

Development & Validation of Bioplex Assays for Cytokine Detection in Clinical Samples

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Outline of Presentation

- Introduction
- Advantages of Multiplexing
- Overview of Multiplexed immunoassay (MIA)
- 2-Panel Formats -Plasma
- 6-Panel Format - Culture Supernatant & Serum
- Caveats of Multiplexing
- Acknowledgements

Introduction

- Emerging trend to evaluate surrogate biomarkers in Phase I/II clinical trials
- Cytokines are considered potential surrogates for various diseases and/ drug activity

Introduction

- Interest in analyzing multiple cytokines in biological fluids and culture supernatants for research and clinical studies

Methodologies for Measuring Multiple Cytokines

- Bioassays (TNF- α , IL-6)
- Enzyme Linked Immunoassays (ELISA)
- Ribonuclease Protection Assay (RPA)
- Polymerase Chain Reaction (PCR)
- Liquid Chromatography- Mass Spectrometry (LC-MS)
- Serum Protein Fingerprinting
- Intracellular Cytokines staining
- **Bead-based arrays**

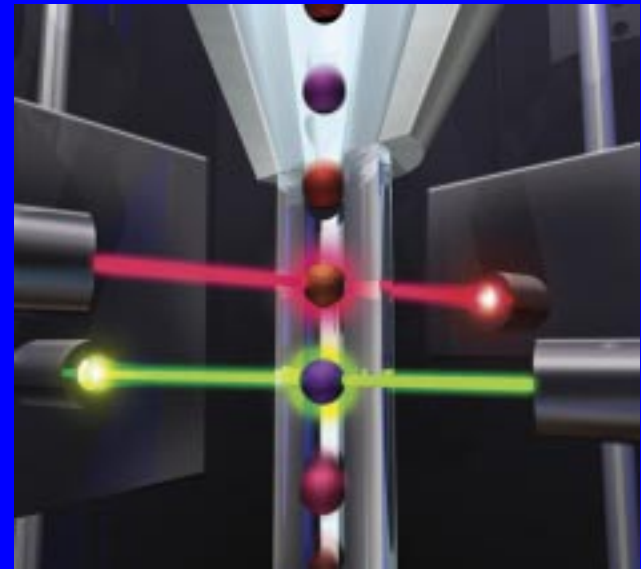
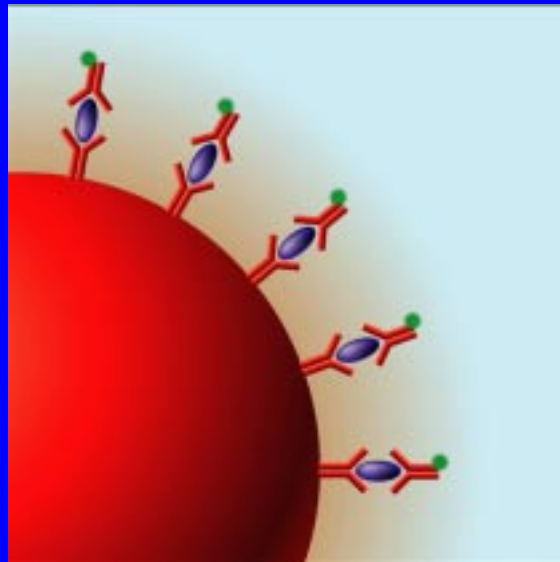
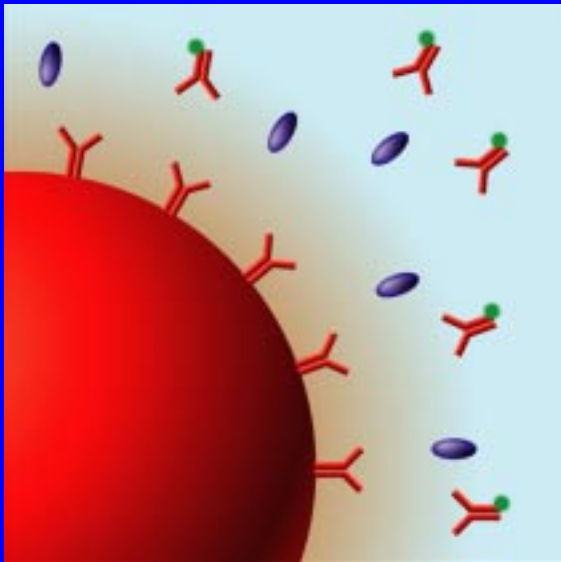
Advantages of Multiplexed Immunoassays (MIA)

- Efficient use of Samples
 - Low sample volume
 - Concomitant measurement of multiple cytokines
- Reduced Time & Cost of Analysis
- Increased Sensitivity
- Greater Dynamic Range

