Biomarkers in Drug Discovery and Development: Pre-analytical and Analytical Considerations

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Abstract Today biomarkers are used widely across the drug development continuum to improve the success rate and cost-effectiveness of rational drug development [1, 2]. These include diagnostic indicators of disease, assessments for biological pathways for new therapeutics, confirmation of target engagement or mechanism-of-action, safety indicators, and as pharmacodynamic (PD) measures of therapeutic efficacy [1, 2]. The application of biomarkers is now commonplace in drug development and has helped usher in a new era of predictive, preventative, and personalized medicine, where therapeutics can be tailored to a patient's unique biology. However, prior to being used for these purposes, there are several practical aspects of biomarker utilization that need to be taken into account. These include biomarker validation and qualification. Analytical validation is the formal process of evaluating an assay to ensure it provides reliable data [1, 2], whereas qualification (also known as clinical validation) is the evidentiary process of linking a biomarker with biology, pharmacology, or clinical endpoint [1, 2]. Analytical validation is important, as issues of quality herein can limit the utility of biomarker data. Moreover, a lack of proper analytical validation can potentially undermine successful demonstration of a biomarker-related pharmacodynamic effect during clinical investigation [1, 2]. Not surprisingly, successful biomarker testing in drug development requires careful consideration of pre-analytical factors that may impact reliable biomarker quantification in biological matrices. Most often, pre-analytical factors include activities associated involving collection, shipment,

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tracking, storage, and distribution of test samples. Analytical considerations include the use of kits versus custom assays, reference standard sourcing, sample preparation strategies [3, 4], instrumentation and the removal of matrix interferences. This chapter will discuss some of the various factors that need to be taken into account when preparing for biomarker analysis.

Keywords Pharmacodynamics (PD) measures · Safety indicators · Validation · Sample collection kits · Storage · Shipping

Biomarkers in Discovery and Development

Biomarkers are useful tools throughout the development cycle of a therapeutic; however, the rigor to which the biomarker assay is tested will change depending on the stage of development as well as the intended use of the data. This idea of a "fit for purpose" validation of biomarker assays is detailed in Lee et al. [5] which defines the validation of a biomarker assay as an iterative process, where the extent of validation increases as the therapeutic moves down the pipeline. Validation is broken into two phases, exploratory method validation and advanced method validation. The exploratory phase is less rigorous and would be used for biomarker in the preclinical and early clinical stage. An advanced validation would be undertaken when a biomarker is in late phase clinical, especially when the biomarker is used as a primary endpoint of a clinical trial. Biomarker assays used in discovery would often have very little characterization, however, would give an indication of up and down regulation of various biomarkers.

Pre-analytical Considerations

The ability to accurately detect and report biomarker results relies on executing several pre-analytical activities in a manner that limits their inherent variability. From the collection of the sample at the clinical site, all the way through shipping, storage and analysis there are several operational and logistical steps that require particular attention so that the sample conditions are consistent and traceable. Each point along the sample supply chain, no matter how insignificant it may seem, presents potential opportunity for inconsistent handling or mismanagement. In order to mitigate the risk of variability, the complete supply chain must be validated to ensure the appropriate steps are in place for every point along the sample's lifecycle.

Collection

The biological sample will almost always be collected at a clinical site. At this phase it is necessary to ensure the staff have proper training in the desired method of collection, labeling, and outbound shipping.

Sample collection kits can be supplied to the site staff which ensures the proper equipment and supplies are used for specific sample collection needs [1]. Kits should include specific collection tubes for the type of matrix that is needed. They can also include needles, protective supplies for the phlebotomist, Band-Aids and gauze, and sample shipping materials.

Proper labeling of the collection tubes are going to ensure the specimen is tied back to the patient and not inadvertently switched after collection. Labels come in a variety of sizes and styles. Typical components include an accession ID, some iteration of subject and study identifier, the visit ID and a barcode so that registration can be streamlined. Tubes can come prelabeled or unattached within the kit and each comes with its own advantages.

Also included in each kit, should be a manual or set of instructions. The manual should clearly identify the visit and collection schedule, a list of components in the kit, instructions on how to use the components, and how to process, store and ship the samples. The manual should be very clear about when samples are collected throughout the day. Since many biomarkers are found to be dynamic, varying in concentration throughout the day [2], and can change with dietary influence, this need to be covered specifically in the manual. Variation in collection times can lead to variation in analytical results.

