Challenges and Solutions with Bioanalysis of Soluble Biomarkers: A Case Study for Non-Invasive NASH Biomarkers

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Key Terms:

Soluble Biomarker: Endogenous analytes measured in biospecimens such as blood and urine as an indicator of normal biologic processes, pathogenic processes, or responses to a therapeutic intervention.

Context of Use: A clear statement that describes the manner of use, interpretation, and purpose of use of a biomarker.

Parallelism: Demonstration that dilution of the endogenous biomarker in a sample is parallel to the standard curve with recombinant biomarker and thus functionally similar.

Introduction:

The demand for detection of biomarkers in bodily fluids (soluble biomarkers) is growing rapidly with the changing landscape of drug development. As drug development becomes more personalized and biologically complex, clinical studies are increasingly using biomarkers for analysis of toxicity, surrogate endpoints, and lead candidate screening. However, bioanalysis of biomarkers is significantly different from bioanalysis of compounds in pharmacokinetic (PK) assays. In this paper, we describe these challenges and solutions that can be used to accurately and efficiently measure these compounds that hold tremendous potential for drug discovery. We then detail a method development and validation process for a panel of soluble biomarkers for clinical studies of nonalcoholic steatohepatitis (NASH).

Challenges:

Analytical and biological differences between biomarker assays and traditional PK assays present challenges that require adapting current bioanalytical practices. First, biomarker assays are used in many stages of drug development answering different clinical questions. For each Context of Use (COU), a term borrowed from FDA qualification of biomarkers in clinical studies, there are unique needs for the bioanalytical assay (accuracy, precision, cost, speed) which present new complexities for method development and validation. Moreover, while FDA guidelines on bioanalytical method validation were established in 2001, these guidelines did not include guidance for biomarker validation. Since then, draft guidelines for biomarkers were published in 2013 and collaboration between the FDA and the industry to formalize guidelines are in progress.

Biomarkers are endogenous compounds and thus present biological complexities complicating their bioanaylsis. Many biomarkers have roles in several disease states and confounding factors can affect their measurement. The COU of the biomarker may also require use of special matrices such as CSF, saliva, or cell lysates. Many biomarkers undergo degradation or changes in isoforms and thus consideration must be given for sample stability from the point of collection through sample analysis.

Reference materials used in biomarker assays are poorly characterized compared to their PK assay counterparts. They are typically recombinant proteins expressed and purified from prokaryotic or eukaryotic expression systems. Their structure, folding, and functional activity can differ from the endogenous human protein. The recombinant proteins can also vary from different vendors and their purity and activity are often poorly characterized.

Finally, biomarker analysis relies heavily on commercially available kits and often multiplex several biomarkers together into a panel. This also presents unique challenges in reagent consistency, specificity, selectivity, calibrators, and quality controls (QCs) during method development, validation, and sample analysis.

Solutions:

Celerion bioanalytical services utilize a collaborative multidisciplinary approach for each biomarker project to customize the assay to the needs of the study. Our process uses clinical and analytical expertise to identify the appropriate biomarkers, select the best analytical methodology (e.g. LC-MS, hybrid LC-MS, ELISA, electrochemiluminescence (ECL)), develop and validate the assay mindful of the regulatory needs of the biomarker study, and maintain assay performance through the duration of the study.

The primary challenge of biomarkers is adapting method development and validation to the timeline of drug development because biomarkers are used for different requirements. To address this, it is beneficial to use guidelines from Clinical & Laboratory Standards Institute and Clinical Laboratory Improvement Amendments (CLIA) which have been regulating biomarker measurement for over 40 years. First, the COU must of the biomarker must be established. The COU is a statement that succinctly describes how the biomarker will be used during drug development (e.g. screen for a drug target, assessment of risk, assessment of prognostic outcome, asses a toxic or safety concern) that will dictate the level of validation. We define three

tiers of validation to be used along the entire drug development spectrum: Exploratory Validation, Partial Validation, and Full Validation (Figure 1, Table 1). The tier of validation is dependent on whether the COU involves a regulatory body using the biomarker to make a decision. Early in the drug development process where biomarkers are used for screening to select a target for a drug, a less intensive exploratory or partial validation is recommended to obtain rapid yet reliable information. Later in the process, where decisions of patient safety and drug efficacy are made, a full validation in accordance with FDA bioanalytical guidelines must be performed regardless of whether the study is clinical or non-clinical. For example, if the COU statement is "[Biomarker X] is a measure of non-clinical toxicity used for demonstration of the best drug candidate among several candidates", a full validation is required for regulatory body decision making. Regardless of the validation tier, all studies are conducted following GLP-like regulations.