Overview of MIA

- Beads are labeled with a distinguishable fluorophore
- Capture antibodies, specific for the cytokine of interest, are covalently linked to beads of a unique fluorescent region
- The secondary antibody is labeled with another fluorescent probe (PE)

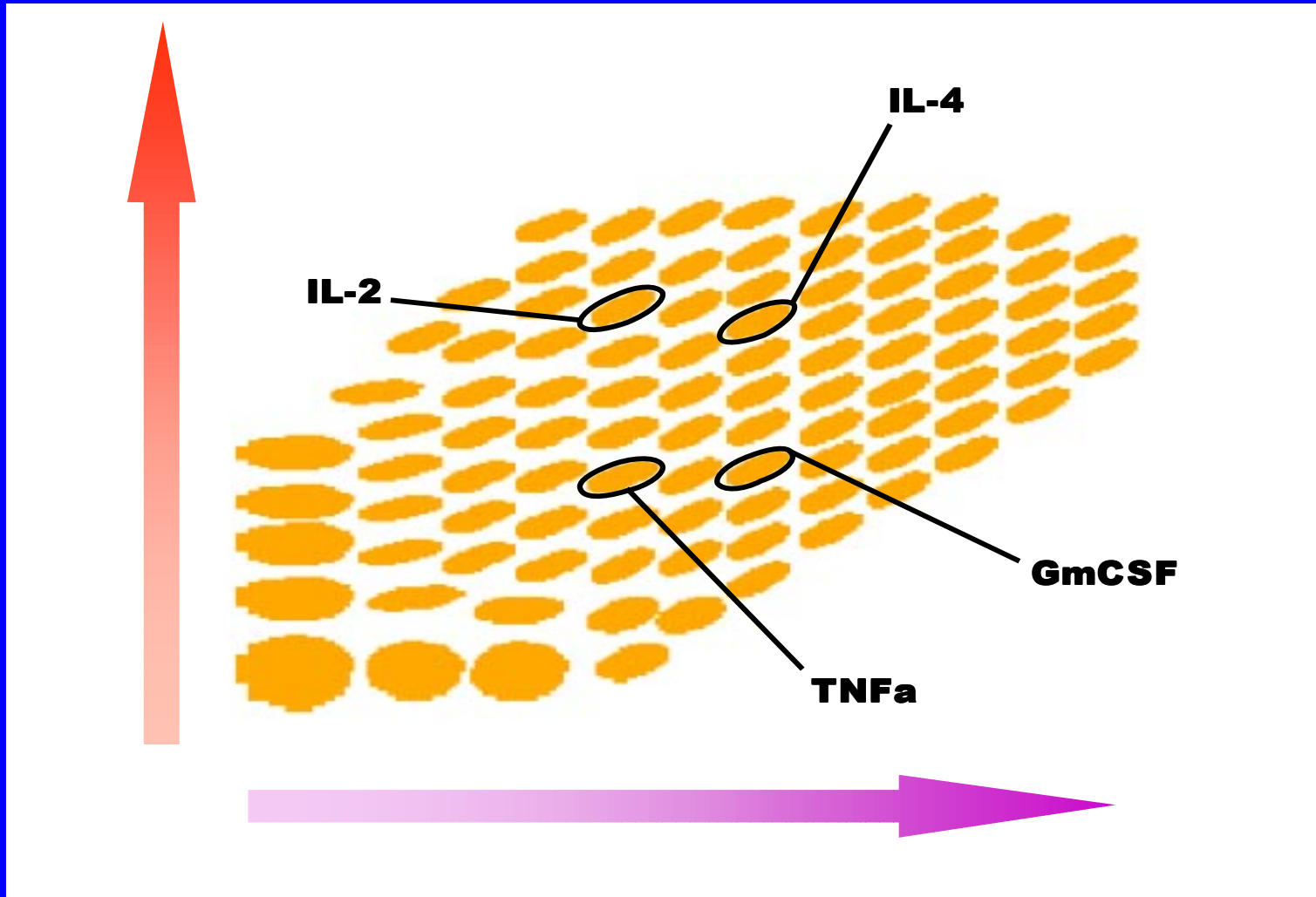
Overview of MIA



- A 635 nM Laser classifies the bead
- A 532 nM laser detects fluorescence

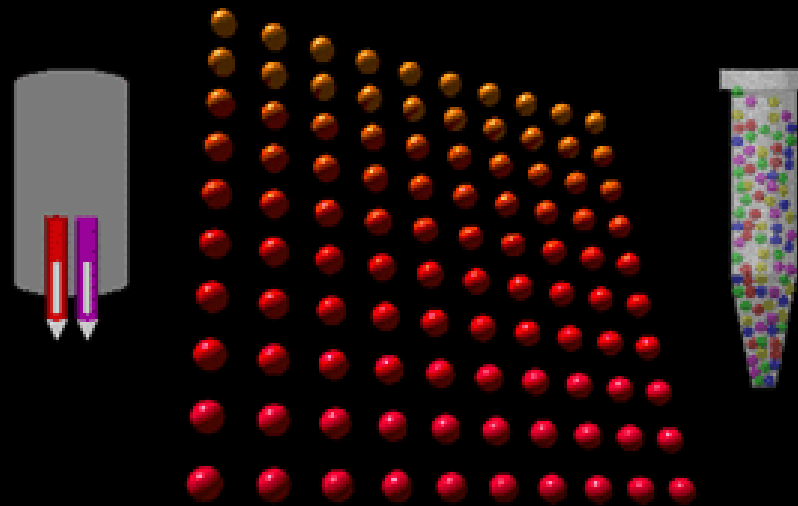
Instrumentation Identifies Microsphere Sets Based on Fluorescence

a



Overview of MIA

**100 Color-codes =
100 Simultaneous Tests**



Using this method, over 100 distinct
microsphere sets can be created.

Need for Establishing Good Antibody Pairs

- Custom-based coupling of beads
- Ready-made bead sets
- ❖ Critical to identify good antibody pairs that do not cross-react with other analytes/reagents in the system

2-panel Format -Plasma

- 3 X Two-panel cytokine panels for $\text{TNF-}\alpha/\text{IL-8}$; $\text{IL-1}\beta/\text{IL-8}$ and $\text{IL-1}\beta/\text{IL-6}$
- Microspheres from Luminex; recombinant cytokines; capture and secondary antibodies antibodies from R&D Systems; Luminex 100TM and XYPTM platform from Luminex
- Samples: Plasma from LPS stimulated blood (from normal volunteers) following Triton X-100 treatment