Biomarker collections come from all over the world. Providing the sites with a consistent set of supplies and instructions helps to lessen the variability and ensure a high level of confidence in the results.

Shipping

The method of shipping needs to be properly qualified prior to the samples being collected. Different shipping containers are rated to allow for specific temperature variations. The location of the site and shipping route can greatly affect the materials needed for shipping. Whether the samples are required to be shipped ambient, refrigerated, or on liquid nitrogen; it is important to understand the tolerable temperature limits of the sample. With those limits in mind, the selection of shipping container will need to withstand both high and low fluctuations [6].

Ensuring the shipping conditions are within the limits of the sample can happen prospectively and retrospectively. A prospective qualification of the shipping means data is collected prior to the shipment of the samples to better understand the environmental factors that will influence the container. You must map the fluctuation of temperature throughout the day and night and since conditions change with

the seasons, yearly temperature readings need to be included in the assessment. Since the supply chain of samples is global, measurements must be made along the whole shipping route. Alternatively, data loggers can be included in the ongoing shipments, providing complete traceability of the sample throughout the trip.

Shipping biological samples internationally comes with the risk of encountering customs delays due to transportation regulations. When the shipping containers are selected, ensure there is adequate room on the surface to display the appropriate warnings and labels necessary. A container that is held up at customs due to incorrect labeling will not necessarily be in a position to be re-filled with ice pack or dry ice. For this reason, a shipment notification system should be in place to effectively communicate the departure and expected arrival of these packages.

Storage

The sample will likely spend most of its time sitting in storage. For that reason, it is important for the environment or unit to be properly temperature controlled and the location of the sample be clearly defined. Whether the samples are stored ambient or at −80 °C, the controlled areas will need to be monitored 24 h a day. Additionally, the monitoring system needs to have temperature thresholds that are connected to audible and traceable alarms notifications that record any temperature deviation.

The recording of temperature deviations in a system gives the ability pin down exact moments of temperature variation in the storage life of the sample. Know biomarker stability data can be cross-referenced against the temperature log, in case of questionable results. For this reason, the IT system that is used needs to be username and password protected, ensuring an accurate audit trail of the sample. The system should also be connected to a calibrated temperature probe that is separate from the unit's original probe.

Even within enclosed temperature controlled environments, there can be significant variation throughout the space. Temperature mapping takes into account the temperature fluctuation of the area. Calibrated temperature data loggers should be used to record temperature in predetermined points with the 3D space over the course of 24 continuous hours. Data should be collected at different points along the height, length and depth of the unit and the total amount of probes is dependent on the total volume of the area [7]. Finally, the data should be compared to the set point of the unit, and the monitoring probe should be placed in the location of the unit where there is the highest level of variance. Placement of the probe in this location ensures the samples will be within the allowable limits of the temperature settings. The location of highest fluctuation will likely be different in every unit, thus the monitoring probe will be in a different position as well. If the probe is not placed in this precise location, the result will be samples potential exposed to temperatures outside of the allowable limits without any indication from the temperature alarm system.

Sample Tracking

The samples need to be tied to specific data that can be tracked along the lifecycle of the sample. The system that is being used to track the samples will also need to capture associated data such as aliquot quantity, volume, and concentration (if necessary). If there are known analyte stability issues as a result of freezing and thawing, the sample may be aliquoted prior to being placed into storage. Having a system in place that accommodates the need of connecting sample lineage, will make organization of the samples easier. If aliquots are necessary, any data associated with the parent sample will need to be related to the aliquot as well.

Any changes in the data should be racked through a verifiable audit trail. Changes in subject information, label information, and sample data can lead to confusion at the time of analysis and results can be misassociated with the wrong subject [8]. Sample locations should also be tracked down to the most specific locations they can be. Pulling many samples from storage at once can be tedious and error prone, particularly if the exact locations are not known. Storage units should be situated with unique positions that allow for single samples to be placed and easily identified when they are later pulled. Providing specific locations allows for quicker access in and out of the freezer, reducing the amount of time the doors are ajar exposed to outside temperatures.

Any movement of the sample should be demonstrated in a chain of custody report. The chain of custody report needs to have detailed information such as personnel ID's of individuals working with the samples, confirmation of sample receipt, storage locations, shipment locations, and time in and out of possession. This data confirms the correct storage and handling of the sample at each step along the lifecycle.