Figure 1. Flexible Biomarker Method Validation Tiers Based on Context of Use Optimize Cost, Speed, and Data Delivery During the Drug Development Spectrum

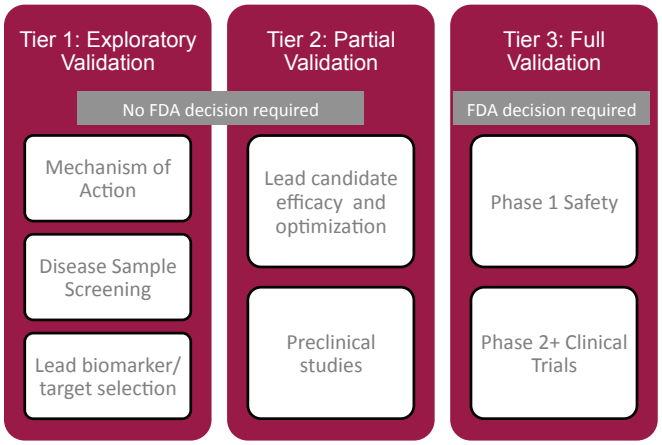


Table 1. Test parameters for tiers of Biomarker Validation

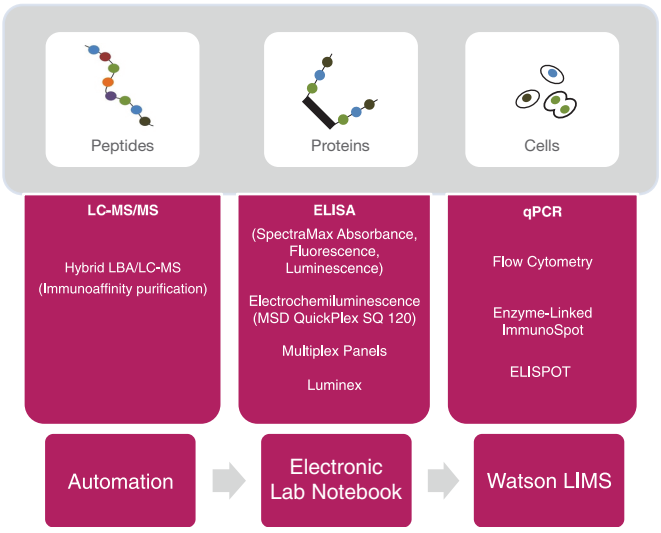
Parameter	Exploratory Validation	Partial Validation	Full Validation
Reference Material	Maintain and monitor quality of reference material. Reference material should be compared to WHO/ international reference material when available.		
Calibration Curve	≥6 calibrators using surrogate or study matrix spiked with reference material. Adjust calibrators in quantitation range as necessary for COU.		
Parallelism	Recommended	Required	Required
Selectivity/ Matrix Effect	Spike recovery at 2 levels with 6 lots of matrix from normal and COU disease subjects each	Spike recovery at 2 levels with 10 lots of matrix from normal and COU disease subjects each	Spike recovery at 2 levels with ≥10 lots of matrix from normal and COU disease subjects each
Specificity/ Cross-Reactivity	Recommended	Recommend	Required: compare structurally similar compounds
Precision and Accuracy	3 analytical runs with analytical QCs	3 analytical runs with analytical QCs and endogenous QCs.	6 analytical runs with analytical QCs and endogenous QCs.
QC Samples	Analytical QCs in surrogate matrix (LLOQ, low, mid, high, ULOQ)	Add endogenous QC pools from normal and COU disease subjects. COU disease endogenous QCs required only if difference in selectivity shown with normal subjects.	Add endogenous QC pools from normal and COU disease subjects. COU disease endogenous QCs required only if difference in selectivity shown with normal subjects.
Dilution Linearity, Hook Effect	Recommended	Recommended	Required
Stability	Scientific judgement	STS and FTS of reference material, analytical and endogenous QCs	STS, FTS, LTS of reference material, analytical and endogenous QCs
Lot to Lot variability	Recommended	Recommended	Screen ≥3 lots for long term studies or source single lot
Method Robustness	Recommended	Recommended	Multiple analysts, instruments, full 96 well plate
Biomarker Work Plan	Required		
Validation Plan / Validation Report	Recommended	Required	Required
Abbreviations: COU : Context of Use, LLOQ: Lower Limit of Quantitation, ULOQ: Upper Limit of Quantitation, STS: Short Term Stability, FTS: Freeze-Thaw Stability, LTS: Long Term Stability, QC: Quality Controls			

Celerion also establishes a biomarker work plan with our clients prior to method development answering questions such as the COU, the concentration range expected in healthy and disease population, and the length of the study (Table 2). These criteria are used to guide decisions on the validation tier, assay platform, sensitivity, accuracy, precision, and data reporting requirements.