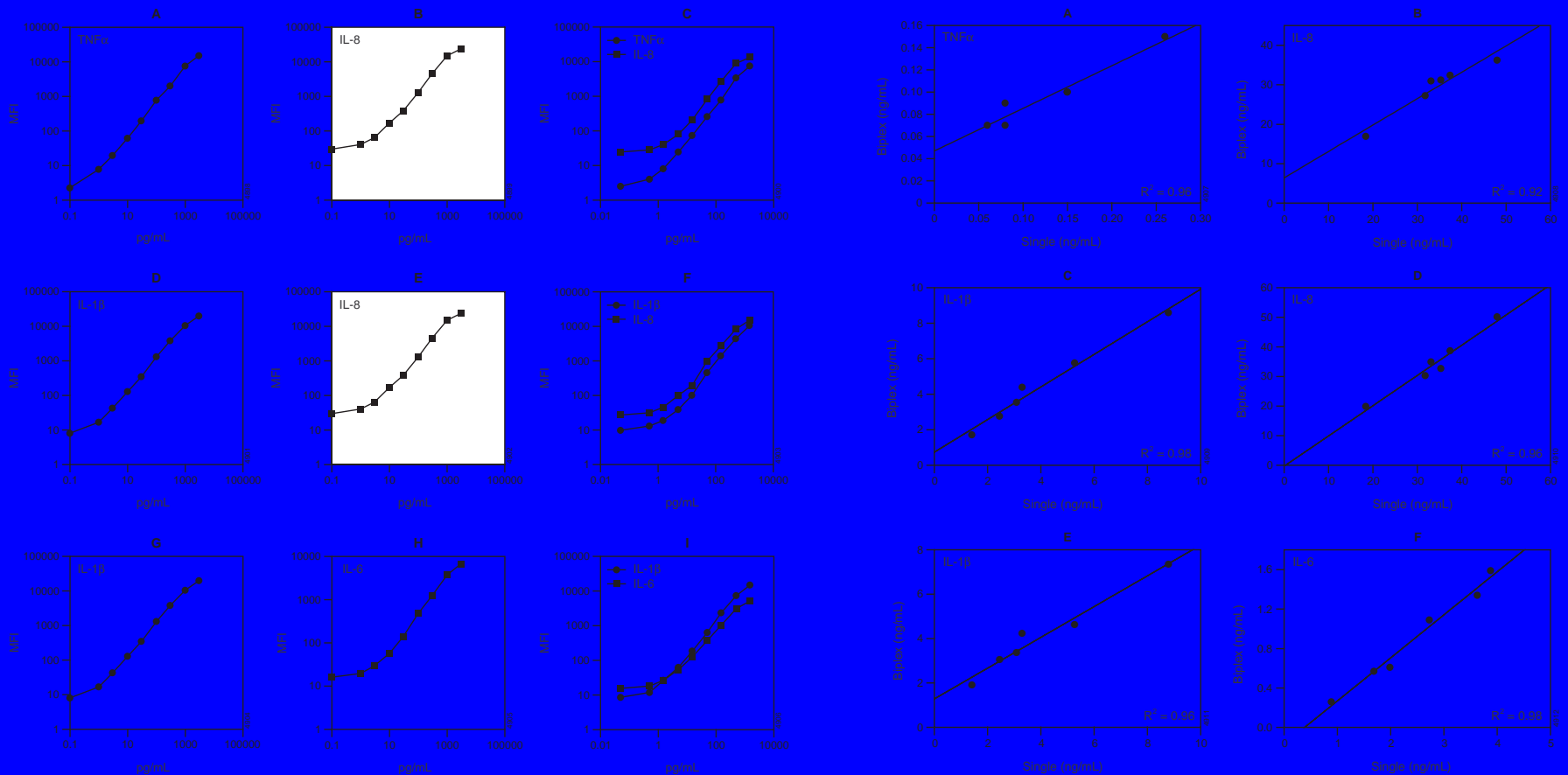
Validation of Assay

- LOD: Limit of Detection
 - $(3XSD + \text{Average MFI of the 0 standard})$
- LOQ: Limit of Quantification
 - $(6XSD + \text{Average MFI of the 0 standard})$
- Precision
 - Inter-Assay Variability
 - Intra Assay Variability

Improvement in LOD/LOQ

Cytokine	LOD (pg/mL)		LOQ (pg/mL)	
	2-Panel	ELISA	2-Panel	ELISA
TNF- α	0.43	1.00	0.68	1.60
IL-1 β	0.36	2.49	0.62	3.06
IL-8	0.48	10.40	0.83	14.00
IL-6	0.68	0.16	1.31	0.20

Single or Two-panel Formats Result in Reproducible Cytokine Values



