

Using Multiplex Assays and Assay Development to Enhance your Research Program

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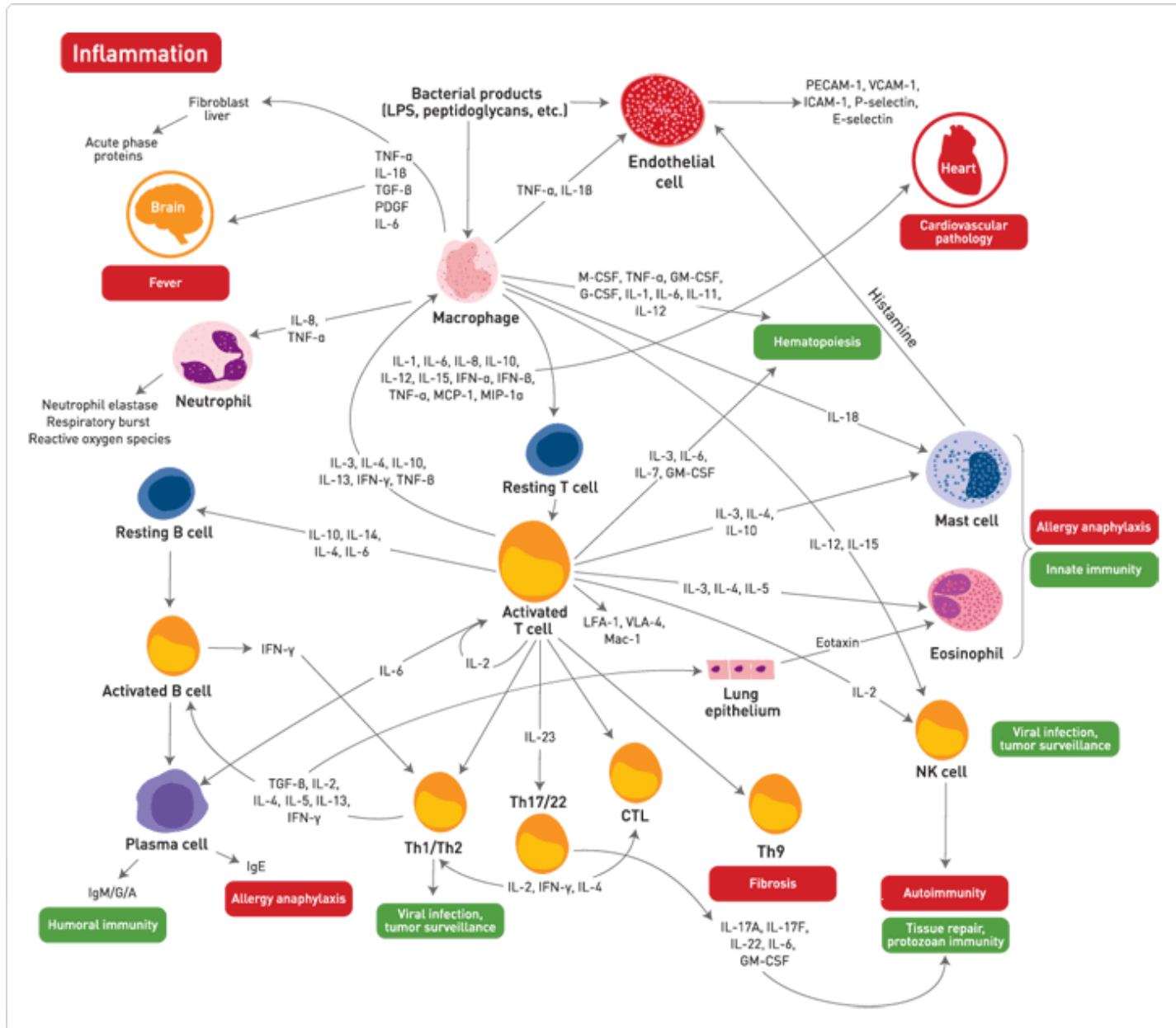
Overview Presentation

1. Research interests and reasons for using multiplex assay.
2. Why and how we began using multiplex assays for our research.
3. Luminex assay basics and strategies for developing custom assays.
4. Examples of how we use and take advantage of multiplexing.

Our Research Projects

1. The effects of traumatic injuries on the immune system
 - Complex response that involves multiple immune cell types and mediators
 - Mouse and human studies
 - Systems Immunology
2. Treatments that enhance immunity and restore immune homeostasis after traumatic injuries and severe infections (sepsis).
 - Immuno-monitoring by profiling immune cell phenotypes by flow cytometry and cytokine profiling.
 - *In vivo* response to injury, infection, and treatments.

Increased knowledge requires increased data



“Old school” single-mediator assays are good, but multiplexing is more in tune with current scientific needs

1. Difficult to interpret findings and publish results without including more than one mediator or biomarker in dataset.
 - Inflammatory responses and T-cell immune regulation for example
2. More financial demands on research funding requires increasing efficiency and data collection strategies.
3. We need to gather as much information as possible from expensive mouse models and limited amounts of human samples.

History: ELISA to Flow Cytometry Bead Assays to Luminex Assays

1. ELISA – basic principle behind all immunoassays
 - Plate-bound antibody – antigen – antibody sandwiches with color absorbance detection
 - Usually a single analyte approach
2. Flow cytometry-based bead assays
 - Same principle as ELISA but on a bead with a specific detection character for gating on a flow cytometer
 - Limited number of beads can be run as multiplex
 - Not automated or customizable
3. Luminex bead assays
 - Same principle as ELISA using beads with a specific detection character
 - High number of specific beads for multiplexing
 - Customizable and automated

Why do multiplex assays enhance research efficiency?

1. ELISA requires large sample volumes – 50 uL for single analyte detection.
 - More samples needed to generate data
 - More reagents needed
 - Need to do multiple wells if more than one analyte
 - If assay does not work, less material for repeat
2. Multiplexing requires much less sample volume for multi-analyte detection.
 - Less sample needed – 20 uL or less
 - Less reagents needed
 - Not necessary to do multiple wells
 - Capability to run multiple assays if needed and for future assays from stored samples

Reasons for developing “in-house” multiplex assays

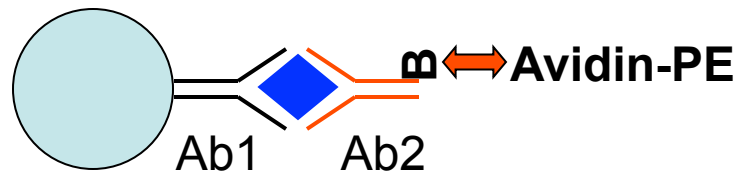
1. Purchased Luminex instrument in 2007 and found discrepancy in results between older technology (BD CBA) and Luminex assays.
 - Several cytokines that were detected by ELISA or CBA were not detected in Luminex assays.
2. Tested several different available kits and found inconsistencies in cytokine detection.
 - Not surprising since assays are likely built with different specific antibody pairs.
3. Some kits detected recombinant cytokines, but not natural cytokines

More reasons for developing “in-house” Luminex assays

1. Confidence that results will be accurate.
2. Reduce overall costs of performing Luminex assays for our immuno-phenotyping studies.
 - Kit costs were \$100-\$200/cytokine/96-well plate.
 - Discovered that making in-house assays in bulk would be less expensive.
3. Flexibility for future expansion of panels and for other multiplex assay development – opens up capabilities to make a variety of different biomarker assays.

