

# Multiplexed Biomarker Methods

*Platform and Assay considerations for appropriate analytical validation*



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# Multiplexed biomarker methods



## Scope

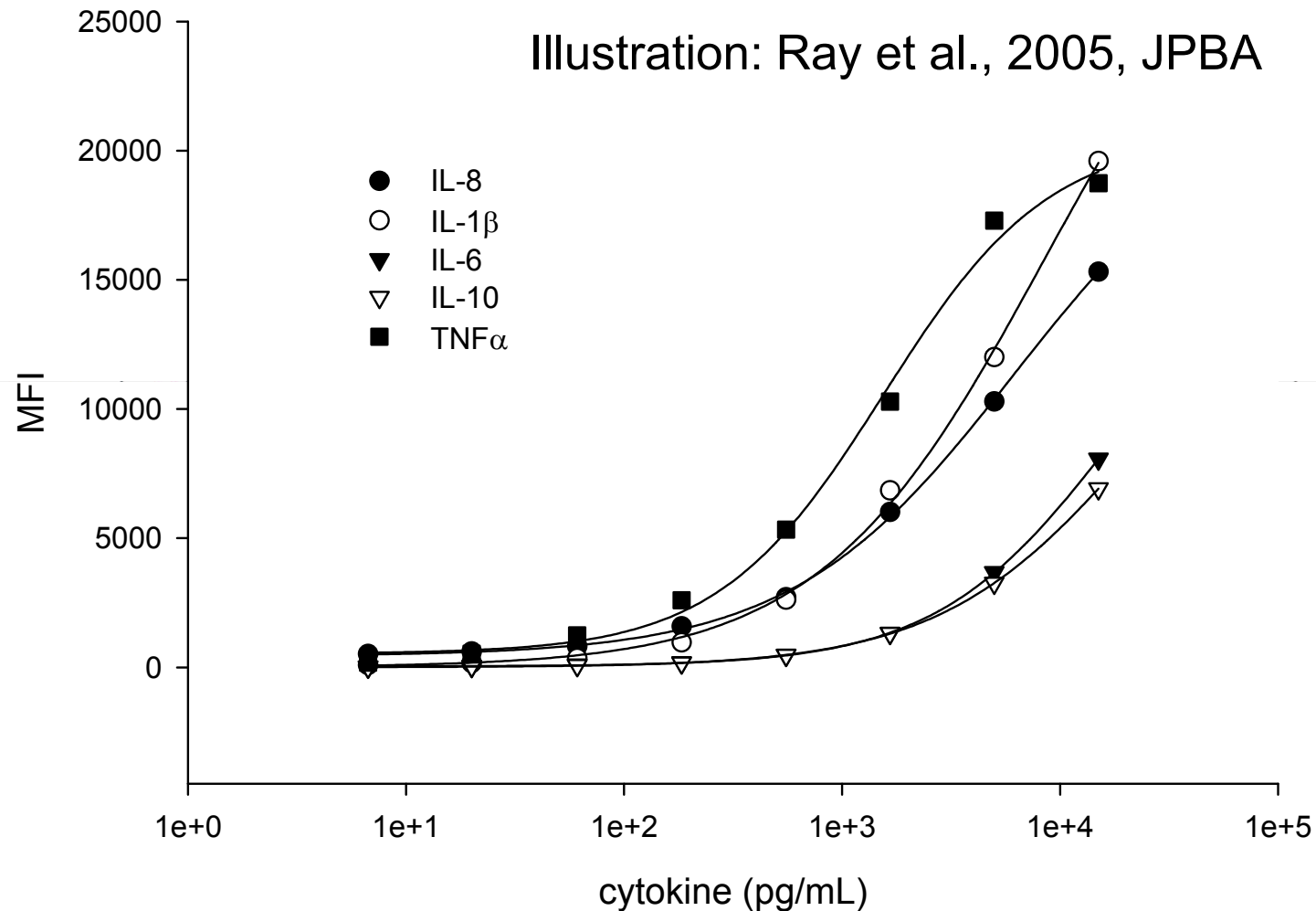
- Introduction
- Multiplexed Platform Technology
- Pro's & Con's of Multiplexing
- Method Validation – special considerations
- Summary

# Multiplexed biomarker methods



## Multiplexing – what do we mean?

Multiple assays simultaneously in a single reaction vessel (ie micro-well/tube)



# Multiplexed biomarker methods



## Technology : “Established”



Luminex®  
L100/200™



Luminex®  
FLEXMAP 3D®



Luminex®  
MAGPIX®



Mesoscale  
Discovery



Aushon  
Cira™plex



BD  
FACSARRAY

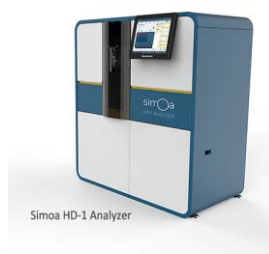
RANDOX  
EVIDENCE



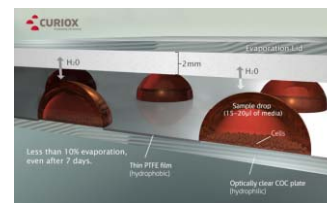
## New “Emerging”