A wide spectrum of assay platforms should be considered due to the variety of biomarkers, ranging from peptides to large receptors to genetic markers, and variety of matrices, ranging from blood to CSF to cells. At Celerion, our scientists specialize in methods that can measure all types of biomarkers using multiple small and large molecule approaches. When using commercial kits, our close working relationship with leading vendors is crucial to adapting kits for biomarker validation and maintaining quality and consistency of reagents for long-term studies.

Regardless of the platform chosen, the use of automation, an electronic laboratory notebook, and a laboratory information management system (LIMS) during method development and validation allows our scientists to organize and monitor reference material, reagents and commercial kits, and variability in data being produced to maintain quality over the life cycle of the study and verify studies are being conducted appropriate to the COU.

Figure 2. Multiple State of the Art Biomarker Platforms Validated to Speed Data Aquisition and Analysis



The Celerion Solution:

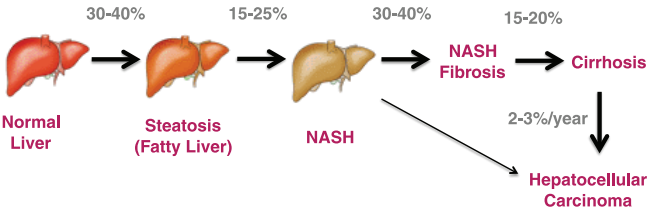
Utilize both ligand-binding assay (LBA) and LC-MS platforms for the ability to measure all types of biomarkers. A fully paperless and GLP environment allow monitoring of reagents and assay performance to bring fast and high quality answers to biomarker questions.

Case Study - Biomarkers for Nonalcoholic Steatohepatitis:

We developed a soluble biomarker panel for nonalcoholic steatohepatitis (NASH) to demonstrate solutions in the bioanalysis of biomarkers. NASH is a severe form of nonalcoholic fatty liver disease (NAFLD) associated with hepatic inflammation and cellular injury. Approximately 80 million Americans are diagnosed with NAFLD and 25% progress to NASH equating to 5% of the US population. NASH can lead to cirrhosis, end-stage liver disease and even hepatocellular carcinoma. In addition, NASH is currently the second leading indication for liver transplant. The rising incidence of NASH is expected to increase as the disease is strongly associated with diabetes and obesity, which have both reached epidemic proportions. Currently there is no FDA-approved therapy for NASH, nor is there a routine, reliable, non-invasive soluble biomarker for diagnosis and clinical management of NASH, leading to a 10 year mortality rate of 60%. Therefore, the need for validated NASH biomarkers is instrumental to advance drug development and patient care for the treatment of this chronic disease.

While the full etiology of the disease is still unknown,

Figure 3. The Spectrum of NAFLD



proinflammatory cytokines are thought to be the driving force behind lipotoxicity observed in NASH development (Figure 4). Lipotoxicity refers to reactive lipids that can induce apoptosis, necrosis and endoplasmic reticulum stress, all culminating the development of fibrosis. Therefore, inflammatory cytokines IL-6, IL-8, and TNF-α are analytes of interest and a biomarker work plan was created for the method development and tier 2 validation of a multiplex panel measuring the cytokines in human serum based on

