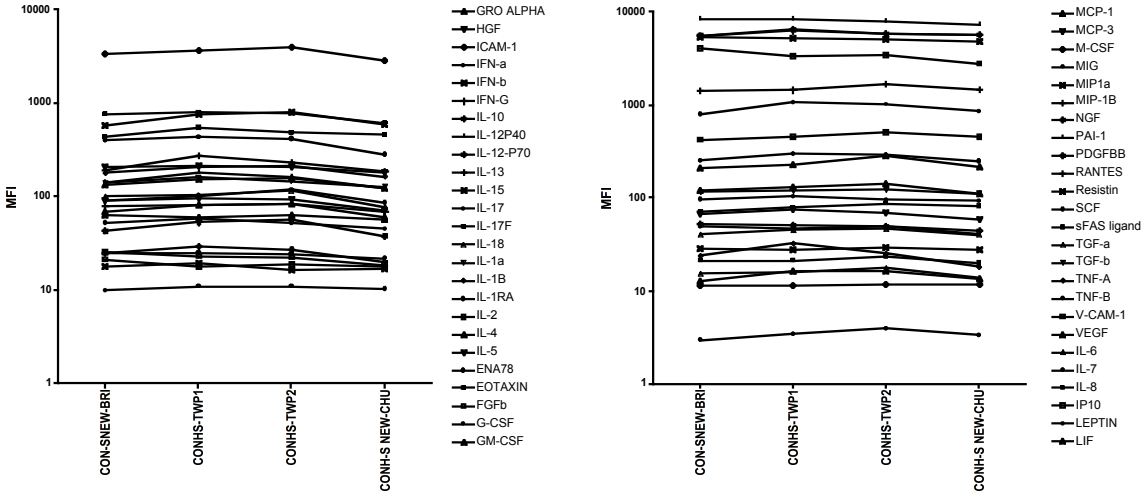

Figure 1. Reproducibility of MFI of a control serum over four consecutive assays using the 50-plex Luminex kit. 25 analytes are shown on each graph. The mean C.V. across all analytes was 10.5%.

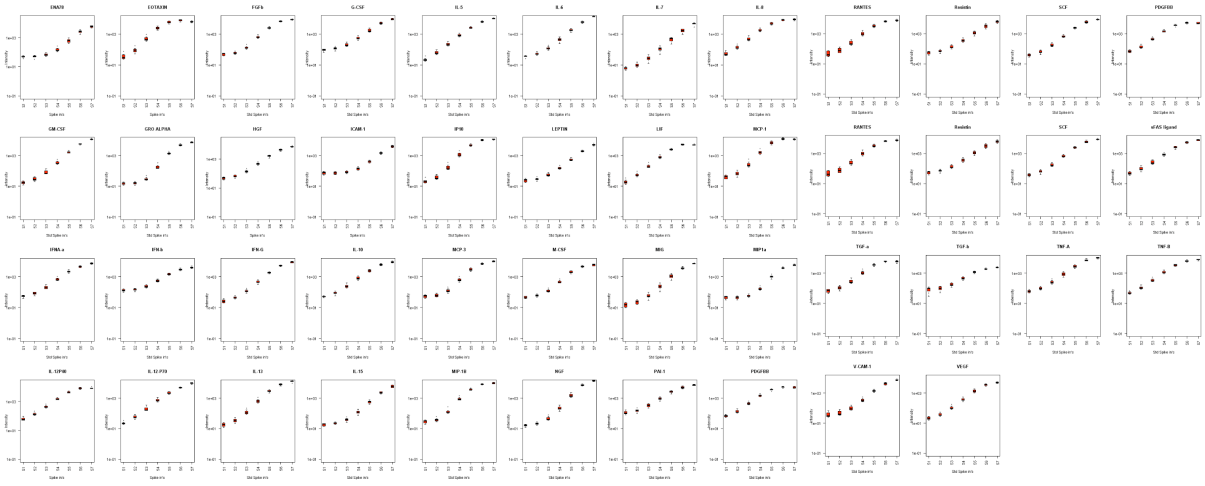

Figure 2. Reproducibiity of standard curve MFI for each of 50 analytes across 30 recent assays using the 50-plex Luminex kit. Each analyte is shown in a separate plot, and the mean, SD, and 95% CI are shown in the form of box-and-whisker plots for each point on the standard curve.

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Sensitivity and validation against other methods. While we have not directly compared each analyte in the Luminex 50-plex to an ELISA assay for that same target, we have sought to maximize the sensitivity and linear range of each analyte by working with the supplier to optimize these kits. For most analytes, we have an apparent limit of detection of 1-10 pg/mL using cytokine standards diluted in a serum standard buffer. We have also compared the sensitivity of selected cytokines in the Luminex kit with another platform that uses electrochemiluminescence detection (MesoScale Discovery). We found roughly similar sensitivity for 5 of 10 cytokines that we compared, with increased sensitivity by Luminex for 3 cytokines (IFNg, IL-5, and IL-13) and increased sensitivity for electrochemiluminescence for 2 cytokines (TNFa and IL-8).

Comparison studies of Luminex and ELISA technology have also been published [1-6]. DuPont et al. [1] found excellent correlations between ELISA and Luminex for 7 of 9 cytokines analyzed, including IL-1b, IL-4, IL-5, IL-6, IL-10, IFNg, and TNFa. Pang et al. [3] also found generally comparable results between two different multiplexed bead assays and ELISA. Dossus et al. [2] found good correlations between a multiplex assay and ELISA for some analytes (IL-1Ra, sCD40L, and CRP), but not for others, which were found at low concentrations in serum (IL-6, TNFa, IL-1b). However, other authors have found multiplexed assays to be more sensitive than ELISA for certain analytes, including IL-4, IL-10, TNFa [5], and vasoactive intestinal peptide [7]. Finally, data collected by our Luminex kit supplier (Affymetrix) has shown good correlation between a TNFa ELISA and the same analyte in their Luminex kit (other cytokines were not tested in that study).

In conclusion, there is evidence that a multiplexed, fluorescence-based readout can be equally sensitive or more sensitive than traditional ELISA. Many variables confound comparisons across these platforms [6], but optimization of the Luminex assay has allowed us to achieve highly sensitive readout of the majority of cytokines, using a tiny fraction of the sample required for a similar number of ELISAs, and with much less cost and effort.

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