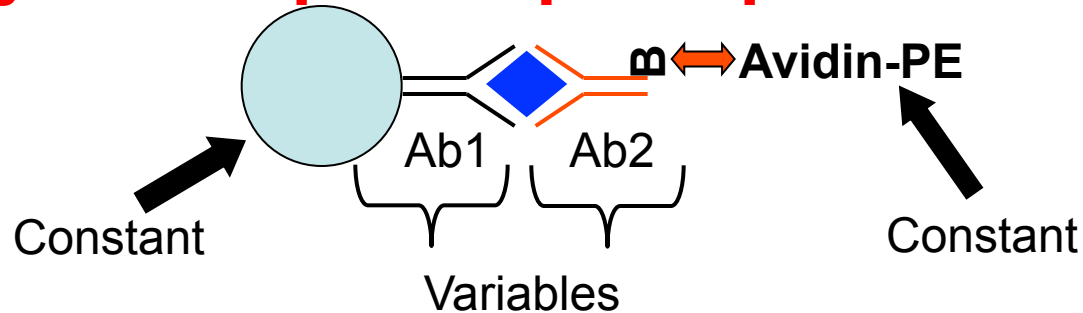
Assay development principles and variables

Immuno-assays based on sandwich ELISA principles



- High specificity due to dependence of signal on bi-molecular binding reagents
- Quality and specificity of assay reaction depends on the nature of Ab1 and Ab2.
- Antibodies remain the most important component of these and other types of immunoassays

Assay development principles and variables



1. Bead coupling reaction – Ab1 Concentration, reaction buffers and chemicals, reaction time, centrifugation washes, blocking buffers
2. Antibody pairing – bead-bound (Ab1) versus detection (Ab2), trial and error
3. Detection Ab and Avidin-PE – Titration optimization to minimize reagent use and prevent non-specific background
4. Standards - Recombinant versus natural cytokine detection

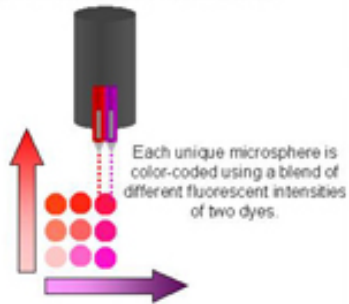
40,30,20 Luminex bead assay protocol

1. Prepare standard dilutions in buffer similar to sample
2. Prepare mixture of Luminex beads
3. 20 μ L of standards or samples added to 96-well plates
4. Bead mix added to plate.
5. Incubate for **40** minutes on rotary shaker.
6. Wash by centrifugation, filter plate, or magnet (MAGPIX).
7. Add mixture of biotinylated-Ab2.
8. Incubate **30** minutes.
9. Wash
10. Add streptavidin-PE
11. Incubate for **20** minutes
12. Wash 2X
13. Read on Luminex instrument – FlexMap3D, LX200, or MAGPix

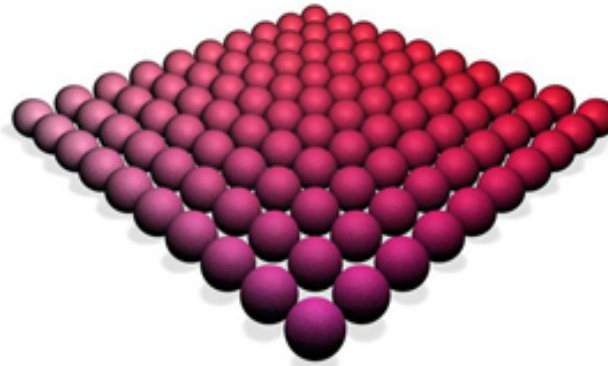
Luminex assay principles and readout

- 5.6 μM microspheres are dyed to create distinct colors and can be delineated based on red/infrared content
- Surface is modified to allow coupling of antibodies, proteins, peptides, or oligonucleotides
- Immunoassays are based on sandwich ELISA

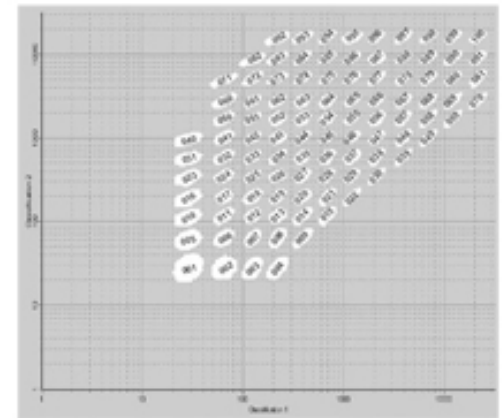
Each Color-code Is A Precise Blend of Two Colors



Each bead is dyed with a specific combination of two dyes to give a distinct spectral address.



100 colour-codes is equivalent to 100 different simultaneous analytes.



Each bead has a specific region on the map.

Data output example: Human IL-4

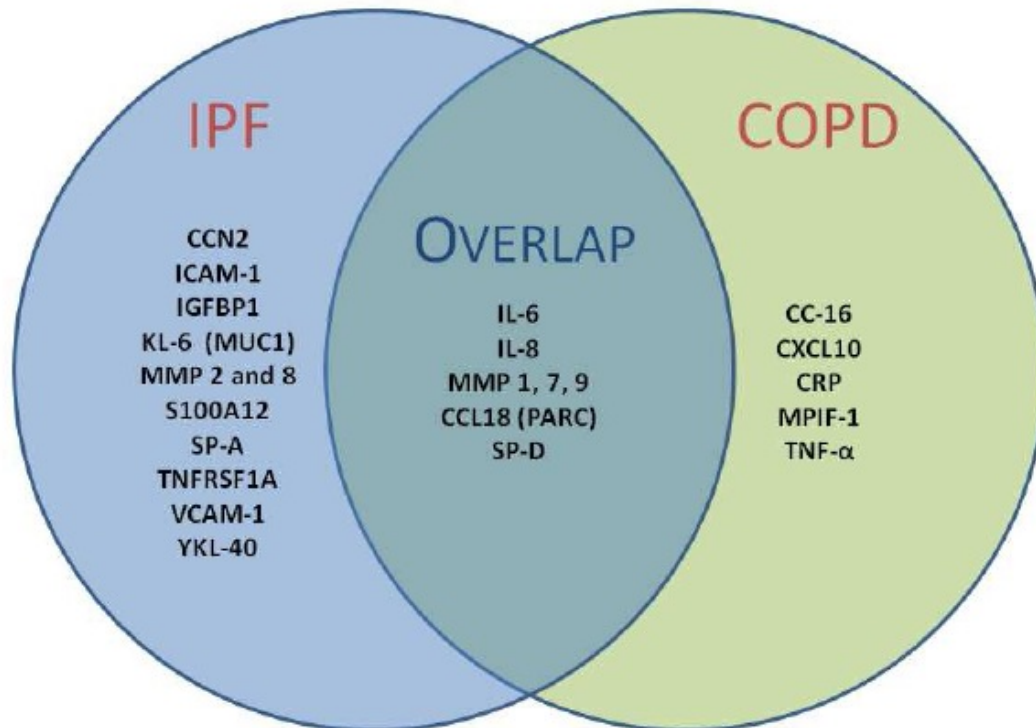
1. Standards run to calculate sample concentrations
2. Readout is mean fluorescence intensity (MFI)
3. Data output from instrument as .CSV files that input into MS Excel
4. Plotted to visualize data

SampleID	Well Location	MFI
Std01 (0.62 pg/ml)	A1	49
Std02 (1.25 pg/ml)	B1	73
Std03 (2.50 pg/ml)	C1	105
Std04 (5.00 pg/ml)	D1	175
Std05 (10.00 pg/ml)	E1	329
Std06 (20.00 pg/ml)	F1	573
Std07 (39.00 pg/ml)	G1	1027
Std08 (78.00 pg/ml)	H1	1695
Std09 (156.00 pg/ml)	A2	2593
Std10 (312.00 pg/ml)	B2	4439
Std11 (625.00 pg/ml)	C2	7267
Std12 (1250.00 pg/ml)	D2	11025
Std13 (2500.00 pg/ml)	E2	15787
Std14 (5000.00 pg/ml)	F2	20073
Std15 (10000.00 pg/ml)	G2	22369
Std16 (20000.00 pg/ml)	H2	22839
	1 A3	67
	2 B3	1399
	3 C3	175
	4 D3	1209
	5 E3	33
	6 F3	25
	7 G3	1341
	8 H3	1447
	9 A4	23
	10 B4	1411

Recent biomarker development project for lung disease diagnosis – COPD vs IPF

NIH development project to construct biomarker sets to distinguish patients with chronic obstructive pulmonary disease (COPD) vs. interstitial pulmonary fibrosis (IPF).

