Bioanalysis ZONE

GYROS PROTEIN Technologies

Single-analyte biomarker assays for pharmacodynamic or safety assessment during biotherapeutic development

fsg

Contents

Technology Digest: Single-analyte biomarker assays for pharmacodynamic or safety assessment during biotherapeutic development

by Ellen Williams
Digital Editor, Bioanalysis Zone

Biomarkers and biotherapeutic development

In a US FDA-supported effort to further the success of new therapeutics in clinical studies, the use of biomarkers in biotherapeutic development has been steadily increasing in recent years. Biotherapeutics are often designed to disturb specific steps in biochemical pathways; the therapeutic target molecule – or molecules involved in subsequent steps in the pathway – can serve as biomarkers, with changes in their concentration being indicative of pharmacodynamic (PD) effects of the biotherapeutic [1]. Biomarkers used in biotherapeutic development also monitor drug safety and efficacy by helping to guide the dosing regimen, i.e., the frequency and dose at which a drug is to be administered to maintain optimum drug exposure to maximize efficacy and minimize side effects [2]. In this Tech Digest, we review multiplex and singleplex assays for biotherapeutic development and provide an overview of the Gyrolab® microfluidic immunoassay platform for biomarker assays.

Multiplex *vs* singleplex

Measurement of clinically relevant analytes such as biomarkers is often achieved through ligand binding assays (LBAs), which were initially designed to detect a single analyte in a biological matrix. These assays, referred to from here onwards as singleplex assays, typically capture and detect analytes using antibodies as critical reagents and can utilize enzymes, chemiluminescence, radioactive isotopes, fluorescence and electrochemiluminescence [3].

When used to make decisions concerning regulatory submissions and drug development, it is essential that biomarker assays deliver high performance and reliability. To meet the demands of biomarker discovery, assay technologies to measure biomarker proteins in blood, other biological fluids and tissues have evolved to have significantly higher sensitivity, as well as the ability to measure increasing numbers of analytes concurrently in a single sample via multiplex assays. This has resulted in the development of a substantial number of premade, commercially available multiplex assay kits, helping to reduce sample volumes and time spent in the laboratory. However, researchers can find themselves employing assay kits that are not specifically designed for their intended study. This requires adaptation of the kit before the assay can be validated, as the kits are usually intended for drug discovery or clinical diagnostics rather than drug development [3]. Performance and feasibility testing should therefore be conducted to ensure the assay is fit for the intended application and researchers should be aware that adaptations may be required for use with a different sample matrix or to meet regulatory requirements [4].

Other challenges with quantitative ranges, optimal sample dilution, cross-talk, sensitivity, specificity and lot-to-lot variability restrict multiplex kits and create additional hurdles for researchers [4]. Therefore, there is an unmet need for well-defined commercial kits that are suitable for biotherapeutic development [3]. Although some commercial kit manufacturers have begun classifying their kits according to higher levels of characterization, there has not been an industry-wide effort to improve the overall quality of these kits for use in drug development. Standardized qualification criteria is also lacking, as manufacturers use their own specific requirements for kit characterization and documentation.

Singleplex assays carry their own unique challenges. In addition to limitations in the number of analytes that can be measured at one time, singleplex assays often require higher sample volumes due to the increased number of measurements required. Obtaining large sample volumes can be difficult, particularly when concerning the sampling of infants or critically ill patients. Singleplex assays are also often less cost-effective than multiplex assays. However, when used for validation of regulated studies, singleplex assays are preferred. Acceptance criteria is likely to be more flexible to accommodate for multiplex assays, or an analyte may have to be removed from the panel if these criteria aren't met [5]. Whereas, for single-analyte assays, the acceptance criteria are clearer and easier to achieve. Whether validation is for exploratory purposes or to inform critical decisions, fit-for-purpose validation of these assays is critical [5].

During biomarker discovery, multiplex platforms are favored as large numbers of potential biomarker candidates can be screened simultaneously. Once these biomarkers have been identified and qualified, the need to screen a high volume of analytes at once diminishes and the ability to screen fewer analytes with greater robustness becomes critical. As development progresses through preclinical and clinical phases, the value of high-quality, well-characterized single-analyte assays with suitably high sensitivity, reproducibility and broad dynamic range becomes essential to the utility of these assays as surrogate endpoints, efficacy markers or safety biomarkers and for validation of regulated studies. Therefore, single-analyte, well-characterized assays are typically developed for use and favored in later stages of development.

Cytokines as biomarkers for biotherapeutic development

Cytokines – encompassing interleukins, interferons, growth factors and chemokines – are a diverse group of soluble proteins and are key modulators of immunity, regulating responses to proximal events of inflammation, immune response and repair [6]. Cytokines are secreted by immune cells, including monocytes, macrophages, T-cells, B-cells and natural killer cells, as well as some nonimmune cells. Cytokine concentration changes regulate the tumor microenvironment, change the proliferation and differentiation of immune cells and even influence the metastasis of cancer cells. These, among other factors, make cytokines apt candidates for therapeutic biomarkers, enabling the establishment of safe yet maximized starting doses for biotherapeutics [7].