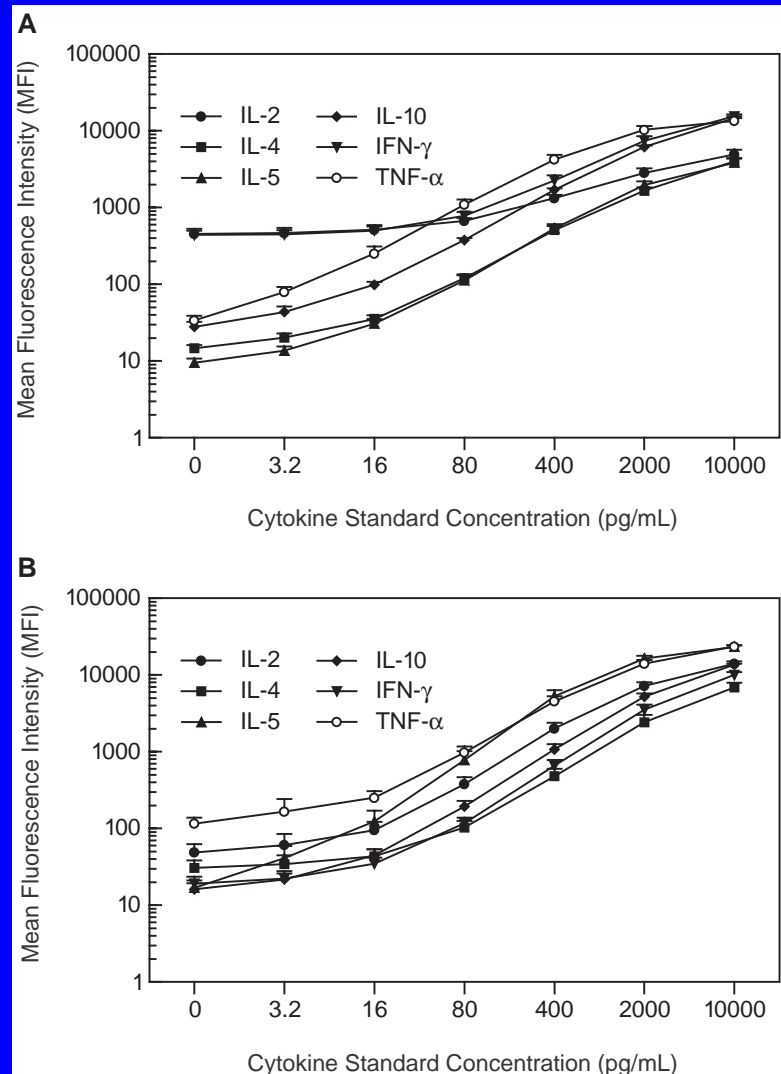
Six-panel Format: Culture Supernatant & Serum

- Ready-made kits for human Th1/Th2 cytokines were from Linco
- Culture supernatants were obtained from human PBMCs treated for different time intervals with various stimulating agents
- Serum was obtained from normal volunteers or from patients with various diseases

Validation of Assay

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 - Inter-Assay Variability
 - Intra Assay Variability
- Accuracy