Distribution

Whether the analysis is being performed in close proximity to the storage location or the samples are being shipped, a detailed manifest should accompany the package. The manifest should include position within the package, and an identifier that corresponds to the tube label. If samples are inadvertently placed in the wrong location, the manifest data and tube can be reconciled against one another and sorted out.

Sometimes, samples are stored in a 96-well plate format and given the emergence of automated analytical technologies, the samples can often be delivered in the same format. There are some 96-well plates that hold individual tubes that can be placed in varying orientations. These tubes typically have enough surface area to support a labeling system or a 2D barcode on the bottom of the tube that can be tied to the manifest. In the cases where individual labels are not available for each well, a rigorous QC step need to be employed so that the sample location are accurate and traceable from the manifest.

Analytical Considerations

Several analytical characteristics need to be examined when utilizing a biomarker to support drug discovery. One question to investigate prior to beginning lab work is the use of a commercial kit versus a custom built assay. Commercial kits are attractive due to the perceived ability to use them "off the shelf"; however, one must test these kits to determine if they are truly fit for their intended purpose. Often times the kit must be modified to gain acceptable performance, however, this can be difficult when critical information is not known, such as concentrations of antibody pairs and the components in the kit assay buffer. When sourcing commercial kits and reagents, enough material should be purchased to limit the number of lot-to-lot changes during the course of a study. The manufacturer of the material is also important, as some vendors are more reputable than others. While the development time needed for a custom assay is typically more, there is greater flexibility with the custom assay. The antibody pairs can be optimized for the desired quantitative range of the assay and an assay buffer can be developed which helps to reduce interferences in the matrix of interest. Another consideration prior to starting lab work is the endogenous concentration of the biomarker. While some biomarkers are high in concentration, most are relatively low and require a sensitive assay for measurement. This is critical when selecting an instrument platform to be used. While a standard ELISA with absorbance detection is simple and cost effective, it may be impossible to obtain the desired sensitivity. One solution is to change out the enzyme substrate for a more sensitive detection scheme. When using horseradish peroxidase as the enzyme detection system, one can switch substrates from TMB (3,3′,5,5′-Tetramethylbenzidine) to ADHP (10-Acetyl-3,7-dihydroxyphenoxazine) for fluorescence detection or luminol for chemiluminescence detection. The same process can be applied to other enzymes as well. If even more sensitivity is needed, one can use the Meso Scale Discovery platform with electrochemiluminescence detection.

The source of the biomarker reference standard is also an important point to consider. Unlike small molecules, which are synthesized and have a defined purity, biomarkers are endogenous with various post-translational modifications and the extent of glycosylation can differ from patient to patient. Thus, it is impossible to have a reference standard which is indicative of the analyte be measured in vivo. Testing spike-recovery and assessing parallelism can be of great importance, helping to determine the extent of homogeneity between the endogenous biomarker and reference material. The desired matrix must also be explored, as serum, plasma, and different anticoagulants can have an effect on your assay. If stability is a concern, one should steer away from serum, as the length of time needed to clot can cause stability concerns, and it is not amenable to protease inhibitors. Na/Li heparin plasma has been shown to cause interferences in ELISA assays and is rarely used. Potassium EDTA (both K_2 and K_3) are commonly used, however, keep in mind that K_3 EDTA plasma has been shown to effect pH and both can have a negative effect when alkaline phosphatase is used as the enzyme system. In addition, additives to

the matrix need to be assessed, especially for smaller proteins and peptides. Some peptides can be especially prone to proteolytic degradation, and thus the addition of a protease inhibitor cocktail is crucial for reproducible results.