Ivan Rosas and Fernanda Golzarri, BWH Pulmonary Critical Care and Lovelace Respiratory Research Institute

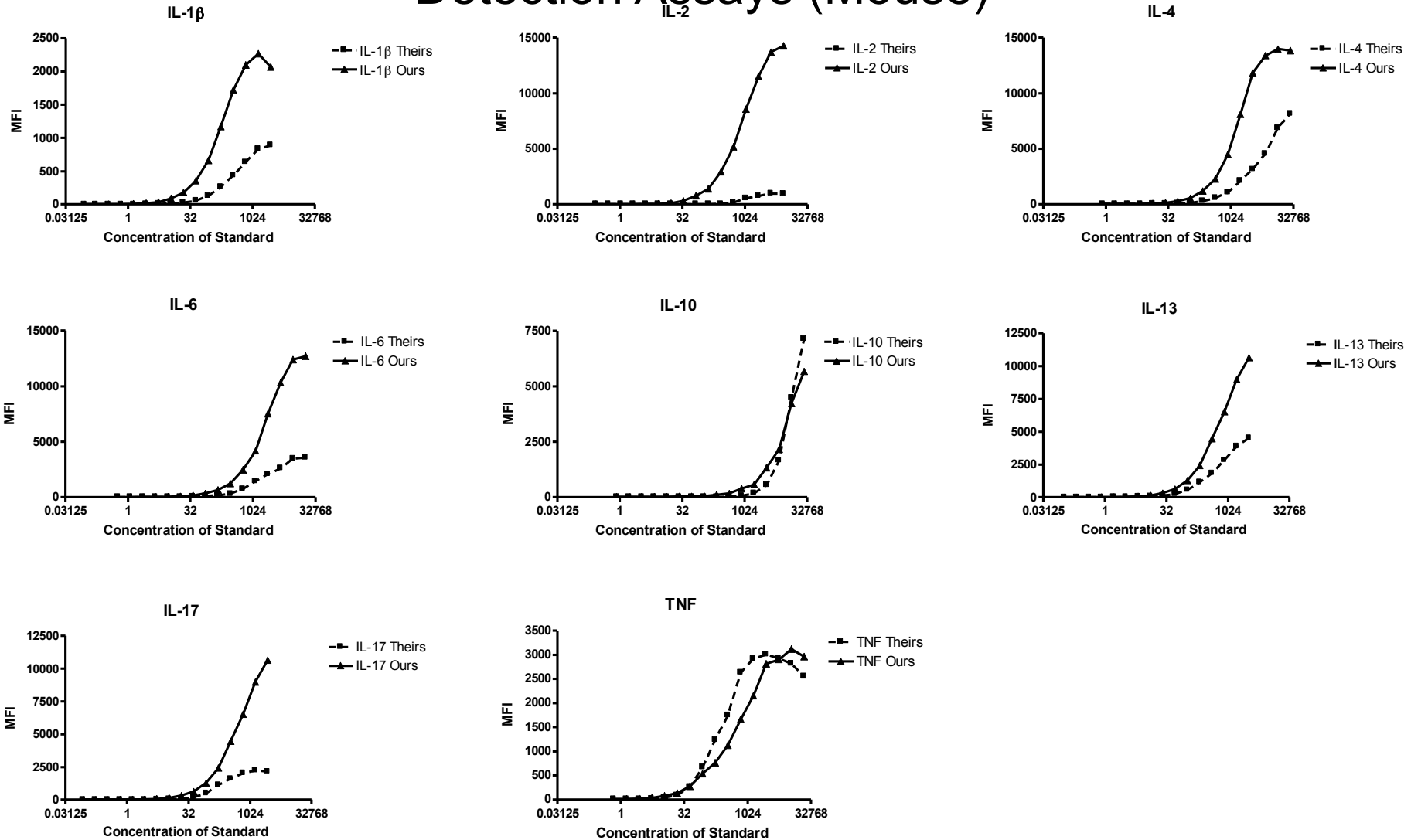


Steps to Building the COPD/IPF panel

1. Identify target analytes – list from literature
2. Search for the needed reagents – Ab1, Ab2, standard
3. Find standard
 - Can be difficult for less studied proteins or complex proteins – *e.g.* surfactant protein A (SP-A)
4. “micro-batch” testing to optimize bead coupling reaction
5. Biotinylation optimization for Ab2, if needed

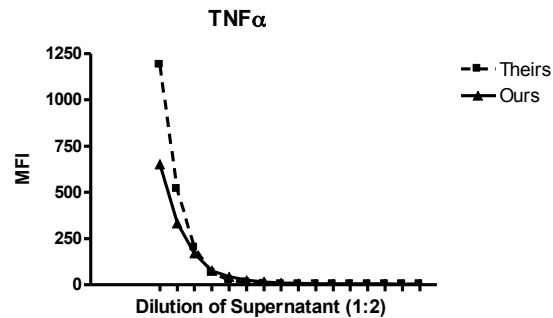
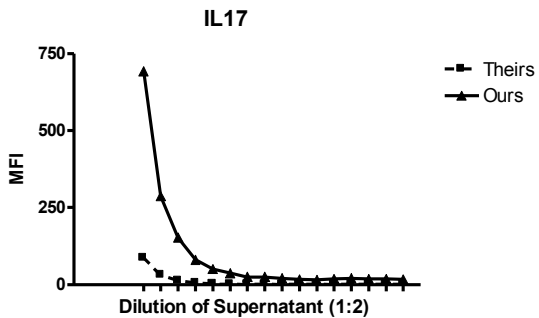
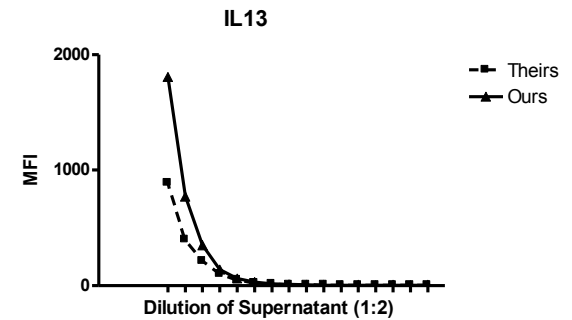
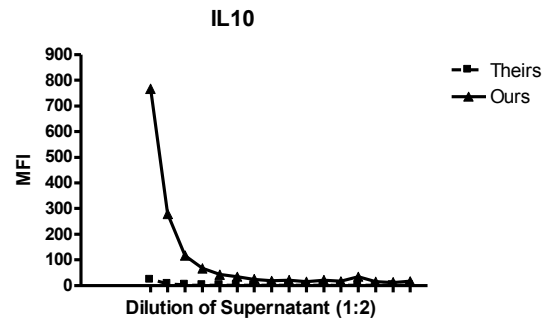
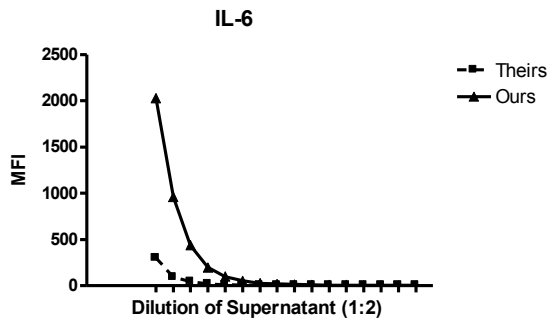
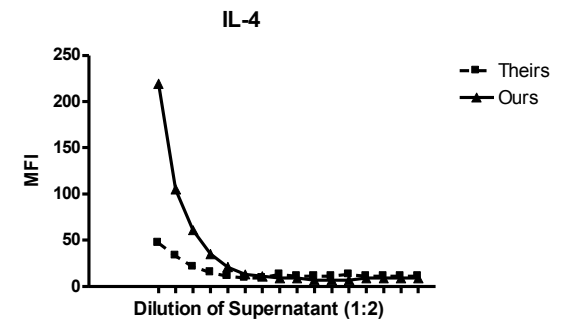
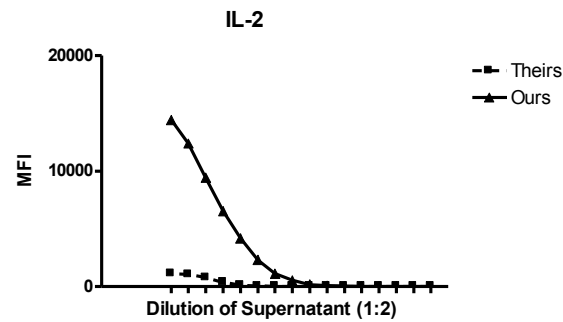
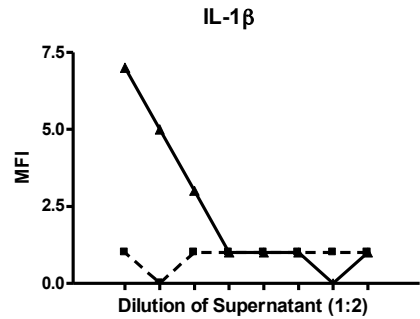
Examples: Cytokine Assays – “In-house” mouse cytokine assay