Comparison of Standard Curves Between A: Culture Supernatants and B: Serum



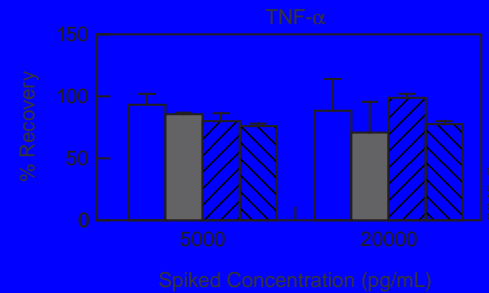
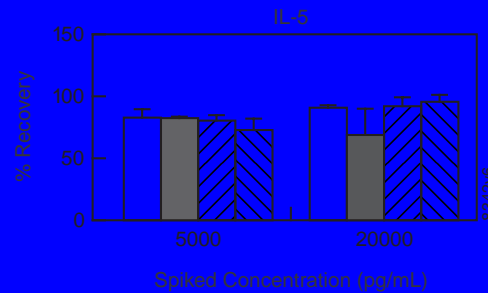
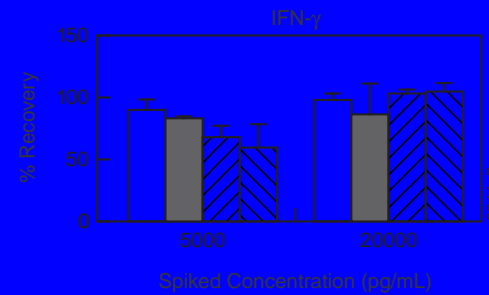
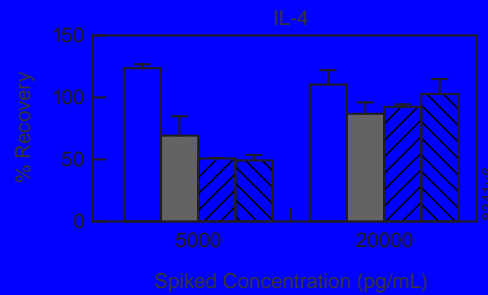
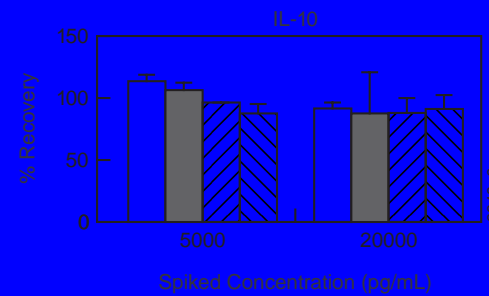
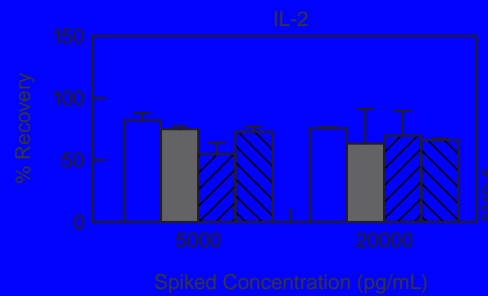
Lot to Lot Variability in MFI

Lot 1	Mean Fluorescence Intensity (MFI)					
Standard (pg/mL)	IL-2	IL-4	IL-5	IL-10	IFN- γ	TNF- α
10000	5713.00	4329.80	4216.30	15141.30	17380.30	15050.30
2000	3063.00	1798.50	1935.30	6083.50	8718.00	11944.50
400	1357.00	556.50	543.00	1723.30	2894.30	5141.00
80	715.50	136.80	119.50	387.00	932.80	1243.80
16	623.00	40.50	32.00	100.00	597.50	330.30
3.2	430.00	21.00	15.50	40.80	444.50	91.50
0	586.30	16.50	9.00	28.50	507.80	38.80
Lot 2	Mean Fluorescence Intensity (MFI)					
Standard (pg/mL)	IL-2	IL-4	IL-5	IL-10	IFN- γ	TNF- α
10000	25357.00	17813.00	27376.00	22687.30	22362.80	23343.00
2000	15745.30	8605.50	26290.50	11408.00	11424.50	18794.50
400	6498.50	2667.30	16349.50	3808.50	4001.30	9749.30
80	1752.80	623.30	4244.30	828.80	967.50	3547.30
16	467.00	139.00	722.50	183.30	217.80	1004.00
3.2	160.80	45.50	112.30	46.80	76.30	321.80
0	80.00	24.00	8.00	17.30	45.50	177.80

LOD/LOQ: Culture Supernatant

	IL-2	IL-4	IL-5	IL-10	IFN-γ	TNF-α
LOD (pg/mL)	24.35	3.22	1.70	2.41	10.46	1.34
LOQ (pg/mL)	54.53	5.77	3.52	3.70	30.44	1.83

