MULTIPLEX IMMUNOASSAYS IN CLINICAL BIOANALYSIS: THE PROMISE AND CHALLENGES TO IMPLEMENTATION IN CLINICAL DEVELOPMENT

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There is no doubt that the implementation of biomarkers in drug development programs is a costly business in terms of resources, time, and money. However, with the advent of new multiplexing technologies for screening multiple biomarkers from the same sample, the question is increasingly changing from "can we afford to have it?" to "can we afford *not* to have it?" A number of important challenges must be overcome though, before biomarkers can be successfully implemented in a clinical research program. This article reviews the most common multiplex platforms available and highlights both their promise and the challenges.

INTRODUCTION

We have come a long way in the last decade in the recognition for the need of biomarkers in drug development. If nothing else, there is at least alignment between pharmaceutical companies and the regulatory agencies about the critical position of biomarkers in the development of safe and efficacious drugs. However, despite the recent advances, tremendous efforts are still needed in order to deliver on the promise of biomarkers to enable go/no-go decisions to be made early and with confidence. Thus, while currently it is almost *de rigueur* to incorporate a biomarker strategy to parallel the development of new drugs, the challenge remains to have the right biomarkers in place at the right time and at the right cost to justify the required investment in resources, time, and money.

THE PROMISE OF MULTIPLEXING TECHNOLOGIES

Progress in biomarker research shows that in many complex diseases, changes in multiple biomarkers rather than single biomarkers are required to provide a better understanding of the drug effect. Prior to the development of multiplexing technologies, the task of measuring many different analytes, although desirable, was both laborious and cumbersome. Key obstacles include:

- Different analytes may require separate samples to be taken. This results in complex logistics for multiple collections, storage space, and shipping. Many clinical sites struggle to support these activities as the research support staff are often not skilled to process and aliquot samples as required for all the assays needed
- The volume of specimen collected is limited, especially for studies when multiple time-points are required and/or in those patient groups where sample volumes may be restricted (eg, pediatric subjects)
- High cost for shipping and analysis
- Time pressure to do all the analysis on time.

As a consequence, each additional analyte potentially increased both the workload and cost incrementally. In this respect, multiplexing analysis offers great potential to overcome many of these obstacles: only one sample is required per timepoint, sample processing is standardized, and measurement is becoming more automated, thus enabling the generation of exponentially more biomarker data almost in the same time as it used to take for a single analyte.

However, the successful adoption of multiplex assays in a clinical research program requires an upfront investment and the following sections highlight key considerations that must be taken into account.

TYPES OF MULTIPLEX ASSAYS

Platforms for multiplexed determination of soluble biomarkers can be grouped into two main categories:

1. Immunoaffinity-based multiplexing, which is mainly antibody-based
2. LCMS-based multiplexing using multiple-reaction monitoring (MRM), which is currently expanding and offers a good alternative when antibodies for developing an immunoassay are not available

Immunoaffinity-based multiplexing or Multiplexed Immuno-Assays (MIA) are generally based on building multiple ELISAs in one assay format. Hence, MIA can be considered as a step forward in the evolution of the ELISA assay in adapting to the high-throughput assessment of soluble biomarkers. As the ELISA method is the gold standard for measuring soluble biomarkers and widely accepted in clinical practice, this review will focus only on MIA and provide an overview on how MIA can be adapted to assess biomarkers in clinical samples

Multiplexed immunoassays are divided into two main classes: planar and suspension microsphere MIAs. These MIAs are mainly represented by the platforms from MSD (planar) and Luminex and Partners (suspension microarrays). Both classes offer various advantages and limitations: planar MIAs offer a wider dynamic range and better signal-to-noise (higher sensitivity), while suspension MIAs offer a bigger potential for multiplexing with an acceptable imprecision.

These technologies have benefited greatly from the close collaboration of biomarker scientists and technology providers such that there is now a wealth of biomarker data in the public domain produced using MIA platforms. In particular, CRO laboratories with expertise across the different platforms and their collaborations with both platform providers and pharmaceutical sponsors continue to play an important role in helping to mature these multiplexing platforms. Consequently, the bead-based multiplexing (Luminex and partners) and the ECL MSD platforms are now widely known and applied in clinical studies from a range of drug development programs such as anti-cancer therapeutics and vaccines, as examples (Backen et al., 2009; Marchese et al., 2009).

THE CHALLENGES OF MULTIPLEX IMMUNOASSAYS IN CLINICAL STUDIES

It must not be assumed that MIAs can be used off-the-shelf for any new application. As is the case for any other analytical method, MIAs must be optimised to demonstrate an acceptable level of performance and robustness before being applied to analyse clinical samples. The unique challenge for MIAs, however, is that this must be done for all of the biomarkers to be analysed in the same sample. It is therefore important to emphasize the need to allow sufficient time upfront for the development and/or optimisation of these assays, ideally even before the clinical trial protocol is finalized.

In this process, the role of the biomarker scientist (or the outsourcing specialist) is key to ensure appropriate communication between the Sponsor's clinical trial team, the investigator, and the assay provider (e.g. CRO). As for any assay, the choice of the sample matrix to be collected and pre-analytical processing of clinical samples should be carefully chosen to suit the requirements of the assay to be used. Sometimes it may happen that there is a disconnect between the assay provider and the Sponsor resulting in decisions on the matrix collected and sample handling and processing being made regardless of the assay to be used. Likewise, the assay provider should have a good understanding of the intended use of the results to be considered when deciding about the assay type. For example, if the treatment is expected to inhibit or stimulate the production of the biomarker, then the expected range of biomarker concentrations in the study population and the expected volume to be obtained need to be known in order to help the assay provider tailor the assay parameters to deliver to the expected requirements.

