

Luminex Assay Validation

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Luminex Technology

- Luminex technology is a bead-based sandwich ELISA based on the principles of Flow Cytometry.
- It uses a single 5.6 micron size microspheres that are dyed with red and infrared dyes in different combinations to create 500 uniquely colored microspheres.

Each bead region is conjugated to a specific target analyte

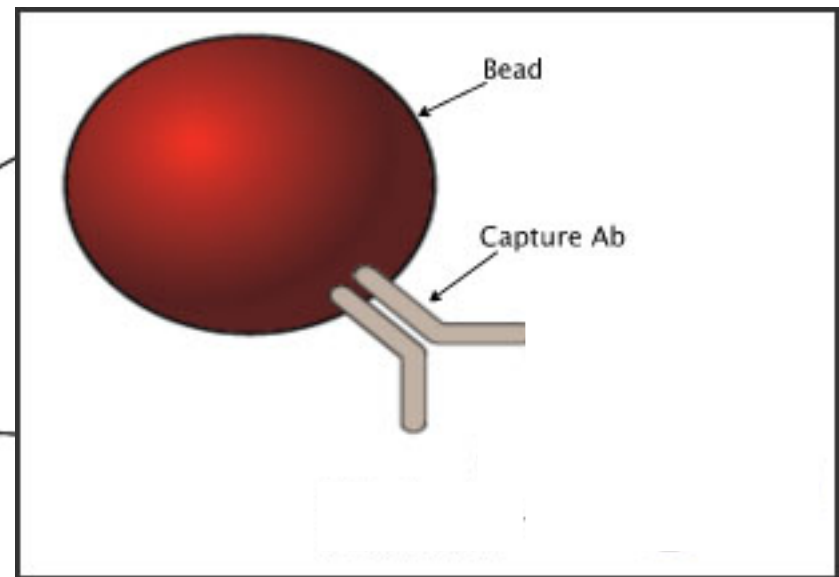
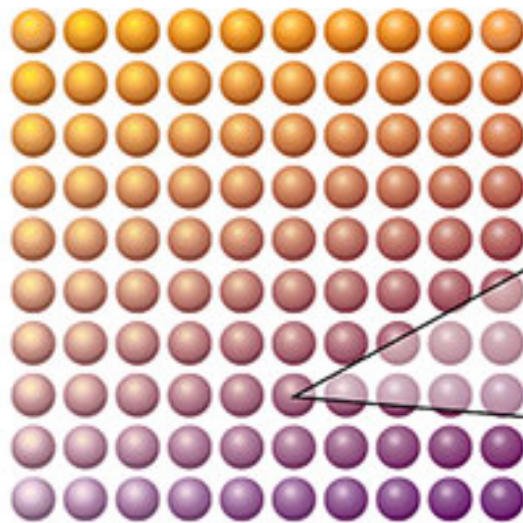
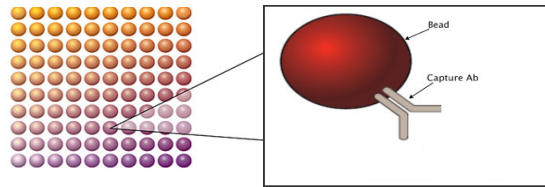
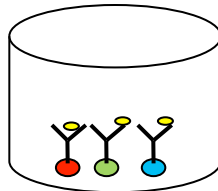


Image Adopted from Bio-Rad

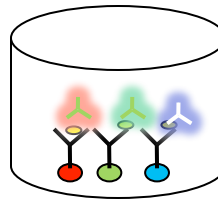
Step 1



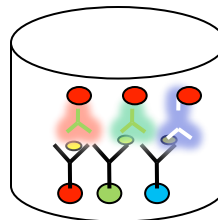
Step 2



Step 3



Step 4



Choose analyte-specific beads and design your assay.