Bioscale  
ViBE®



Quanterix  
SiMOA



Curiox  
DropArray™ Plate



Genalyte  
Maverick



BIOSIMS  
Dynaxi

# Multiplexed biomarker methods



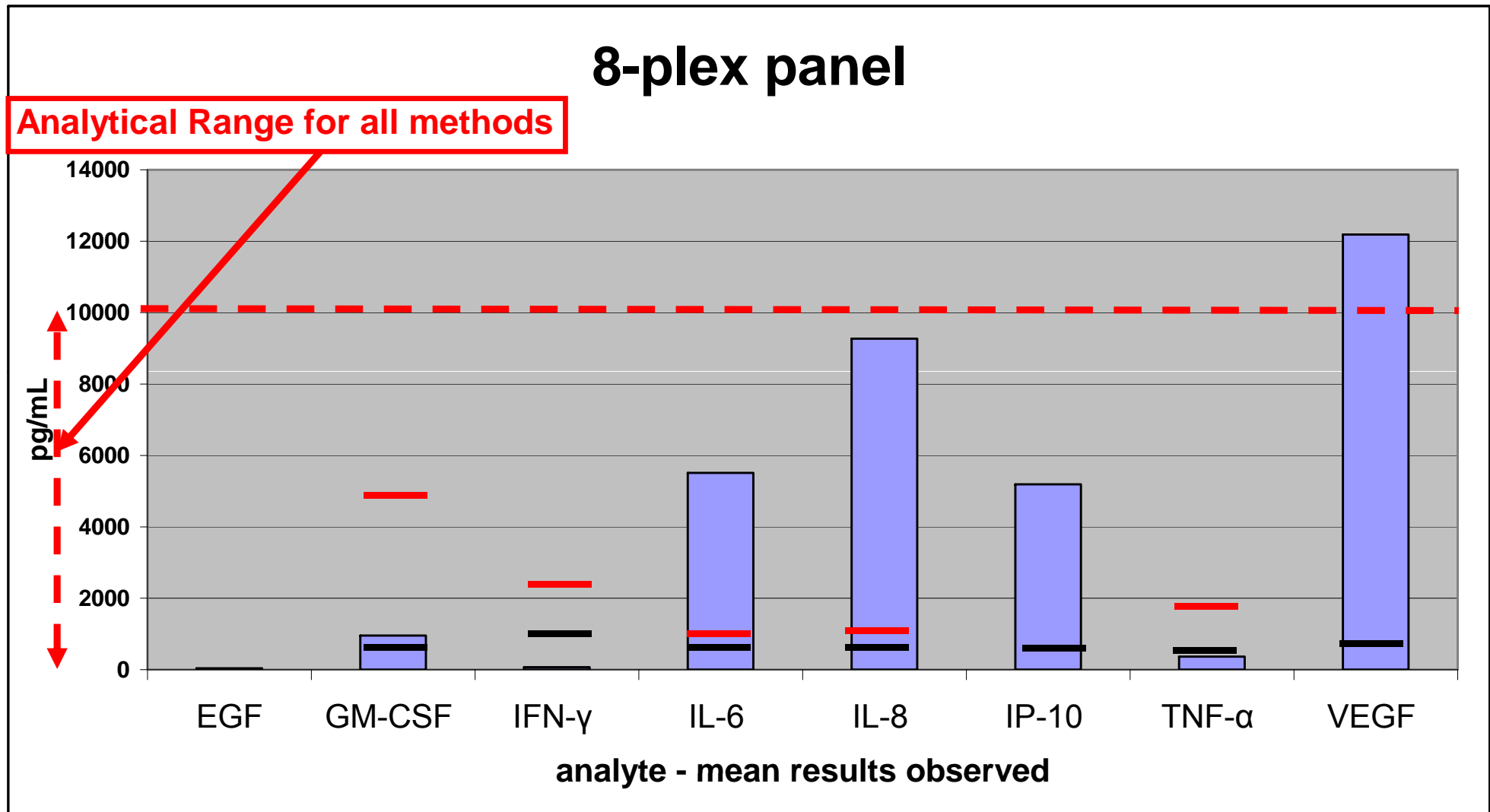
- Why Multiplex?
  - Sample volume restrictions
  - Potential for more analytical data
  - Cost

# Multiplexed biomarker methods



- Limitations? (Past / Present?)
  - In “qualitative” or research work – very good
  - For fully validated quantitative work – how many assays?
    - (may be platform dependent)
  - Cross-reactivity, Cross-talk, specificity, sensitivity, robustness
  - Often same analytical range for all methods
  - Degree of “Validation” by manufacturer
  - Availability of additional materials to enable method validations
  - Standardisation of Calibration Reference materials
  - Number and volume of QC’s
  - Lack of Biological Matrix data and QC’s
  - LOD vs LLOQ data