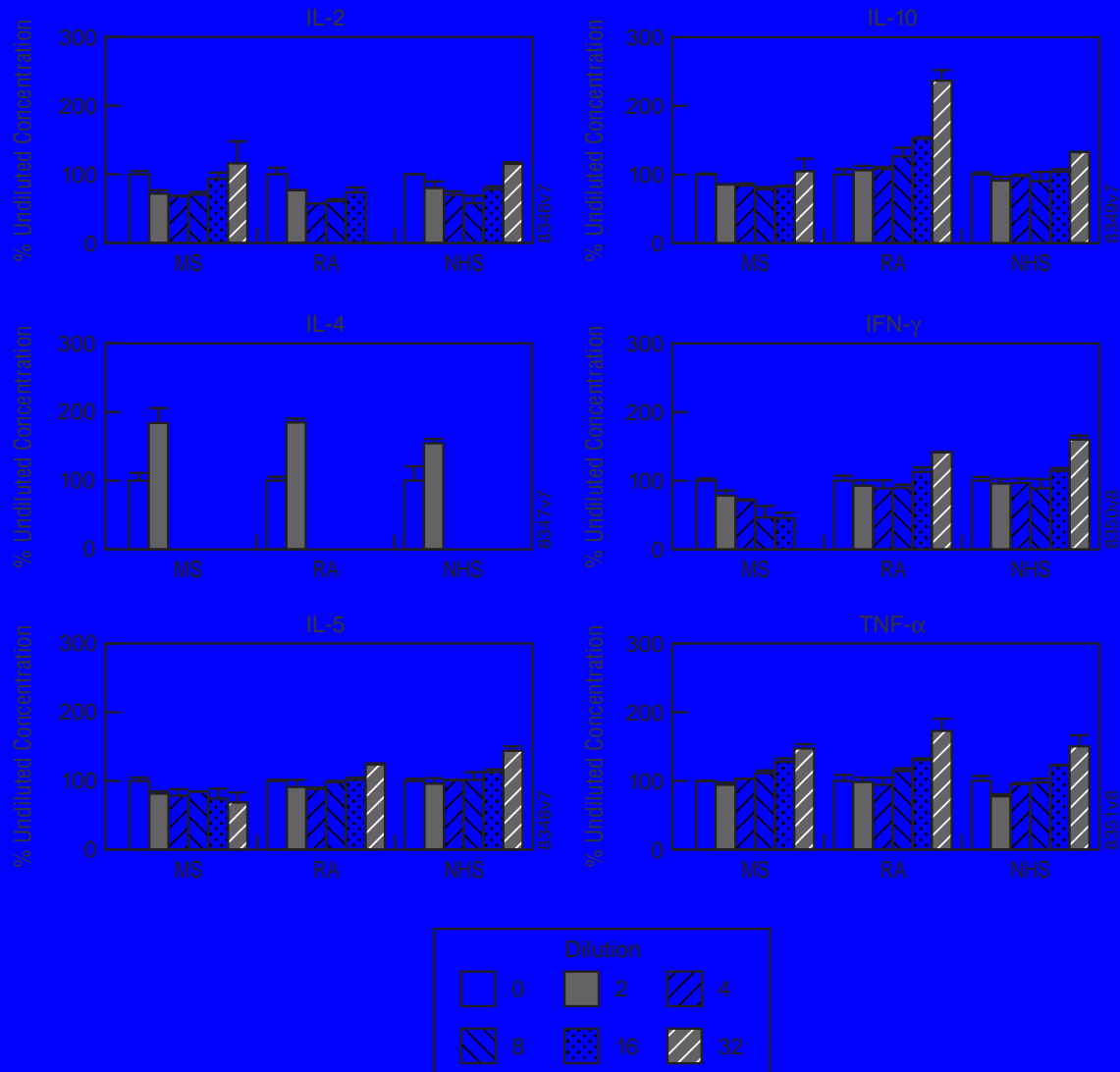
Accuracy & Precision in Culture Supernatants



