Development, Optimization and Validation of Luminex based cytokine assays

Introduction

Luminex is a fluorescence covalent microbead immunosorbent assay, in which up to 500 analytes can be multiplexed in a single well. The advantages of Multiplexed bead-based immunoassays, which make it an attractive option to other bioassays, such as MSDs and ELISAs, are speed and high-throughput, small sample size, accuracy and reproducibility, versatility, flexibility, and powerful software support via the xPONENT® software.

The evolution of a robust Luminex assay typically follows the following flow-chart:
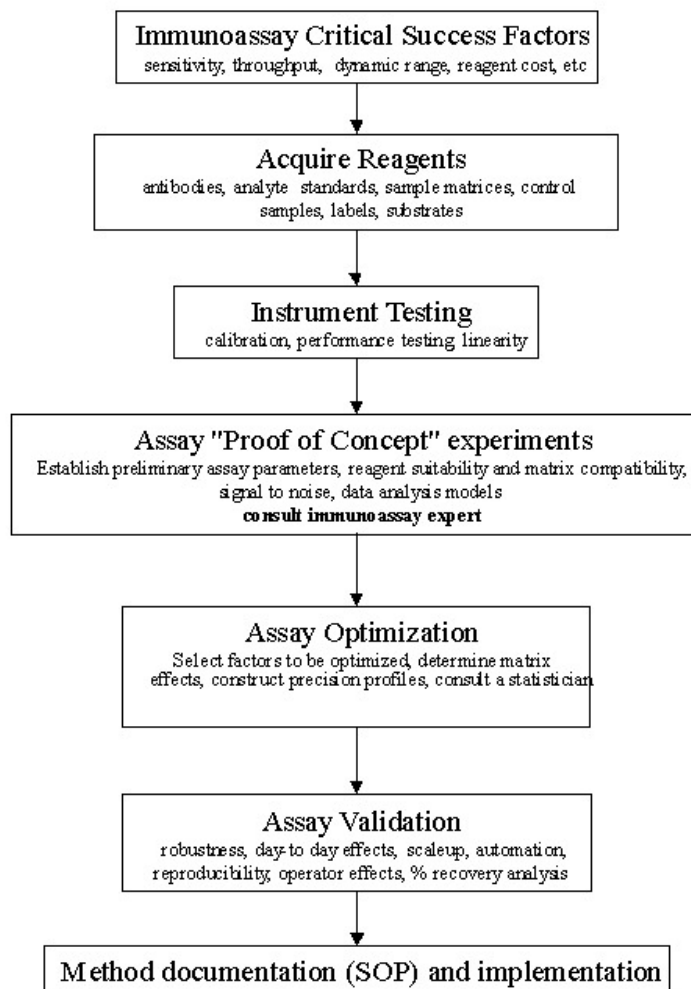
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Development

The first step to any immunoassay development, is to establish the assay critical success factors that will decide whether a particular immunoassay would be feasible. This would include evaluating factors such as the desired sensitivity of the assay, the preferred dynamic range, and even practical factors such as reagent costs, all of which will determine the future success of the assay and project.

De novo development of Luminex kit

For development of *de novo* Luminex assays, a good way to start is with researching and acquiring:

- Relevant primary and detection antibodies;
- xMAP antibody coupling kits (ABC kits, Luminex Corporation) to immobilize the primary capture antibodies to magnetic or polystyrene xMAP microspheres;
- Recombinant protein that will be used for standards and/or controls; the purity of the reference standard used to prepare standards can affect study data. For this reason, an authenticated analytical reference standard of known identity and purity should be used to prepare solutions of known concentrations (from Guidance for Industry: Bioanalytical Method Validation, FDA);
- Assay buffers (For dilution of detection antibody, secondary antibodies, and streptavidin conjugates),
- Assay diluents (Serum-based diluent for dilution of standards and samples); Streptavidin-Phycoerythrin Conjugate; and
- Appropriate matrix used for development and assay validation, such as serum, plasma, tissue lysates, or cell culture supernatants.

Once the *de novo* kit has been prepared, it has to be tested to see if the antibody coupled microspheres function, as expected. It is also recommended to perform preliminary 'proof of concept' experiments:

- To establish basic assay parameters, such as standard curve dynamic range and standard curve dilution factor;
- To test for suitability of the reagents for the matrix in question, and for the targets that are to be assayed in the matrix (matrix and reagent suitability);
- To establish the signal to noise ratio, which will further aid in determining the lowest standard in the standard curve, and thus the LLOQ of the assay; the lowest standard should be at least five times the response of the assay blank, and
- To decide on the data analysis model that will be used during validation, and in its final form, to assay pre-clinical and clinical samples, as an example.