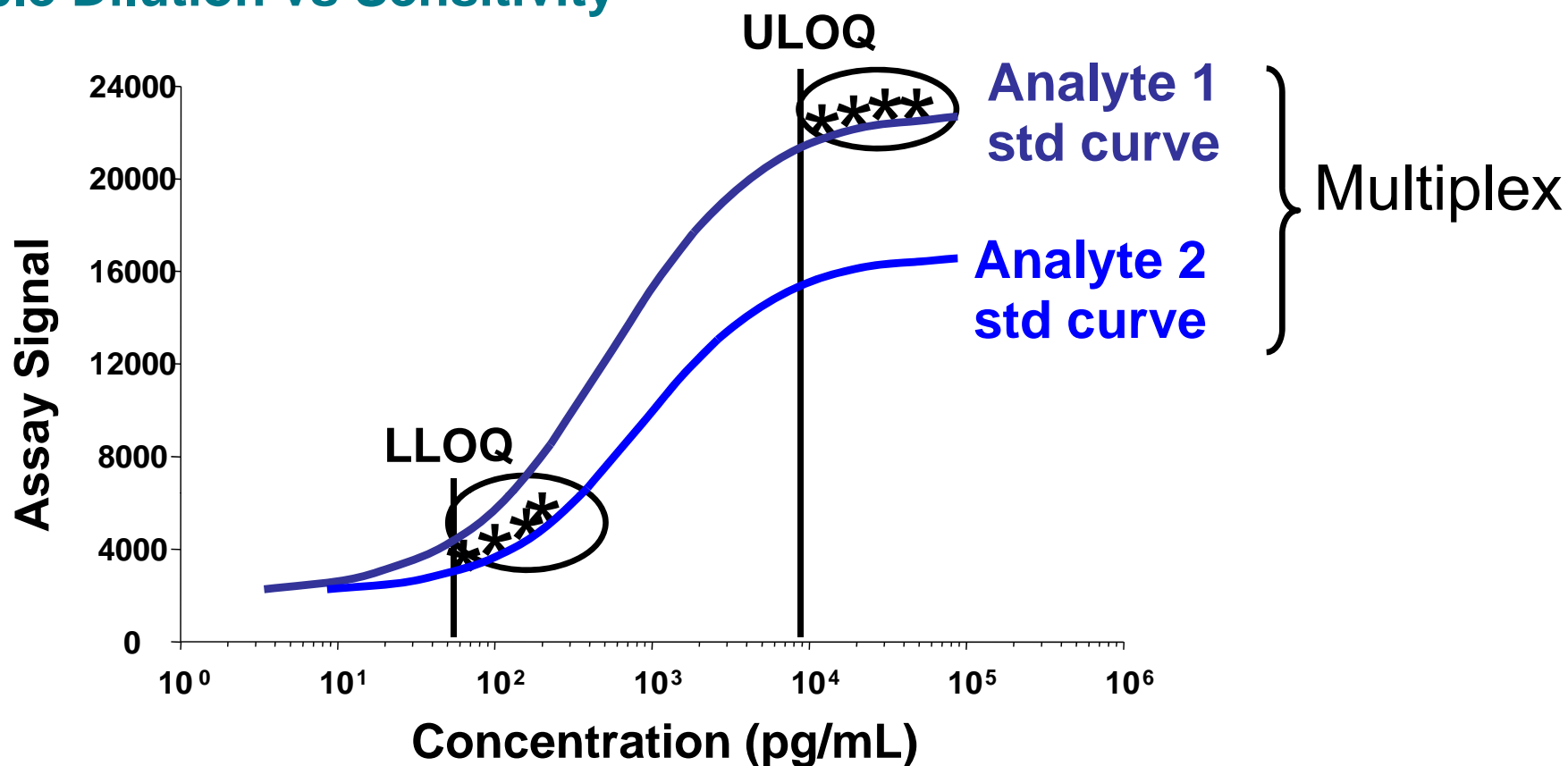
# Multiplexed biomarker methods



# Multiplexed biomarker methods



## Sample Dilution vs Sensitivity



***Dilution of these 4 samples is necessary to measure Analyte 1, but