Standard Curve Plots Comparing Recombinant Cytokine Detection Assays (Mouse)

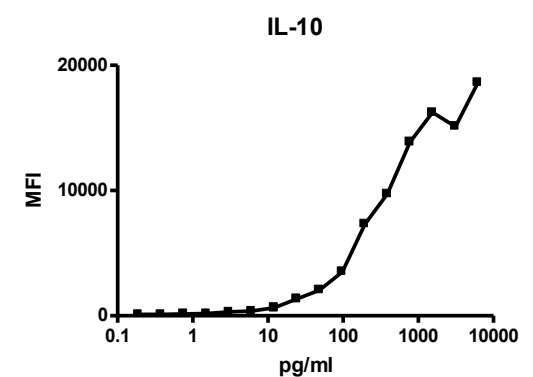
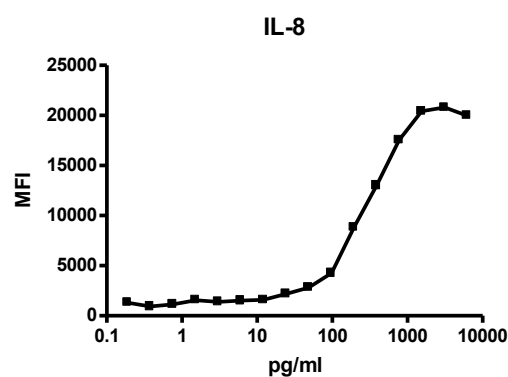
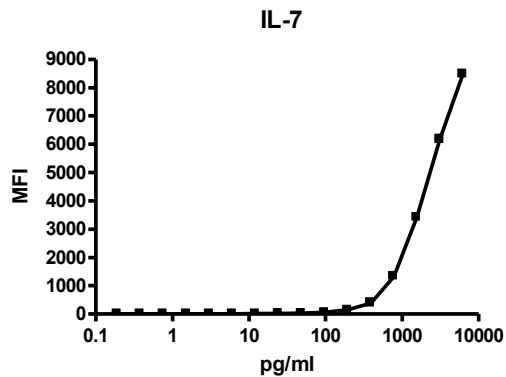
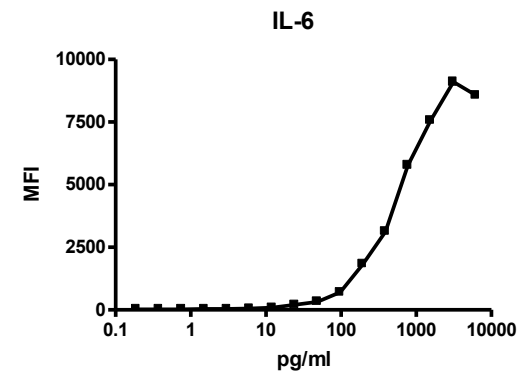
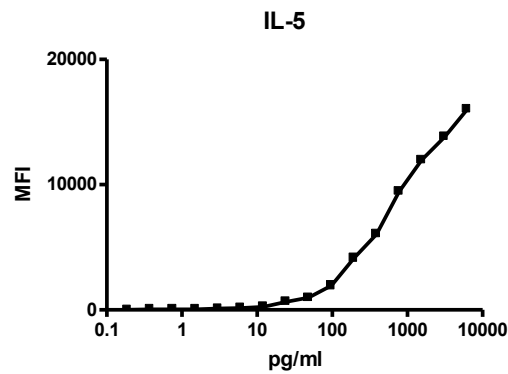
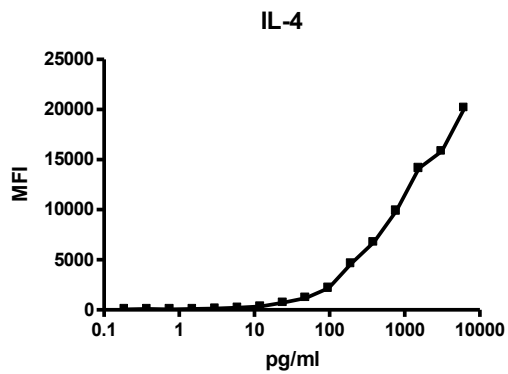
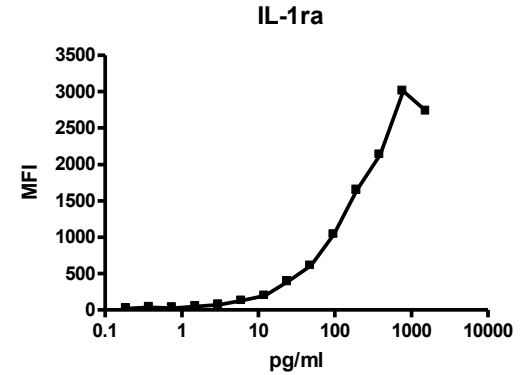
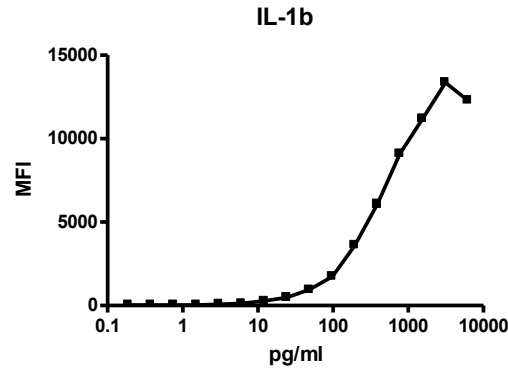
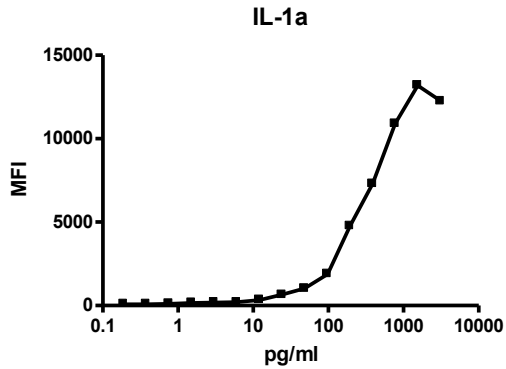