Figure 5. Inflammatory biomarkers mediate the progression to NASH fibrosis

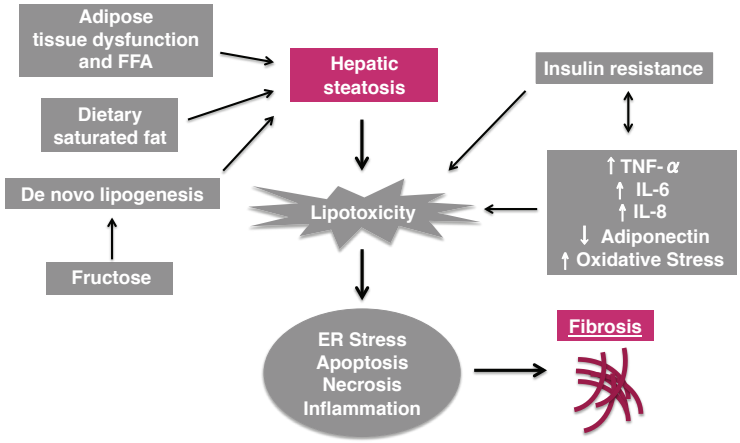


Table 2. Biomarker Work Plan - NASH Inflammatory Biomarkers

Parameter	Requirements
Analyte(s)	IL-6, IL-8, TNF-α
Context of Use (e.g. Disease sample screening Lead biomarker screening Target Optimization Preclinical study Assessment of susceptibility/risk Assessment of prognostic outcome of interest Predict a patient's response Assess or monitor a patient's response Assessment of toxicity or safety concern)	Screen disease sample screening to quantify increase in analytes in NASH
De Novo or Commercial kit	Well characterized commercial kit preferred for cost and time requirements
Multiplexing (Y/N)	Y: Use inflammatory cytokine panels based on physiological and analytical ranges of the analytes and the absence of cross-reactivity and structural similarity
Sample storage/ analyte stability	Stable at -20, 3 f/t cycles, LTS > 6 months
Clinical Ranges and sensitivity required (Normal → Disease)	IL-6: 4 → 8 pg/mL IL-8: 15 → 28 pg/mL TNF-α: 1 → 3 pg/mL
Biological variability	Previously reported standard deviation of IL-6: 5.21 pg/mL
A priori acceptance criteria to quantify anticipated effect	The mean concentration should be within 20% of the nominal values at each QC level (within 25% at the LLOQ and ULOQ level). The CV values should not exceed 20% for the QC samples at each QC level (25% at the LLOQ and ULOQ level).
Sample matrix and volume	Human Serum, 100 ul per sample for 3 analytes in duplicate
Commercial kit adaptations for COU	Add endogenous QCs from matrix pools of normal and NASH human serum. Perform lipemic sample integrity for NASH serum.
Sample number and study length	~100 samples, 6 months
Data Interpretation	Show statistically significant changes with NASH
Rationale for methodology and validation tier based on information gathered	Advanced qualification of a multiplexed MSD ECL assay with a commercial kit using standard bioanalytical accuracy/precision acceptance criteria. Lipemic sample integrity will be performed for NASH serum. T-test analysis with outlier analysis will be performed after sample concentration determination. International reference material not required due to COU.

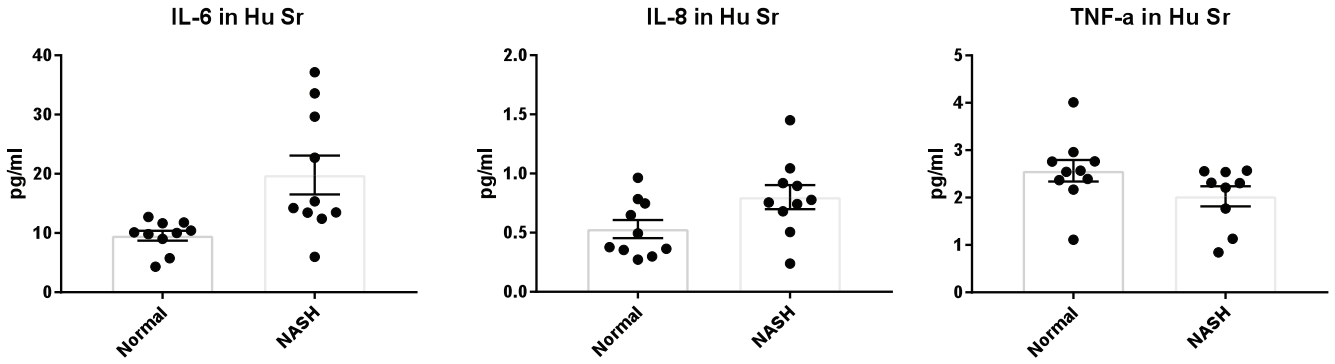
the Meso Scale Discovery ECL platform.

During method development and validation, endogenous levels of each cytokine were assayed in normal human serum (n=10) and diseased NASH serum (n=10). Statistical analysis was performed to remove biological outliers and determine whether statistically significant increases in mean concentrations of IL-6, IL-8, and TNF-α were observed. The robust regression and outlier removal method (ROUT) was designed for detection of outliers in nonlinear regressed data (GraphPad Prism 7). Since concentrations of the cytokines were determined using 4 parameter logistic regression, this method was employed to remove outliers. Subsequently, Student's t-test were performed (GraphPad Prism 7) with outliers removed and reported as mean±SEM for the final reporting of data (Figure 5). Our results indicate that this multiplex panel may be a valuable rapid and non-invasive biomarker panel to identify NASH patients for clinical studies and track progression or treatment of their disease during a study.

Conclusion:

The demand for using soluble biomarkers as a critical part of drug development is rapidly growing. Celerion bioanalytical services works closely with sponsors to find the best biomarkers by understanding the pathology of the disease and addresses the unique challenges of each assay to accelerate during a drug development study.

Figure 5. Concentrations of Inflammatory Biomarkers in Normal and NASH Patient Donor Serum



The Celerion Solution:

40 years of bioanalytical and clinical expertise adapting to find the best biomarkers and best assays for your drug development studies.

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