this will bring Analyte 2 levels below LLOQ***

***These analytes shouldn't be multiplexed if assay cannot be improved.***

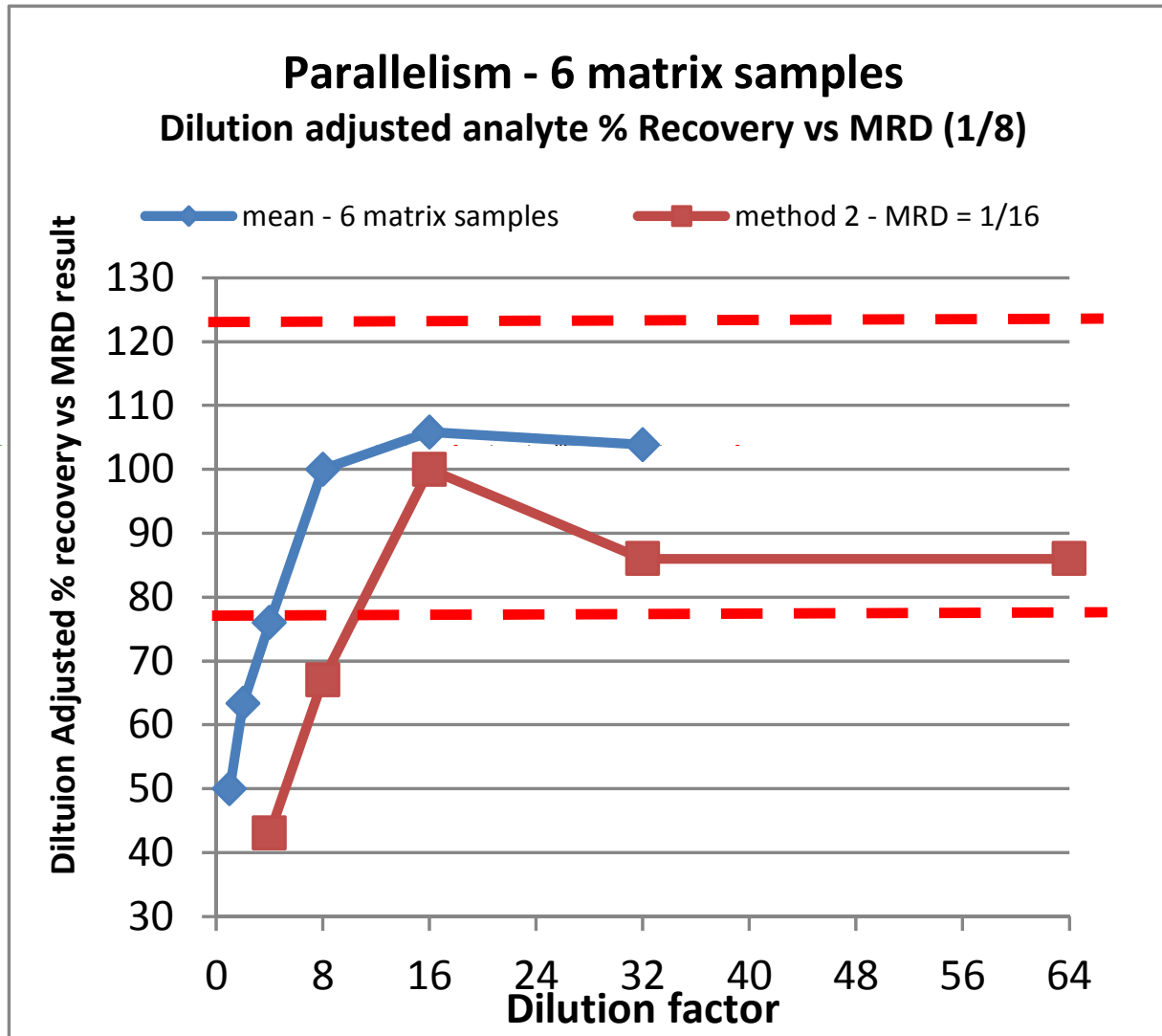


# Multiplexed biomarker methods



## Parallelism & MRD

### Two methods with Different MRD's

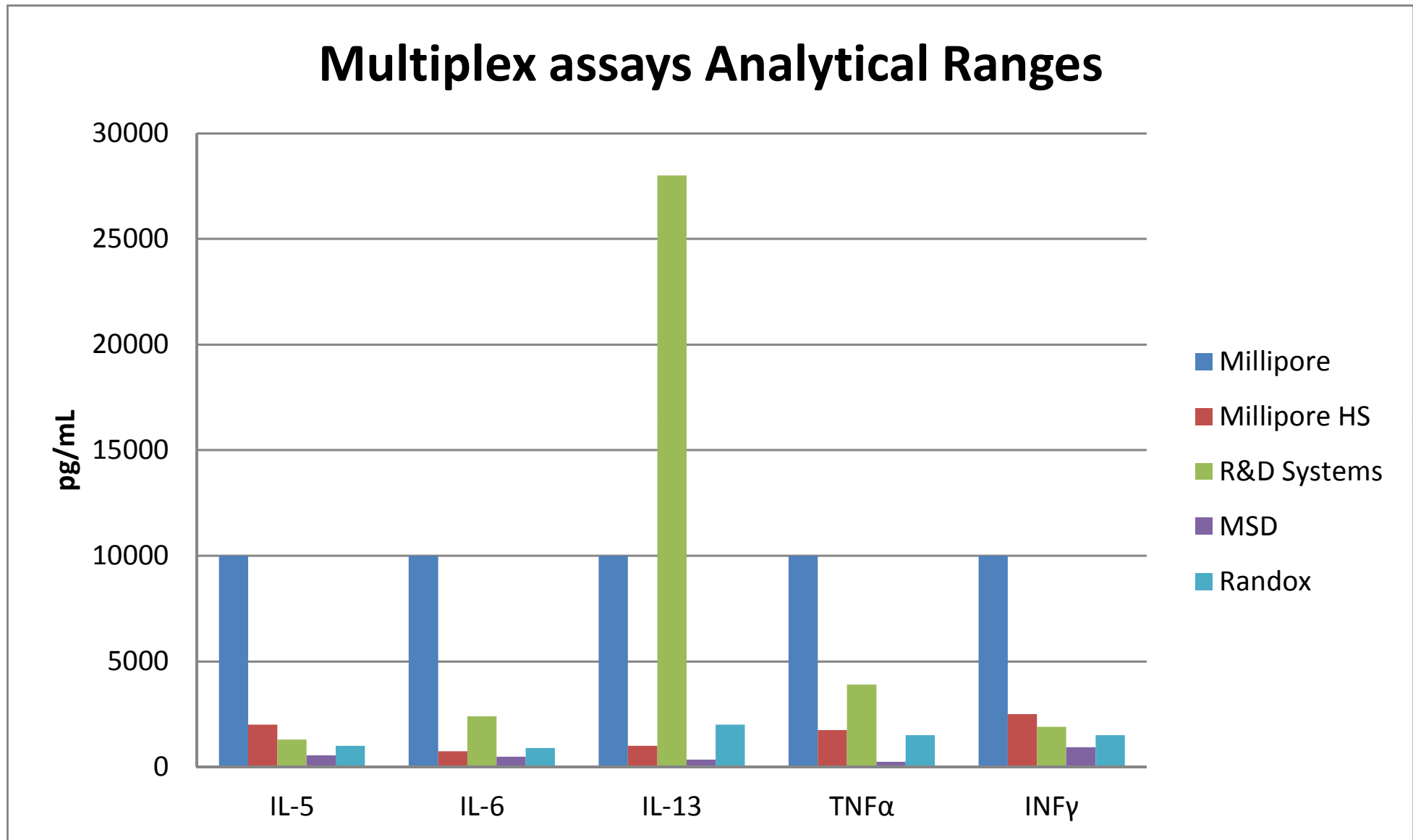


So – this is sample dilution requirements:

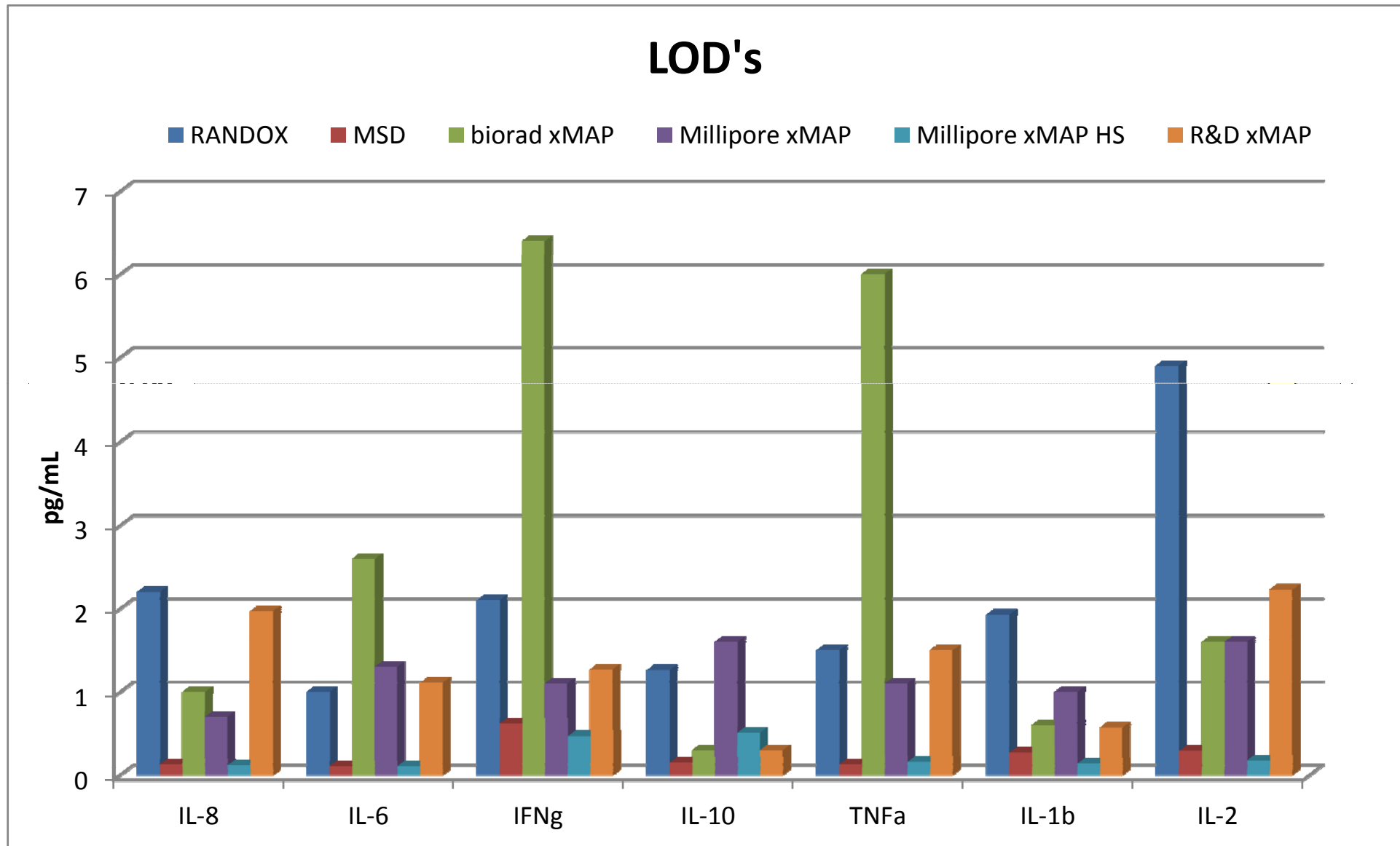
**NOT** due to analyte concentration but,