Mouse cytokine assays

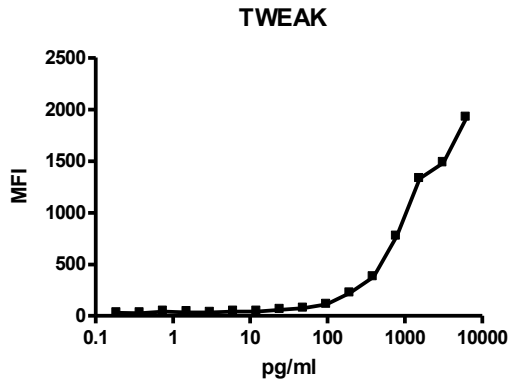
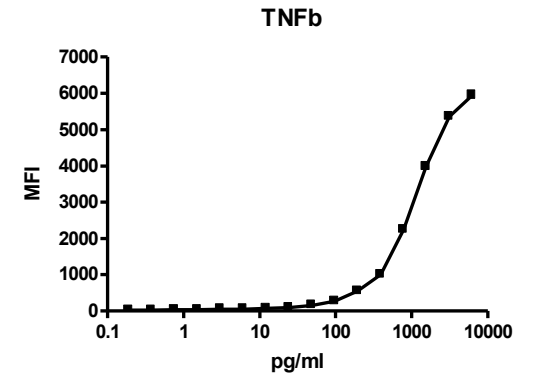
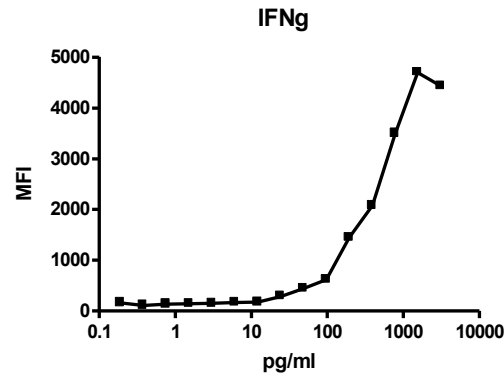
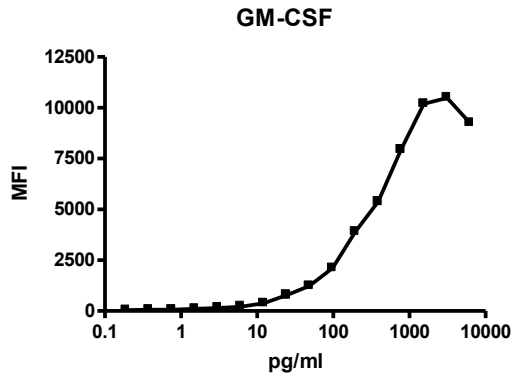
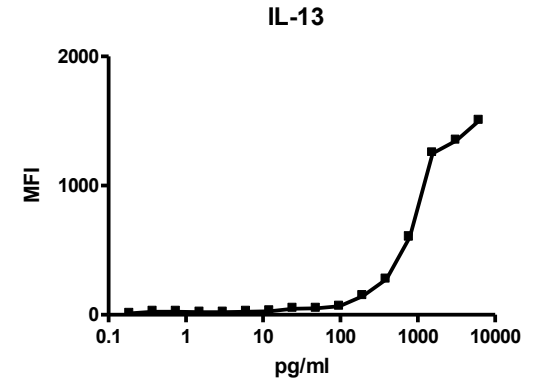
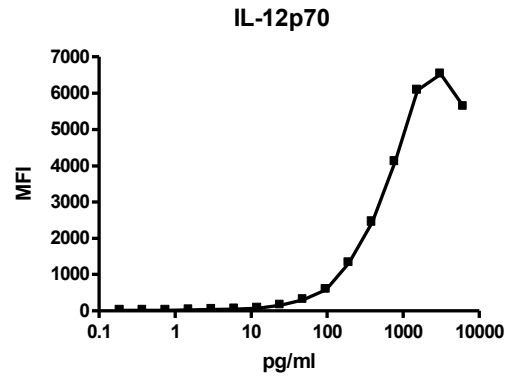
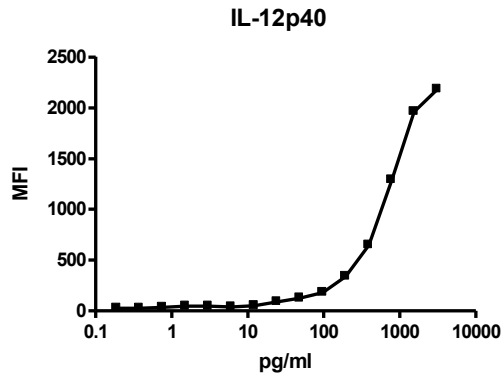
Detection of Naturally-Produced Mouse Cytokines



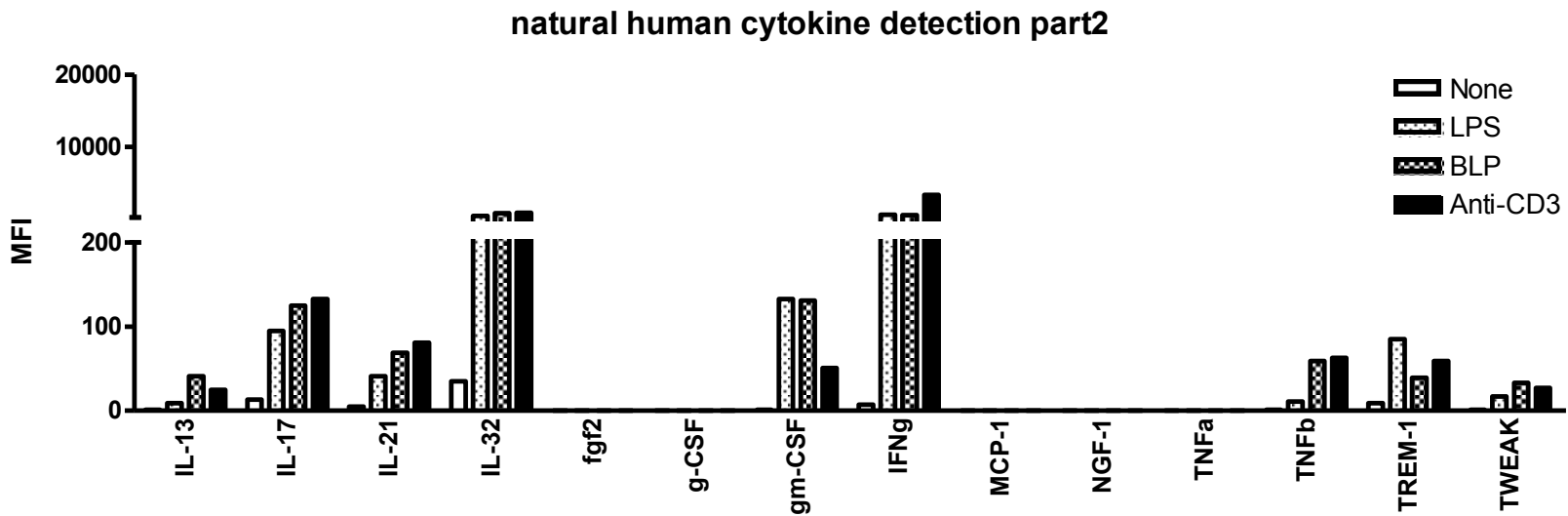
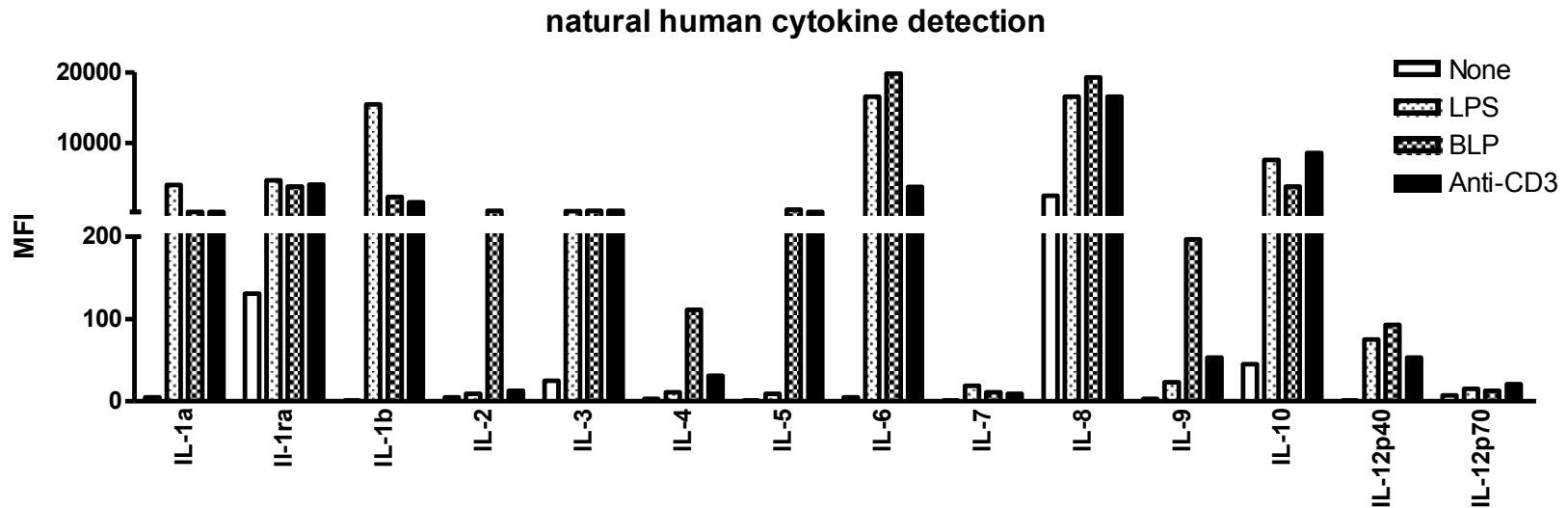
Human cytokine assay development and testing: Recombinant standards