Incubate samples with the beads



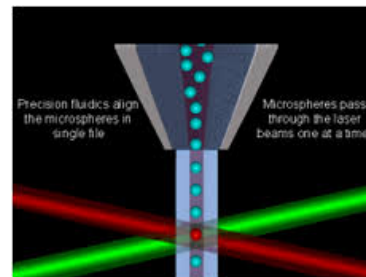
Add Biotinylated Detection Antibody



Add PE-conjugated Streptavidin



Acquire and Analyze Data



Advantages and Applications of Luminex Technology

Advantages:

- Low sample volumes
- High specificity
- High sensitivity
- Cost effective

Applications:

- Clinical Diagnostic
- Life Science and Clinical Research
- Bio surveillance

Role of Cambridge Biomedical In Assay Validation

- Cambridge Biomedical is a Contract Research Organization which provides Bioanalytical Services to Life Science Companies.
- We are a recognized CLIA/CAP/GLP/GCLP compliant Clinical Reference Laboratory.
- Offer services to support clinical trials and post market studies under guidance provided by the FDA.

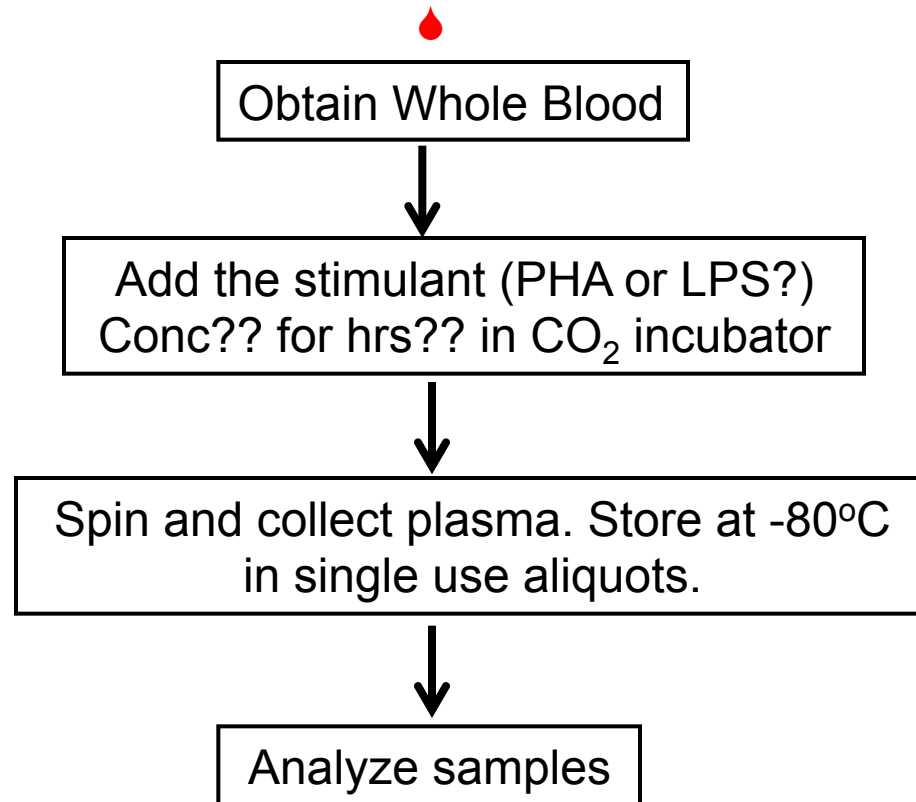
Assay Validation

- **Reagent and Kits:** Ready to use kits from Vendors (Affymetrix, Millipore, Bio-Rad etc) or in-house developed kits.
- **Instrument and Software:** Use of 21 CFR part 11 compliant software.
- **Optimization:** Determine matrix effect, construct precision profile.
- **Validation:** Establish Sensitivity, Precision, Robustness, Recovery, Accuracy, Reference range, Interfering substances, Dilution and Stability.
- **Implementation:** Use in Clinical Sample testing.