**because of Matrix effects** in the assays

# Multiplexed biomarker methods



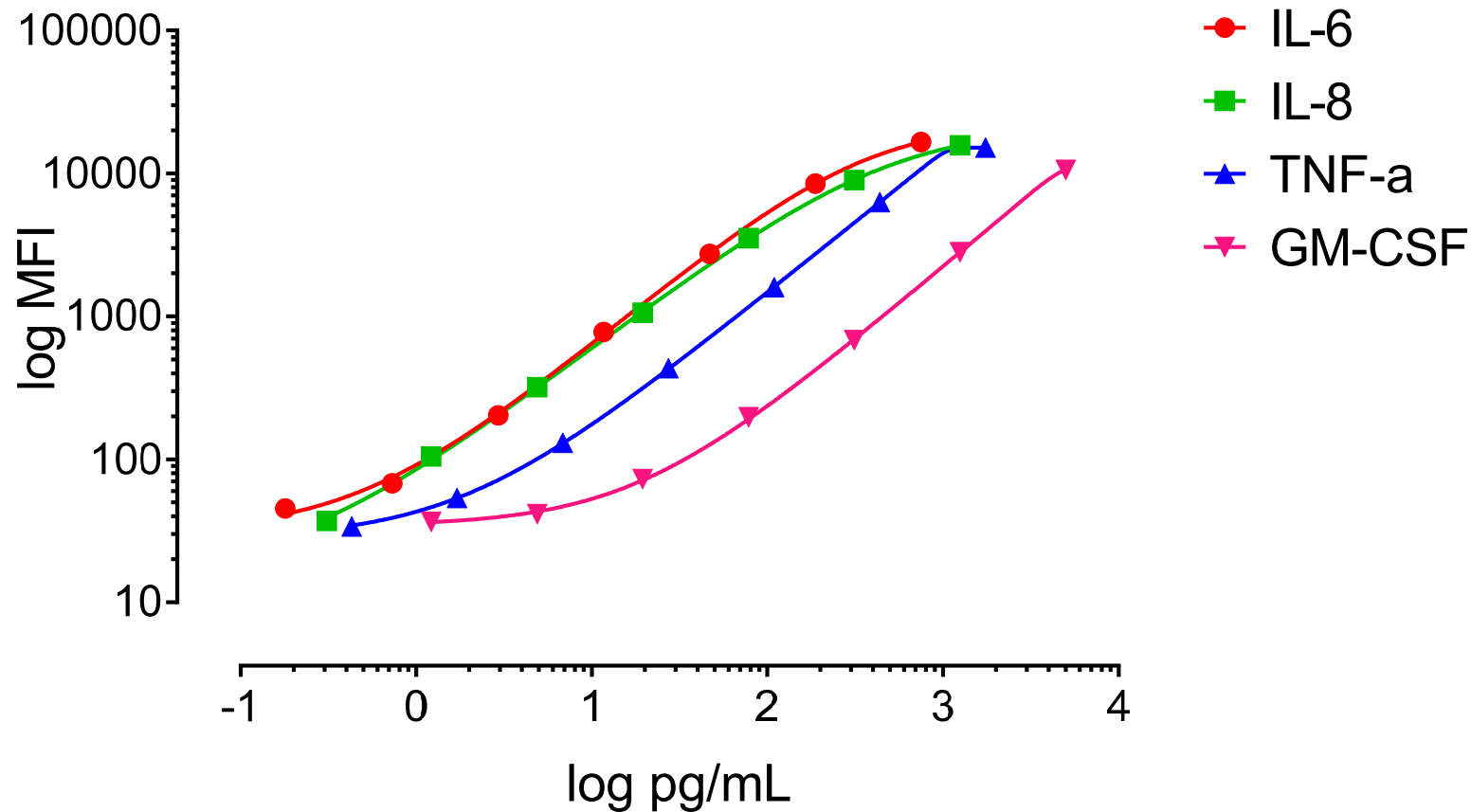
# Multiplexed biomarker methods



# Multiplexed Biomarker Methods



Human HS Cytokine kit MFIs ( $1/y^2$  weighted)



# Multiplexed biomarker methods

## VALIDATION - what do we test?

Total list of performance criteria includes.....

Accuracy..... ***will often be impossible to determine***

Precision

Sensitivity

Linearity

**Parallelism**



Mean different things to different scientists

Spiked Recovery

Dilutional Linearity

Specificity..... ***to T/A + metabolites AND other endogenous molecules***

Analytical Range

**Analyte Stability**..... ***completely different for biomarkers***

Standard and QC Stability

Reagent Stability

Whole Plate Imprecision

**So – what's special about Multiplexed assays?**

# Multiplexed biomarker methods



## VALIDATION –

*what additional precautions for Multiplexed Biomarker Methods?*

These are often the same as for single assays but bring more complexity when they are challenges for Multiplexed assays:-

- **Quantitation ranges and sensitivities**
  - ?matrix specific - check for hook effect (prozone)
  - ?sample dilutions – (ranges and sensitivities)
  - Appropriate for all biomarkers in panel and the clinical utility in the study?
- **Performance in sample matrix vs surrogate matrix**
  - always worse
- **Cross-talk?** (well-to-well or spot-to-spot “carryover”, “bleed-over”, or “leaching”)
  - Requires screening by spiking in one analyte at very high concentration
- **Selectivity?**
  - Check for each project – antibodies (RF,HAMA), TA, Mets., Concomitants, similar molecules
- **LOT-to-LOT variability**