Luminex assay development of premixed xMAP microsphere-based kits

In these kits, all the reagents and Luminex plates needed to run Luminex-based panels are supplied as a pre-mixed kit in a ready-to-use format from various vendors. These kits have

undergone basic qualification and validation by the manufacturer to establish reagent and matrix suitability, as well as sensitivity and repeatability.

We have found in our experience that, despite the manufacturers claims that the pre-mixed and pre-packaged kits are ready to use without any modification, the assay parameters may need to be 'tweaked' or modified from what the technical data sheet or technical insert of the kits suggests, such as

- Standard curve dilution factor might need to be decreased or increased,
- The total number of standards required for a standard curve generation may need to be changed, which can range from 6 to 12 non-zero standards, depending on the anticipated dynamic range, as well as the 'curve fit' that best reflects the analyte/response relationship. For a five parameter fit (5-PL), used by most Luminex acquisition and analysis software, a minimum of six non-zero standards should be used. More standard concentrations are required for a non-linear curve fit, than a linear curve fit.
- Further dilution or less dilution of the sample matrix might be needed,
- The stabilization of the cytokines or other target proteins in the matrix of interest by 'carrier' proteins, such as Bovine Serum Albumin (BSA), or Human Serum Albumin (HSA) in order to improve accuracy and recovery of the target protein in the sample matrix might need to be used for unstable or labile proteins, such as many cytokines.

As an example, a multiplexed Luminex assay developed and validated by us was modified from the protocol mentioned in the manufacturer's technical data sheet, as follows:

- The top standard was dropped, and the second standard was used as the top standard. The reason was that the dynamic range suggested by the manufacturer of the kit was 'stretched' to beyond the working limits of the assay or the instrument, resulting in variability in spiked controls near the top part of the standard curve. Once the top standard was lowered to the second standard, and the dilution factor reduced, the dynamic range was still in the required range of the assay, but the variability of the spiked controls near the top half of the standard curve was abrogated.
- The sample dilution suggested by the manufacturer (1:2) resulted in poor accuracy and recovery of spiked standards in sample matrix. When the sample matrix was further diluted to 1:5, and then spiked with the same standard proteins, the accuracy and recovery dramatically improved, suggesting that the matrix effect of the more concentrated sample matrix resulted in poor recovery of the spiked protein.

In summary, even though a pre-mixed commercial Luminex kit is sold as a *worry-free assay that* will supposedly require minimum manipulation, in reality, the assay conditions will in most instances be needed to be modified to make it 'fit for purpose'.

It is also imperative that the instrument that will be used during validation and sample testing is calibrated, and annual or semi-annual maintenance performed diligently. Also, the acquisition software should be validated for all the necessary and relevant functionalities. This applies to all kits, whether home-brewed or purchased premixed from a vendor.

Validation

Once a working assay has been established with all the necessary parameters put into place, assay validation can commence. **It cannot be stated firmly enough that all the quirks and weaknesses of the assay be ironed out before proceeding into validation.** Since this is a multiplex assay, with up to 500 possible targets that can be detected and quantified by the assay in one assay well, the development can be complex, and hence, should be comprehensive in order to identify and correct short-comings of the assay before proceeding to analytical validation.

The validation of a Luminex assay evaluates the classic assay parameters of any other immunoassay:
- Inter-and intra-assay precision (a measure of within run, and between runs repeatability);
- Sensitivity (Lower Limit of Quantitation or LLOQ);
- Limit of Detection or LOD;
- Upper limit of Quantitation or ULOQ;

- Linearity;
- Accuracy and Spike and Recovery;

- Short and Long term stability; Freeze-thaw stability;
- Matrix interference; and
- Dilutional studies

Since these parameters are universal for all immunoassays, they will not be described in detail here, except for those with issues unique to Luminex multiplex technology.

'Functional LLOQ'

One of the most problematic issues with Luminex assays that we've developed in-house, have been with what we now term as the '**functional LLOQ'**, which differs from the LLOQ according to the standard curve. In a classic immunoassay, the last standard is designated as the LLOQ, if it follows certain criteria (from Guidance for Industry: Bioanalytical Method Validation, FDA):

The analyte response at the LLOQ should be at least 5 times the response compared to blank response; and analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%.