A major complication when setting up a biomarker assay is the effort to remove matrix interferences [9, 10]. There are many things to examine, including the species of the antibodies, degree of post-translational modifications (sugars), and the presence of heterophilic antibodies and rheumatoid factor. The addition of serum components to the assay buffer can aid in the removal of nonspecific interactions. A good first step is to identify the species of the antibodies used in the assay, keeping in mind that the total serum content in the assay buffer should be roughly 5 % [10, 11]. If a noncompetitive ELISA is being used and the capture antibody is raised in mouse and the detection antibody is raised in goat, then the assay buffer might contain 5 % mouse serum and 5 % goat serum. If both the capture and detection antibodies were raised in mouse, then the assay buffer would instead generally contain 5 % mouse serum. Addition of other serum sources can be used as well; we have had good results with the addition of equine serum. Knowing the degree of post-translational modification of your antibodies and types of sugars can give a clue as to additional buffer components. Sugars bound to your antibodies can often be very sticky and the addition of sugar to your assay buffer can help to reduce these interactions [12, 13]. Heterophilic antibodies often cause problems in immunoassays. Heterophilic antibodies are typically low affinity binders and are polyreactive against poorly defined antigens of different chemical composition [9, 10, 14–17]. While they may be low affinity binders, they are present in 20–40 % of subjects [10]. Known generally as Human Anti-Animal Antibodies (HAAA), these anti-species antibodies create both false-positive and false-negative results [13]. This is an area where the addition of serum from the species of your detection antibodies can greatly aid in the reduction of this phenomenon, as the anti-species heterophilic antibodies will interact with the free serum components as opposed to the antibodies. Knowing the typical patient population's disease state can also aid in the choice of assay buffer components. Rheumatoid factor, for example, is often found in rheumatoid arthritis patients, and can cause false-positive results due to bridging of the capture and detection antibody pairs.

An analytical consideration often overlooked by immunoassay scientists is the use of an extraction procedure for sample cleanup prior to the immunoassay. An extraction step is not practical for larger proteins, since extractions are intended to remove proteins, which are often the analyte of interest. However, for peptides, extractions can be useful in removing endogenous interferences from the immunoassay. This can be especially critical for immunoassays of peptides, since specific antibodies against peptides can often be difficult to generate. In addition, there are often matrix binding proteins which interfere with measuring the true endogenous component. Low affinity binders can include albumin and circulating free protease inhibitors. High affinity binders can include soluble receptors and anti-drug antibodies (ADA). There are several extraction reagents which can be used. Extractions with polyethylene glycol (PEG) are an attractive choice, since the extraction reagent is in the aqueous phase, and the supernatant can be directly

applied to the immunoassay. However, sometimes PEG can cause interactions with the antibodies in the immunoassay, reducing a signal, and rendering them useless. Another option is the use of alcohols and acetonitrile. These reagents can be used to crash out the proteins. The resulting supernatant can be dried and reconstituted in the assay buffer of choice. Yet another option is the use of an acid treatment step. The addition of an acid to the samples, reducing the pH to $\sim$2–3, can help to break apart the interactions with binding proteins and ADA. However, care must be taken to ensure the pH of the resulting reconstituted extract is neutral, as the addition of an acidic supernatant to the immunoassay can reduce the biding of analyte with the antibodies. This can be done by increasing the ionic strength of assay buffer or by incorporating a neutralization step prior to the drying down the sample.

As discussed previously, the extent of validation is dependent upon the intended use of the biomarker data. Regardless, there are several experiments that should be performed to ensure the assay is performing adequately. One question that needs to be answered almost immediately is the endogenous concentration of the biomarker in vivo. Often times, a surrogate matrix will be used in lieu of a matrix calibration curve. For biomarkers with high endogenous concentrations, it can be difficult to source matrix with low enough levels to be used for calibration. This surrogate matrix can be stripped to remove the endogenous analyte of can consist of a protein buffered solution. If a surrogate matrix is used, it is important that quality control samples in the matrix of interest are included during the validation to ensure that the biomarker is being recovered from the matrix. Depending upon the extent of validation, anywhere from three to six runs to determine precision and accuracy can be run to determine this important parameter. A selectivity (spike/recovery) experiment should be performed in various individual lots of matrix to ensure that spiked analyte is being recovered. Each lot should be analyzed with both spiked and unspiked with drug to account for the endogenous concentrations. Dilutional linearity should be performed to ensure that samples with high concentrations can be diluted into the range of the curve, and that no hook effect is observed. Parallelism, as discussed previously, is important to ensure that endogenous analyte, when diluted, is parallel to the calibration curve. When possible, the specificity of the critical should be defined, by spiking similar analytes into control samples and assessing recover of the biomarker of interest.

Conclusion

There are many areas to consider when preparing to run a biomarker assay. From the point of collection to the moment of analysis, there are multiple points of variability that can influence the outcome. Taking the time to consider these prior to execution will result in more consistent and reliable data.

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