More recombinant cytokine standard testing



Validate natural cytokine detection: Cytokine production by in vitro stimulated human PBMCs



Current list of validated mouse and human cytokine and other factor assays

<u>Mouse</u>		<u>Human</u>	
IL-1 α	IL-18	IL-1 α	IL-18
IL-1 β	IL-21	IL-1 β	IL-21
IL-2	IL-33	IL-1ra	IL-32
IL-4	IFN- α	IL-2	FGF-2
IL-5	IFN- γ	IL-3	G-CSF
IL-6	TNF α	IL-4	GM-CSF
IL-7	G-CSF	IL-5	IFN γ
IL-10	GM-CSF	IL-6	MCP-1
IL-12(p40)	M-CSF	IL-7	MIP-1 α
IL-12(p70)	FLT3L	IL-8	MIP-1 β
IL-23	SCF	IL-9	NGF
IL-13	MCP-1	IL-10	RANTES
IL-17		IL-12/23(p40)	TNF α
		IL-12(p70)	TNF β
		IL-13	TREM-1
		IL-17A	TWEAK
			sTNF RI
			sTNF RII

Collaborative consumption

- On campus Luminex assay collaborations with reagent replenishment support.
- A need for on-campus Luminex assay development and services – Harvard Catalyst or BWH-BRI

Dr. Andrew Lichtman, BWH and HMS – mouse models of T cell mediated inflammation

Dr. Charles Serhan, BWH and HMS – inflammation research in mice and man

Dr. Gerry Pier, BWH, Channing Lab – vaccine and infectious disease projects

Dr. Pedram Hamrah, MGH – eye inflammation and infections

Dr. Rachel Clark, BWH and HMS – human T cell biology in the skin

Dr. Arlene Sharpe, HMS – mouse models of basic T cell activation mechanisms

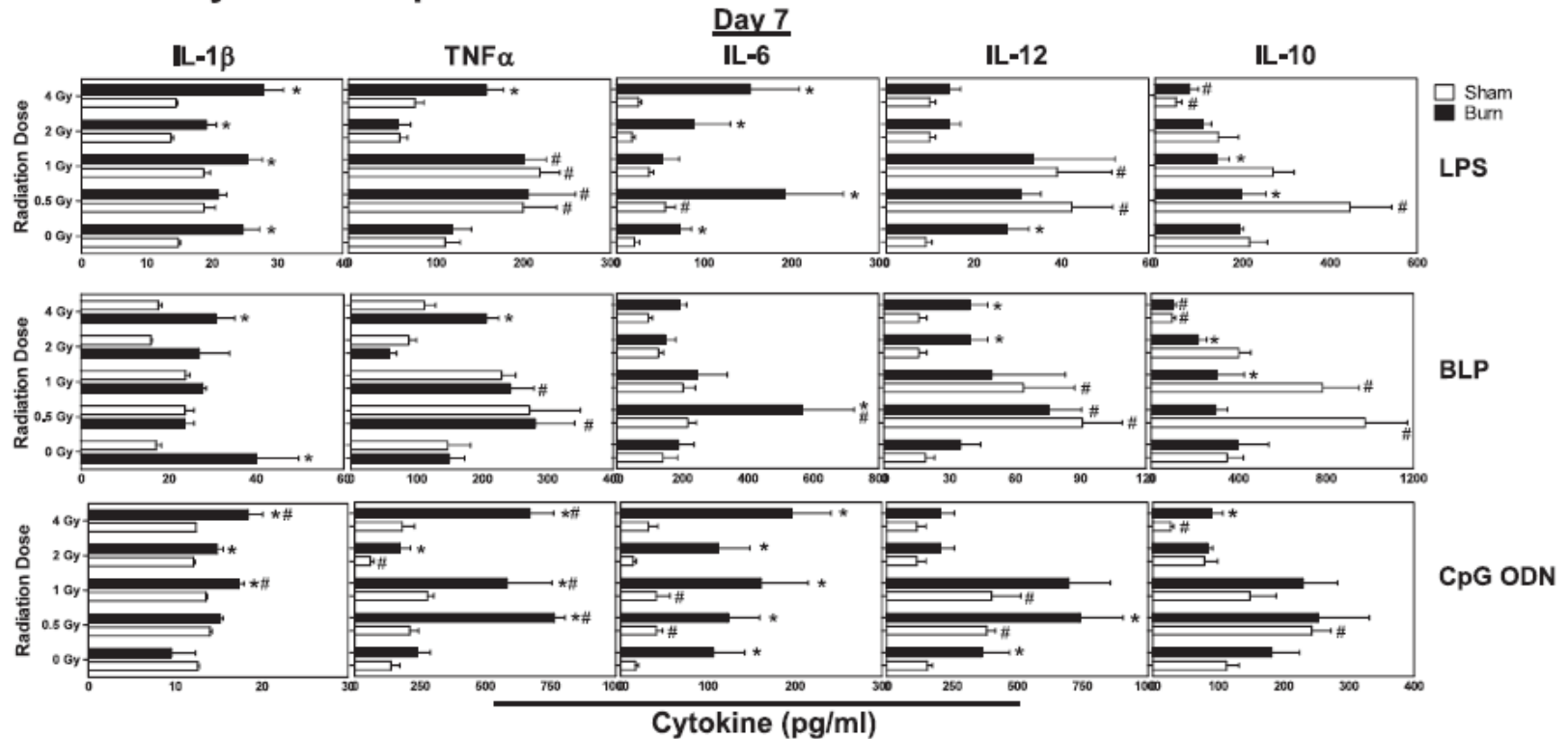
Dr. Mark Perrella, BWH and HMS – mouse stem cells and sepsis responses