We started defining a 'functional LLOQ' of assay as a separate entity from the classically described LLOQ, as defined above, because of a few unique features of the Luminex assay, and the way the software generates pg/ml values from the raw data median fluorescent intensity (MFI) units.

Any target with median fluorescent values below the lowest standard is **extrapolated** from the lowest standard, and then assigned an **approximate, extrapolated pg/ml concentration** value by the software. Because the extrapolation is imprecise, and just an approximation of the concentration of the targets in that particular sample, the results in pg/ml values of the replicates of same sample can be variable, resulting in a high **%CV, and imprecision**.

Similarly, samples are diluted at least 1:2 in assay diluent in most Luminex assay protocols, or in one of our assays, 1:5 in assay buffer. If the dilution uncorrected MFI of the sample falls below the lowest standard, an extrapolated pg/ml concentration is produced by the software, *then multiplied by the inputted dilution factor by the software,* and a final pg/ml value that looks higher than the lowest standard finally generated. However, since these pg/ml concentrations aren't precise, but an approximation, they can vary widely between replicates, giving the appearance of imprecision.

A closer look at the **CSV files** generated by the Xponent software shows what pg/ml values were extrapolated, and what were not. Because of imprecise nature of the extrapolated

concentration results, these results should be used in analysis with extreme caution, and avoided for analysis and reporting of results, if possible.

Also, in the cases where samples are diluted in assay diluent, the **functional LLOQ is 'standard 08X dilution factor'** of all the samples-for example, standard08X2 for a 1:2 dilution, and standard08X5 for a 1:5 dilution. Using this scheme allows us to analyze and report only those results that are accurately read by the software, and aren't an imprecise approximation based on extrapolation. Samples with pg/ml concentrations below the functional LLOQ should be reported as <LLOQ.

Spike and Recovery

The process of validating spike and recovery is complicated by the fact that recombinant cytokines that are usually employed to spike test matrices, behave very differently from native cytokines. This may be due to the three dimensional or tertiary structural differences between native and recombinant cytokines; the interaction of recombinant cytokines with matrix components; and the tendency of recombinant cytokines to aggregate in certain test matrices and in certain diluents, which depends on the cytokine involved. Another factor is spiking a number of different cytokines in the same test matrix sample, which adds another layer of complexity to the spike and recovery process, as the recombinant cytokines in their varied diluents, can also interact with one another.

We have tried many different diluents in order to stabilize recombinant cytokines after reconstitution and spiking into test matrix, including BSA or FBS containing DPBS or cell culture media; sample or standard diluents provided with the kit; and different concentrations of the recombinant cytokines (high, mid and low)-all with variable results for each cytokine tested.

One way around this issue, is **to spike high and low standard dilutions into the test matrix**. This is because the standards that come with the kit are usually well-characterized and relatively stable after reconstitution of lyophilized proteins, and the concentrations of the spiked standard can be accurately calculated.

 Different dilutions of the test matrix to be spiked should be tested, to determine the matrix dilution that offers the minimum matrix interference, and the maximum % recovery.

It is recommended that the **dilution of the test matrix used for spiking standards for spike and recovery, be also used for diluting samples in the assay-**for instance, if a 1:5 dilution of serum is used for spike and recovery, then a 1:5 dilution of the samples be used also.

Conclusion

Luminex technology is a powerful tool in the arsenal of multiplex assays, as it can generate tremendous amount of data from approximately 100 µl of test matrix. However, it's this very

advantage of multiplexing that can complicate development and validation of multiplex Luminex assays. A detailed development and validation process in order to investigate and resolve issues with an assay of this complexity, is critical. Approaching extrapolated data with extreme caution, as well as optimizing spike and recovery before the start of validation, is highly recommended

Sonal Gupta, MD, PhD

Dr. Gupta is a Senior Research Scientist at Cambridge Biomedical Inc. She is a cell biologist and immunologist with specific expertise in developing various assays based on techniques such as flow cytometry (FACS), Luminex, ELISA, Mesoscale Discovery (MSD), immunohistochemistry, and cell-based assay techniques, among others. Dr. Gupta has extensive and broad experience in oncology and inflammatory diseases, hematological cancers, skin disorders, cancer and melanoma research, and biologics and recombinant antibody technology.

She previously worked in these fields at the Massachusetts General Hospital, Abbott Bioresearch Center, and Shire Human Genetic Therapies.

Dr. Gupta obtained her degree in Medicine from India, after which she pursued her PhD in Cancer Biology from the University of Bradford, in the United Kingdom.