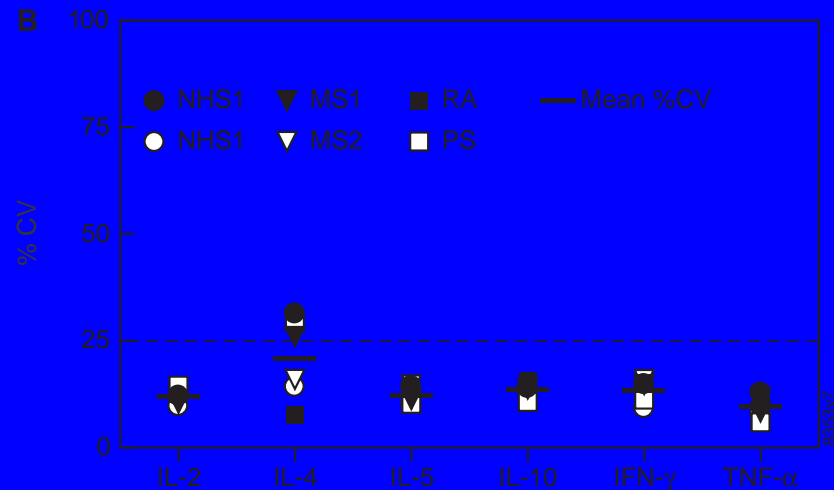
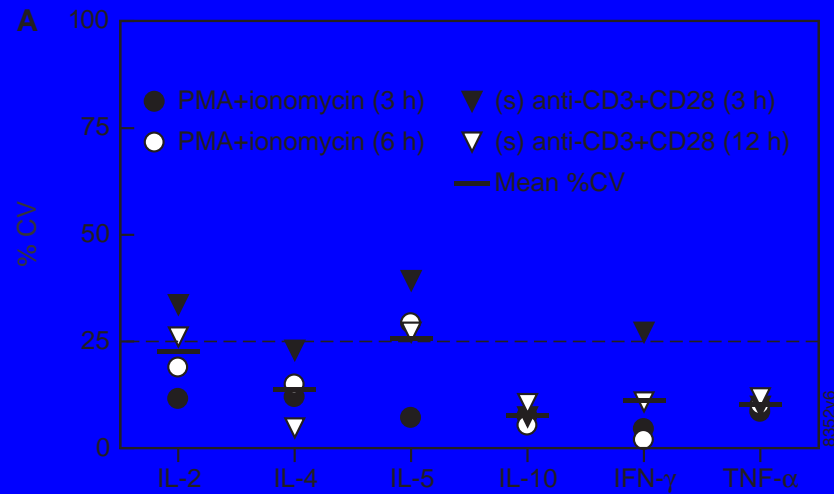
The Source of Serum Affects Accuracy

Sample (n)	Amount of cytokine spiked	IL-2	IL-4	IL-5	IL-10	IFN γ	TNF α
1000 pg/mL							
NHS (2)		94.77	46.16	97.15	96.25	88.55	114.61
RA (1)		59.45	58.65	81.64	98.35	92.41	95.73
MS (2)		83.81	56.39	92.08	88.75	60.93	107.41
PS (3)		105.08	45.44	98.72	91.94	100.62	121.95
100 pg/mL							
NHS (2)		84.02	40.95	106.43	91.69	94.48	126.77
RA (1)		48.91	81.55	90.01	92.63	102.52	99.34
MS (2)		75.54	59.47	105.44	87.35	64.72	126.85
PS (3)		93.52	55.59	117.36	96.26	117.01	121.95
10 pg/mL							
NHS (2)		61.42	34.64	79.43	50.77	51.10	84.82
RA (1)		27.70	91.18	64.20	80.79	62.40	85.03
MS (2)		54.90	94.76	89.42	59.85	34.15	94.01
PS (3)		61.81	67.53	95.30	73.00	83.83	104.75

Precision In Serum Is Reliable

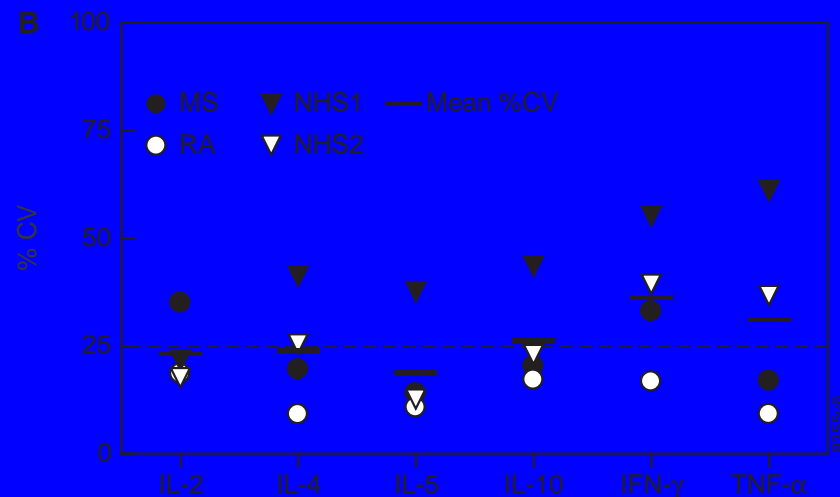
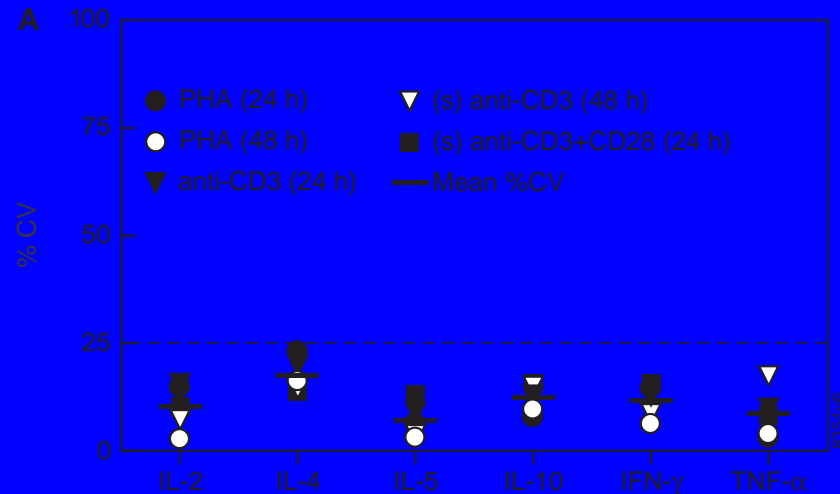