Examples of how to use of Luminex multiplexing to increase research efficiency

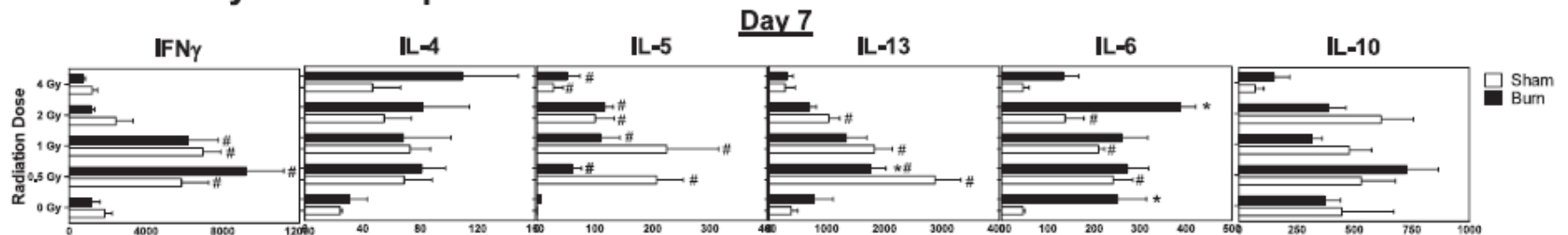
1. Micro-size experiments to gain more information from less cells
2. Test multiple stimulation conditions
3. Develop more efficient assays to save on reagent costs and sample cost – *e.g.* antibody-isotyping of vaccine assays by multiplex
4. Potential to assay any type of cell or tissue extract – *e.g.* human tears, organ extracts, exhaled breath condensate

Immune System Phenotyping of Radiation and Radiation Combined Injury in Outbred Mice

A. Innate cytokine responses



B. T cell cytokine responses



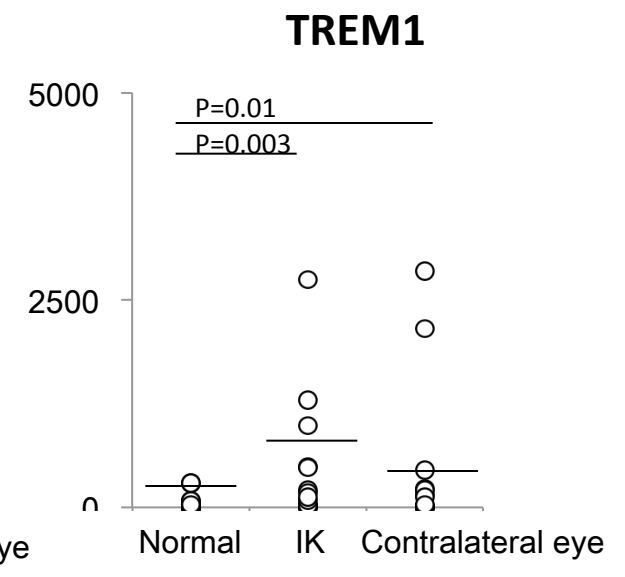
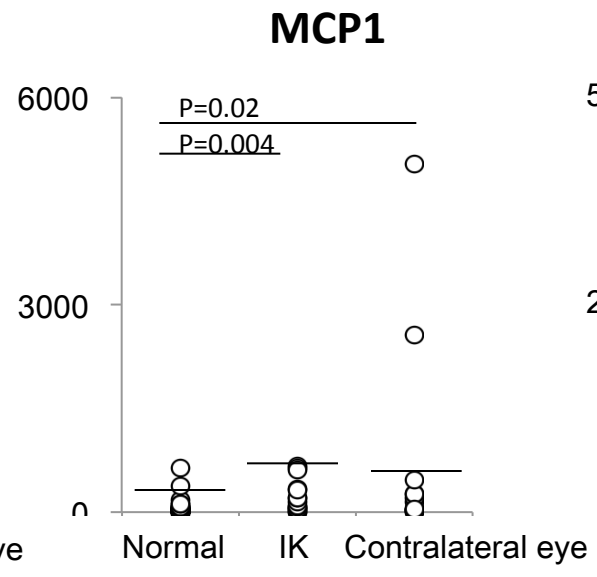
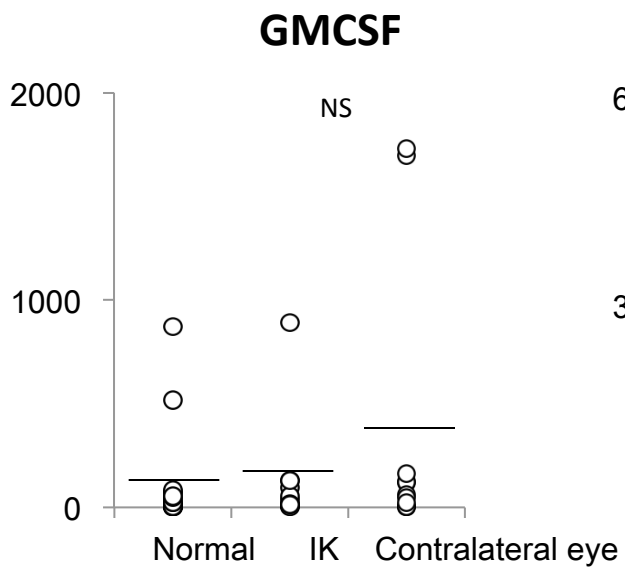
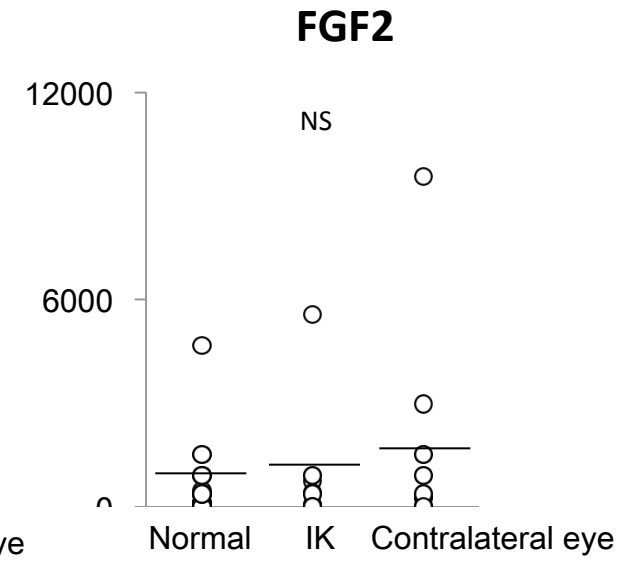
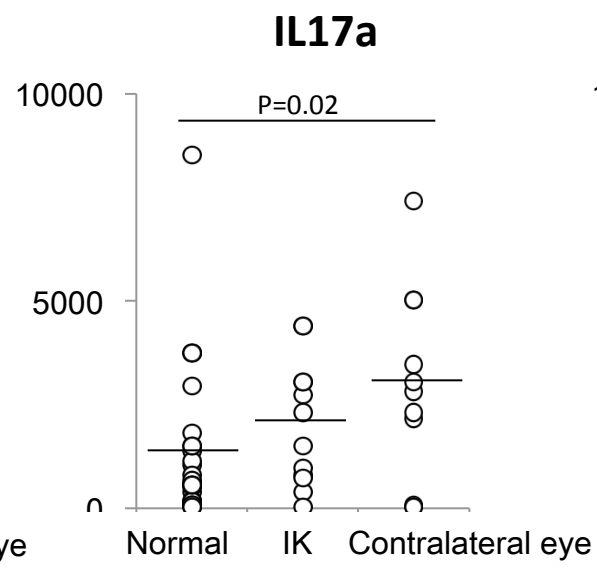
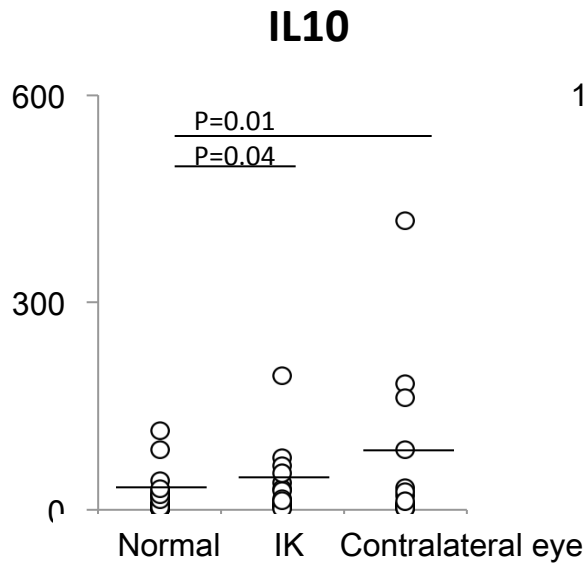
Cytokine Levels in Tears Are Correlated with the Corneal Nerve Density and Dendritic Cell Counts in Eyes with Infectious Keratitis