Luminex Assay Validation: The Challenges and Recommendations

- **Positive Samples:** Screen donors that are positive for *few* biomarkers and cytokines, if not all.
- **Recommendation:** If validating inflammatory cytokines then use standard methods to obtain positive samples.

Whole Blood Stimulation Protocol



Measurement of Cytokine Production Using Whole Blood. Thurm CW and Halsey JF. Current Protocols in Immunology (2005) 7.18B.1-7.18B.12

- **Lower Limit of Quantitation:** According to FDA guidance, the lowest standard on the calibration curve should be accepted as the LLOQ if the lowest standard is *at least 5 times* the response as compared to the blank response.
- **Observation:** FDA recommended “LLOQ be at least 5 times as compared to the blank” does not always apply to Luminex assays, where the LLOQ is very often only *1.5 to 3 times the blank value (or LOD)*.

- **Accuracy:** Determined by spiking a *known amounts of the analyte* into the sample (plasma/serum) and if the recovery is between 70-130% of the spiked concentration, the result is considered accurate.
- Both Plasma and Serum are complex in nature and consist of components other than the analyte of interest. These components often cause a Matrix Effect which hinders the results quantitatively thus affecting the accuracy of the analyte.

Recommendation:

- 1) During the optimization step perform dilution study to detect any matrix effect and use optimal sample dilution during the Validation to obtain accurate results.
- 2) If accuracy fails, run a baseline sample to evaluate the differences between the pre- and post- treatment samples.

- **Precision:** FDA describes Precision as the *closeness* of individual measures of an analyte when the procedure is applied repeatedly.
- The precision of an assay should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.
- **Observation:** Certain analytes consistently fall outside the FDA recommended precision range.

Table 1: Inter Assay Precision of Cytokine 1 and 2

Experiment #	Cytokine 1 (pg/mL)	Cytokine 2 (pg/mL)
Experiment 1	3967.51	4.66
Experiment 2	5200.00	6.12
Experiment 3	3528.52	5.00
Experiment 4	4969.46	5.68
Experiment 5	3141.00	6.88
Experiment 6	4925.21	5.16
%CV	20.05	14.66

- **Recommendation:** Modify the Precision acceptance criteria for Luminex assays to 25% CV except at LLOQ where the precision is 30% CV. Interpret results based on the known precision of the assay determined during validation.

- **Stability:** An analyte is considered stable if the difference between the baseline sample and the stored sample is within $\pm 30\%$ (Relative Error).
 - Stability is dependent of several conditions such as: Storage, collection, transportation, freeze thaw cycles, duration of the study, etc.
- **Observation:** certain cytokines “wobble” from being stable to unstable to stable state.

Table 2: Stability of Cytokine 1 and Cytokine 2 at -80°C

Cytokine	Time Point	Concentration (pg/mL)	%RE
Cytokine1	Baseline	3967.51	N/A
	1 Month	5200.00	31.06
	3 Month	3828.52	-3.50
Cytokine2	Baseline	4.66	N/A
	1 Month	6.12	31.33
	3 Month	5.00	7.30

Table 3: Inter Assay precision of Cytokine 1 and Cytokine 2

Cytokine	Time Point	Concentration (pg/mL)	%CV
Cytokine1	Baseline	3967.51	17.43
	1 Month	5200.00	
	3 Month	3828.52	
Cytokine2	Baseline	4.66	14.52
	1 Month	6.12	
	3 Month	5.00	

- **Recommendation:** The precision of the assay should be considered when interpreting stability results. Use both %RE as well as %CV for Stability studies unless there is trending of the data that shows that the sample is failing stability.

Conclusion

- Multiplexing technology offers great advantages over single-plex assays.
- The advantages include include high throughput, lower cost, and utmost importance, use of smaller sample volume.
- In spite of their advantages over single-plex assays we still need to address challenges that will help us use these new technologies in a regulated environment.
- These recommendations have been accepted by our clients in multiple studies for using the Luminex platform in sample analysis.

ACKNOWLEDGEMENTS

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Thank You!

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