Once the assay format is chosen, sufficient time should be invested to run feasibility studies to produce an assay with optimised parameters for each component on the panel prior to the validation. Depending on the objectives of the clinical study, pre-validation work could range from simply running an off-the shelf assay following the kit instructions, to developing a new assay. If the multiplex data are intended for a regulatory application, then ideally a statistically designed assay optimisation or development plan should be used to determine the optimal settings and conditions for the major controllable factors in the MIA before the validation. It is fundamental to MIA to demonstrate that there is no signal contamination from one analyte with another and no antibody cross-reactivity between analytes. Other parameters to check or optimise are the test system suitability (platform), block-

ing buffer, washing steps, incubation time with the samples, incubation time with the secondary antibodies, sample diluents, secondary antibody concentrations and the matrix interference using the same matrix as study samples. Also, it is at this stage where an idea on the assay sensitivity is determined allowing a go/no go decision on the assay prior to validation. If the feasibility is adequately conducted, then it is almost guaranteed to run a trouble free-validation.

HOW MANY BIOMARKERS TO INCLUDE?

While at the discovery level, biomarkers can be measured in panels of up to 100 for some multiplex immunoassays, efficient throughput at the clinical level requires a much smaller number of biomarkers on the panel (ideally four biomarkers at most) in order to balance between the quality of the data as the assay is easier to validate, and the other advantages offered by multiplexing, such as savings on matrix volume, time and cost. If the list of biomarkers to evaluate is bigger, then it is recommended to split them into smaller panels taking into account other criteria such as the cross-reactivity, grouping based on dilution factors, etc. Often, data from smaller panels are of much better quality and is therefore better suited to help guide the project and make decisions with more confidence.

Since multiplex immunoassays combine many reagents in a single assay, this presents additional issues not usually encountered in single analyte ELISAs. Hence, keeping the multiplex panel small helps overcome a number of challenges, including:

- Increase the chance of finding diluents and assay buffers interacting effectively with all reagents for each constituent under common assay conditions
- Higher likelihood of compatibility in the dynamic range needing one sample dilution
- Ease of checking nonspecific binding due to cross-reactivity between capture, detection antibodies, and non-targeted analytes
- Improved sensitivity, as nonspecific binding can often lead to a larger background signal in larger multiplex panels compared to single plex ELISAs
- Easier to interpret data from multiplex QC and to determine acceptability of the analytical results based on QC data

BE PREPARED FOR VALIDATION AND ANALYSIS CHALLENGES

Clinical trial regulations require that any measurements performed on subjects participating in clinical trials are conducted to a quality standard that encompasses every intervention on the clinical samples from their collection until their analysis (Ezzelle et al., 2008). Whether the biomarkers are for exploratory or for go/no-go decisions, a fit-for-purpose level of validation should be applied which can range from a simple assay qualification check to a full-validation where the precision (intra- and inter-assay), the accuracy, the assay range (sensitivity with lower limit and higher limit of quantitation), specificity and stability are assessed. Depending on the pre-validation work performed and the compatibility of the analytes on the panel, the MIA validation can encounter various challenges. The main issues during the validation are related to the preparation of the quality controls (QCs) and the interpretation of the data from the QCs during the conduct of the validation. For example, how to proceed if the precision and the accuracy for one analyte from the panel are outside the pre-specified acceptance criteria? Depending on the utility of the data from that analyte, the assay scientist and the sponsor representative may decide to apply more flexible acceptance criteria or remove that biomarker from the panel.

During study sample analysis, the main challenges encountered can be summarized as the following:

- Cases when QC acceptance criteria are not met for one analyte on the panel only.
- Does this mean that the run will be entirely rejected for all analytes on the panel or only for that particular analyte failing to meet the acceptance criteria?
- Cases when results from one analyte do not fulfil the pre-set precision.
- Which data from the analytes meeting the acceptance criteria will be reported (ie, from the first run or from the re-run)?

In both cases mentioned above, repeat analysis should be conducted according to the criteria discussed and agreed with the sponsor representative. The most

appropriate response depends on a good understanding of the importance of each biomarker on the panel in the decision to be made from the clinical study. Having a fair level of flexibility in setting up acceptance criteria accordingly for each biomarker on the panel often helps overcome most of these issues. For example, if a biomarker is expected to show a big change following a treatment, then a certain level of assay imprecision for that biomarker could be tolerated.

CONCLUSION

Multiplex assays can offer a very efficient way to maximize the amount of biomarker data from a limited number or volume of biological samples. Recent innovations and developments in this area signal that these technologies are reaching a stage of maturity when they can be successfully implemented in a clinical development program to enhance go/no-go decisions. However, successful implementation does require forward planning and upfront investments to enable the suitable platforms are available with the right validation on time for clinical trial sample analysis. Although this work obviously has an impact on time and resources, it is nevertheless crucial to ensure all the components of the assay are optimised to the best performance. Thus, despite the additional outlay upfront, this is more than repaid once the assays are validated and used to analyse clinical samples. Good communication and close collaboration between all the interested parties, especially between those who design the studies to collect biomarker samples and those who validate the assays and analyse the samples, cannot be overemphasized.

With innovative study designs, optimal facilities and strong regulatory intelligence, SGS can favorably impact client's drug development timelines and decision-making process.

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