Takefumi Yamaguchi, Bernardo Cavalcanti, Pedram
Hamrah

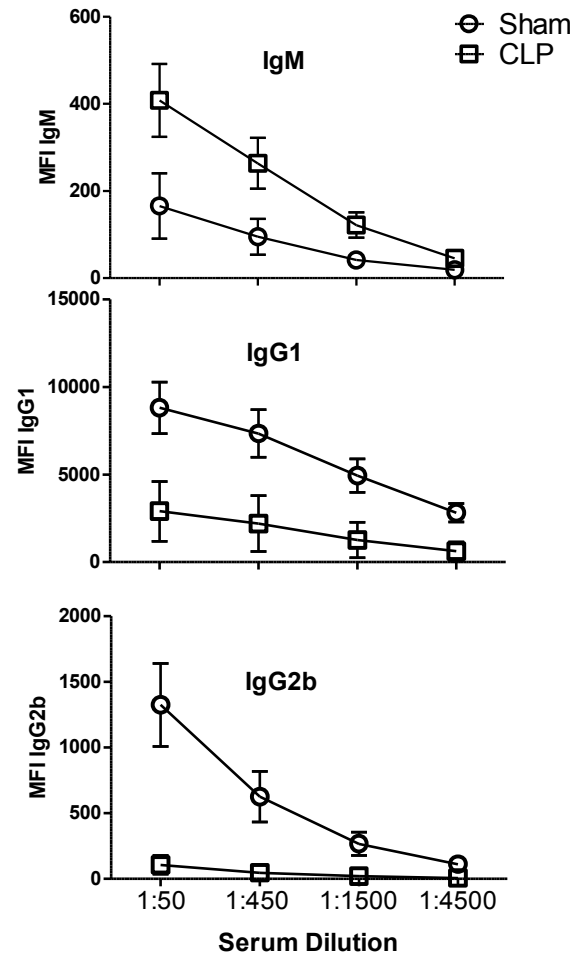
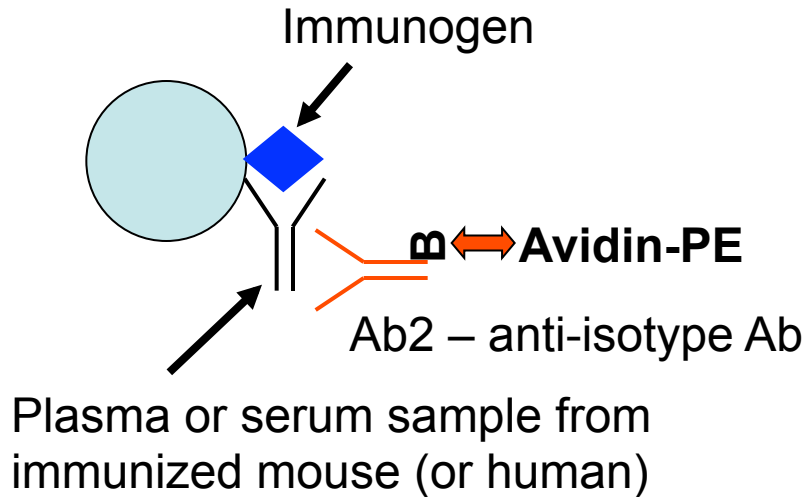
Mass Eye and Ear Infirmary

Shizu Ishikawa, Akinori Osuka, Kentaro Shimizu, James
Lederer

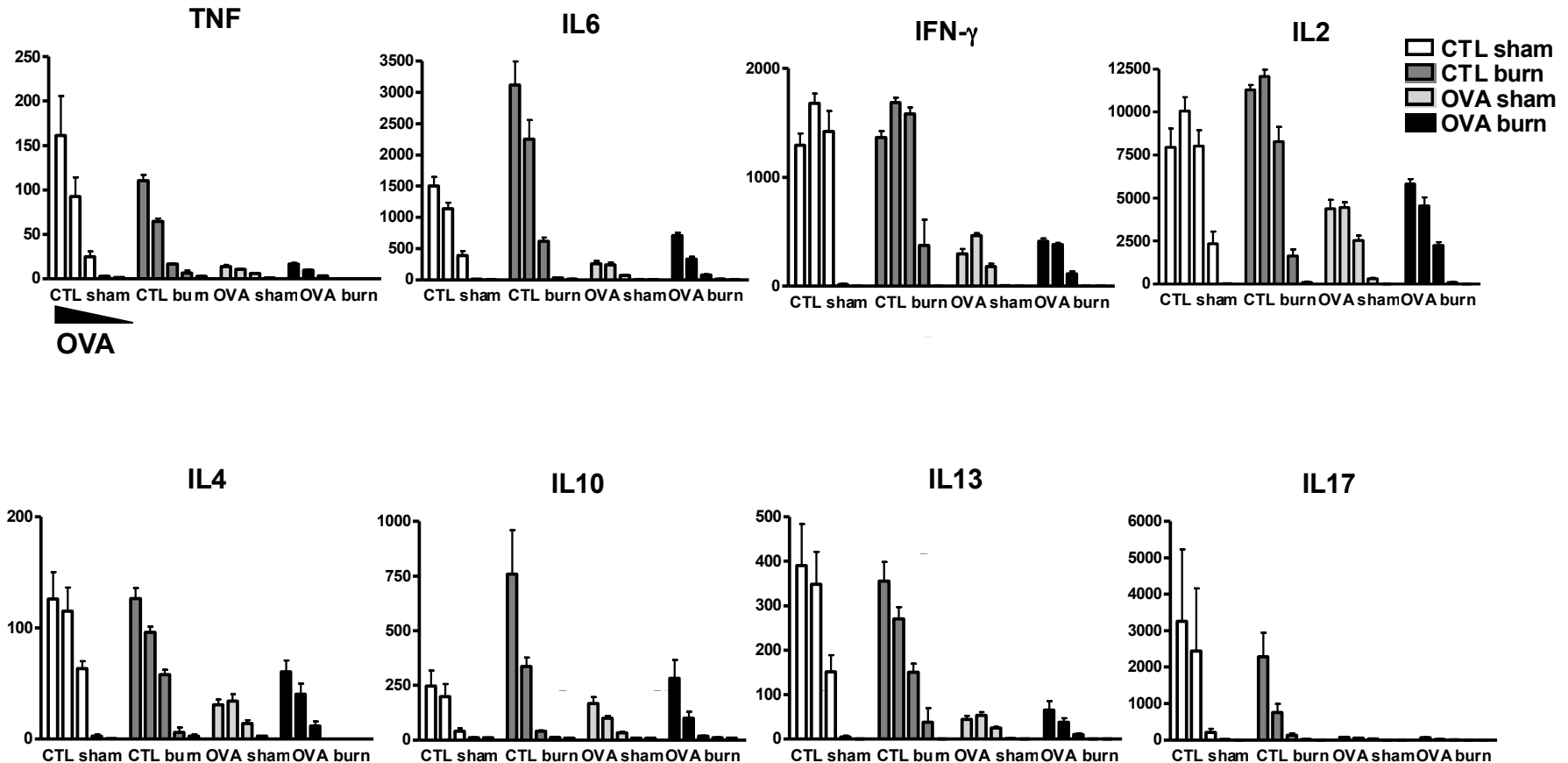
Department of Surgery, Brigham and Women's Hospital



Multiplexing antigen- or immunogen-specific antibody assays for vaccine testing



Ovalbumin-peptide induced cytokine production from TCR transgenic T cells, *in situ* detection with MagPix beads



Summary

1. Development of custom multiplex assay is feasible and cost effective through careful optimization and validation.
2. Provides an opportunity to develop new assays that are not commercially available.
3. Opens opportunity for collaboration.
4. Recent development of VeloceBio LLC as a collaborative biomarker development company and potential partnering with Cambridge Biomedical to offer assay development and Luminex assay service.