# Multiplexed biomarker methods



## VALIDATION –

*what additional precautions for Multiplexed Biomarker Methods?*

- **Matrix Parallelism:**
  - Difficulty in obtaining matrix with single analytes of interest – much more difficult for multiple Biomarkers
  - Multiple samples will be required
  - May often be impossible to cover all analytes therefore.....
- **Spiked matrix dilutional linearity**
  - Likely to be required in many cases
  - Requires obtaining additional and multiple calibration reference materials
  - Sometimes not available from kit manufacturer
- **Biomarker Stability**
  - Collection/storage etc will be dictated by the least stable.
  - However, availability of suitable matrix is the same problem here as for parallelism
- **Precision & Accuracy**
  - Each assay will potentially perform differently, therefore different acceptance criteria for each is appropriate – drive by clinical utility & statistical significance

# Multiplexed biomarker methods

## SUMMARY

- Multiplex assays can be powerful tools
- Their use (and the platforms to conduct them on) has grown in last 10 years
- These assays bring an increased complexity in terms of validation
- Assay robustness and degree of “validation” work conducted by the manufacturers have improved but can still vary a lot
- Commonly, the most difficult analytical challenges are:-
  - Analytical ranges
  - Sensitivity of each assay
  - Performance with different biological matrices

**Finally – get the data interpretation right! (statistical analysis plan)**



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## *White Paper*

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### **Recommendations for Use and Fit-for-Purpose Validation of Biomarker Multiplex Ligand Binding Assays in Drug Development**

**Darshana Jani,<sup>1,2,3</sup> John Allinson,<sup>2</sup> Flora Berisha,<sup>3</sup> Kyra J. Cowan,<sup>4</sup> Viswanath Devanarayan,<sup>5</sup> Carol Gleason,<sup>6</sup> Andreas Jeromin,<sup>7</sup> Steve Keller,<sup>8</sup> Masood U. Khan,<sup>9</sup> Bill Nowatzke,<sup>10</sup> Paul Rhyne,<sup>11</sup> and Laurie Stephen<sup>12</sup>**

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**Abstract.** Multiplex ligand binding assays (LBAs) are increasingly being used to support many stages of drug development. The complexity of multiplex assays creates many unique challenges in comparison to single-plexed assays leading to various adjustments for validation and potentially during sample analysis to accommodate all of the analytes being measured. This often requires a compromise in decision making with respect to choosing final assay conditions and acceptance criteria of some key assay parameters, depending on the intended use of the assay. The critical parameters that are impacted due to the added challenges associated with multiplexing include the minimum required dilution (MRD), quality control samples that span the range of all analytes being measured, quantitative ranges which can be compromised for certain targets, achieving parallelism for all analytes of interest, cross-talk across assays, freeze-thaw stability across analytes, among many others. Thus, these challenges also increase the complexity of validating the performance of the assay for its intended use. This paper describes the challenges encountered with multiplex LBAs, discusses the underlying causes, and provides solutions to help overcome these challenges. Finally, we provide recommendations on how to perform a fit-for-purpose-based validation, emphasizing issues that are unique to multiplex kit assays.

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**KEY WORDS:** biomarker; diagnostics; ligand binding assay; multiplex; validation.

# Thank you for your attention!



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# Multiplexed biomarker methods



## Results Data Interpretation.....

- Different methods perform differently
- Method specific acceptance criteria is appropriate...
  - drive by clinical utility & statistical significance

***What impact can method performance have on data interpretation?***

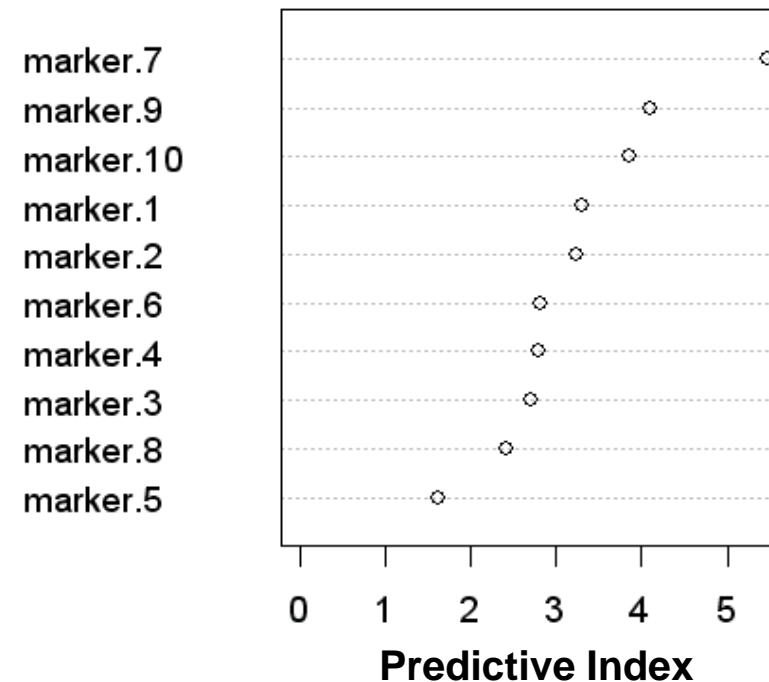
*....2 case studies*

# Impact of Analytical/Biological Variability on biomarker evaluations



	Effect
Marker 1	56% inc.
Marker 2	24% dec.
Marker 3	80% inc.
Marker 4	45% inc.
Marker 5	53% inc.
Marker 6	31% inc.
Marker 7	19% inc.
Marker 8	12% inc.
Marker 9	13% inc.
Marker 10	47% inc.