Inter-assay Variability in Both Culture Supernatants and Serum



Intra-assay Variability

A: Supernatant; B: Serum



Cytokine Secretion in the Supernatants of Stimulated PBMC Cultures

Stimulation	IL-2 (<u>±SD</u>)	IL-4 (<u>±SD</u>)	IL-5 (<u>±SD</u>)	IL-10 (<u>±SD</u>)	IFN- γ (<u>±SD</u>)	TNF- α (<u>±SD</u>)
PHA-P (24 h)	221.93 (32.98)	1806.17 (419.85)	107.55 (7.04)	2161.44 (172.28)	277.75 (40.74)	570.72 (18.21)
PHA-P (48 h)	618.94 (17.64)	791.88 (129.21)	180.63 5.76	2368.33 (230.49)	1428.11 (91.00)	748.81 (30.26)
(s) anti-CD3+(s) anti-CD28 (3 h)	38.79 (5.23)	9.56 (2.07)	6.07 (5.64)	14.5 (1.52)	46.36 (10.09)	64.01 (6.15)
(s) anti-CD3+(s) anti-CD28 (12 h)	785.73 (201.55)	104.37 (17.21)	4.65 (1.58)	234.66 (5.71)	474.30 (45.01)	1054.85 (98.39)
(s) anti-CD3+(s) anti-CD28 (40 h)	548.2 (62.60)	234.44 (34.02)	39.1 (3.44)	1726.79 (214.69)	2718.70 (254.92)	3264.57 (813.31)
PMA+ionomycin (3 h)	1351.73 (271.35)	69.44 (9.44)	10.07 (6.04)	15.45 (1.47)	321.91 (31.36)	369.32 (15.81)
PMA+ionomycin (6 h)	4195.21 (920.45)	142.26 (17.08)	12.13 (5.84)	59.06 (4.39)	967.62 (153.55)	467.49 (40.11)

Cytokine Levels in Serum Samples From Normal and Disease States

Sample	IL-2	IL-4	IL-5	IL-10	IFN- γ	TNF- α
Normal (9) Range	4.68 (0.97) 3.20-5.95	5.94 (5.16) 3.20-16.80	4.56 (1.81) 3.20-7.31	6.63 (3.35) 3.20-13.02	11.15 (9.02) 3.20-30.01	6.88 (3.28) 3.20-11.44
RA (8) Range	12.49 (10.35) 3.20-36.64	50.95 (72.81) 3.20-210-56	7.63 (6.73) 3.20-23.80	22.24 (29.10) 3.20-89.47	45.28 (72.08) 3.20-213.98	6.39 (6.74) 3.20-22.61
MS (3) Range	5.37 (1.93) 3.20-6.89	50.88 (80.58) 3.20-146.24	5.96 (4.78) 3.20-11.48	6.61 (5.91) 3.20-13.43	3.20 (0) 3.20	5.53 (4.04) 3.20-10.20
PS (8) Range	8.30 (5.64) 3.20-20.71	18.10 (28.38) 3.20-75.31	3.20 (0) 3.20	10.62 (16.01) 3.20-49.46	11.61 (16.34) 3.20-46.09	17.08 (8.09) 7.72-30.73

Summary of 6-Plex Assay

- Performance of a method for the simultaneous measurement of six cytokines
- High degree of assay precision, but poor assay accuracy (based on recovery data)
- Assay acceptable for evaluating cytokine expression following activation of PBMC by stimuli in vivo
- Assay acceptable for evaluating longitudinal clinical samples from patients in a single assay

Caveats of Multiplexing

- ‘Real World’ assay performance
 - Inherent variability of certain cytokine measurement
 - Recovery: unacceptable at physiological concentrations of some cytokines (IL-4)
 - Source of serum (interference from other serum factors)
 - Effect of test drug on cytokine measurement
- Assay performance must be evaluated to understand how the results should be used

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