Ranking of Markers for Predicting Clinical Outcome

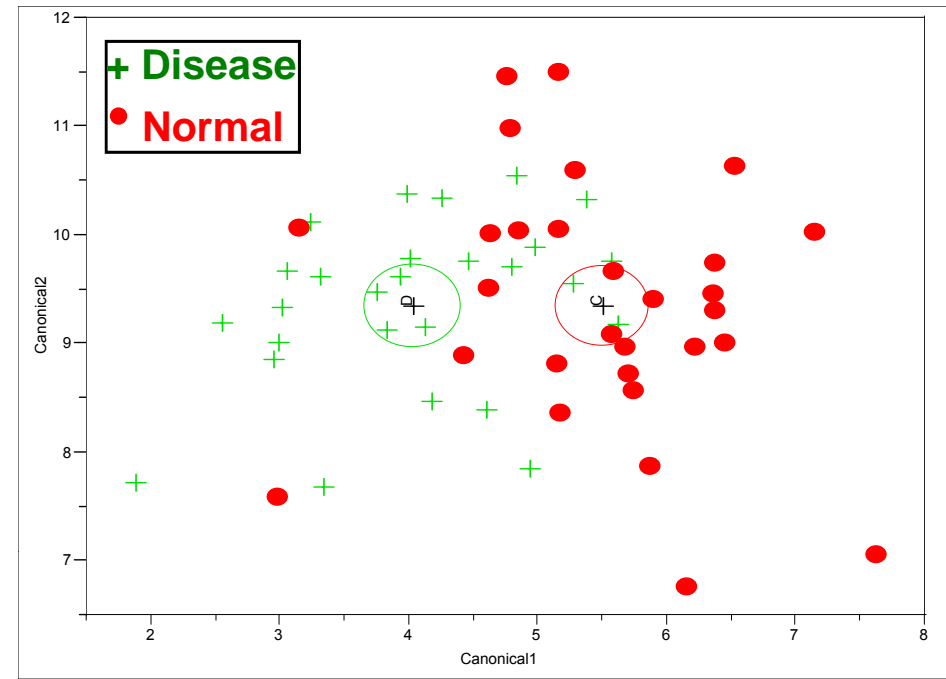
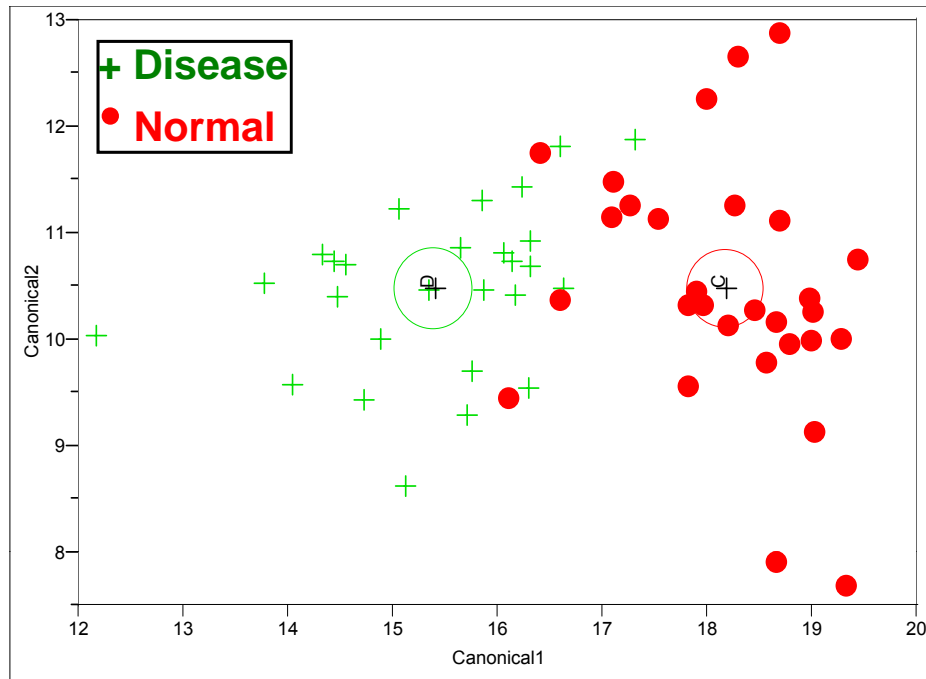


Random Forests Analysis

- Markers 7 and 9 have < 20% effect, but most predictive
- Markers 3 and 5 have > 50% effect, but least predictive

**Important markers may be left out due to assay/biological variability!**

# Impact of Analytical/Biological Variability on Predictive Power



Discriminant Analysis

- Marker X with 15% CV is a key predictor from the multi-analyte panel.
- Prediction Accuracy from cross-validation is 94%

- Same Marker X in the panel from another lab has 35% CV
- Prediction Accuracy from cross-validation drops to 75%

**Biomarker Evaluation & Reproducibility affected by analytical issues!**