In recent years, there has been an increase in the use of single-analyte biomarker assays for PD or safety assessment during biotherapeutic development and cytokines have been one of the many biomarker categories helping contribute to this increase [7].

Numerous platforms now utilize cytokine kits for biomarker quantification, including the Gyrolab microfluidic immunoassay platform. The latest Gyrolab kits utilize five single-analyte biomarker reagents to facilitate the quantification of human inflammatory cytokines IL-4, IL-6, IL-10, IFN-gamma and TNF-alpha in human and cynomolgus monkey serum [101]. Optimized for use on all Gyrolab assay platforms, these reagents demonstrate high sensitivity, matrix tolerance and wide dynamic range to cover the entire spectrum of cytokine levels observed in disease states or PD studies. To meet the requirements of regulated environments and maximize assay reproducibility, the Gyrolab human cytokine assays provide rapid and entirely automated biomarker assays and the use of low sample volumes makes the platform well adapted for biotherapeutic development [101]. The minimal matrix effect experienced in the Gyrolab assays facilitates the shift from traditional serum screening to targeted tissue screening and the introduction of the Gyrolab Bioaffy™ 4000 CD to the Gyrolab platform has improved the overall sensitivity of these immunoassays, extending quantification levels to low pg/mL or high fg/mL levels [102].

Biomarker assay platforms: Gyrolab® Technology

The features of the Gyrolab platform address the key existing challenges within biomarker discovery and analysis. As explained by Ourania Tzara, Research Scientist for Biotherapeutic Development, H. Lundbeck A/S (Valby, Denmark), biomarker assay development can be simplified with the appropriate automated tools:

> *"Gyrolab technology serves as a unique system for rapid development of robust and sensitive biomarker assays for use in drug development programs. As a fully automated immunoassay platform, it enables smooth transfer between analysts and laboratories and the low sample volume requirements allow it to be effectively implemented in the preclinical phase."*

There is a strong need, particularly during preclinical stages of research, for biomarker platforms to be optimized for flexible assay development to adjust to the user's immunoassay design and reagents. This need is highlighted by Kenneth Munroe, Principal Scientist I, In Vitro Pharmacology, Charles River Laboratories (MA, USA);

> "Developing biomarker assays for drug discovery requires an assay platform that is robust and versatile to meet changing demands of early research. The Gyrolab technology not only can be applied in preclinical assays where samples are frequently of low volume and tend to be split during collection, but it also allows for assay formats being adapted to research where several biomarkers need to be evaluated while testing drug effects."

The general movement towards plug-and-play kits has minimized pipetting errors as well as the time needed for assay development, to deliver high-performance immunoassays. Aruni Karunanayake M, Scientist II, Charles River Laboratories, explained:

> "Biomarker assay development is challenged by utilizing the appropriate analytical tool to scientifically justify the choice of a relevant biomarker and its measurement. The Gyrolab technology supports customizing sensitive, robust, and reliable immunoassays for biomarker readouts. The easy-to-use platform allows for minimal sample volume and automated protocol execution, while ensuring precision and accuracy of an analytical method."

As several platforms may be suitable for a particular biomarker application, it is critical that the user selects a platform that is optimized for the intended application, able to meet the demands of preclinical research through clinical studies and delivers sensitive and selective biomarker immunoassays.

Concluding remarks

Although biomarker discovery benefits from the ability of multiplex assays to screen multiple potential biomarkers simultaneously, the accuracy, sensitivity and reproducibility of well-characterized single-analyte biomarker assays is essential for quantification during the later stages of biomarker development. Researchers have a range of tools at their disposal to provide fit-for-purpose methods for quantification of biomarkers, which is essential for the use of these assays to guide efficacy and safety of biotherapeutics.

Sponsorship & disclaimer

This article has been drawn from discussions from a Technology Digest article published in Bioanalysis Zone, sponsored by Gyros Protein Technologies. The opinions expressed in this feature are those of the author and do not necessarily reflect the views of Future Science Group.

Financial & competing interests disclosure

E Williams is an employee of Future Science Group and Digital Editor of Bioanalysis Zone. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

1 Lowe PJ. Applying physiological and biochemical concepts to optimize biological drug development. Clin Pharmacol Ther. 87(4), 492–[LP1] 496 (2010).

2 Agoram BM. Use of pharmacokinetic/ pharmacodynamic modelling for starting dose selection in first-in-human trials of high-risk biologics. Br J Clin Pharmacol. 67(2), 153–160 (2009).

3 Islam R, Kar S, Ritzén H et al. Recommendations for classification of commercial LBA kits for biomarkers in drug development from the GCC for bioanalysis. Bioanalysis. 11(7), 645–653 (2019).

4 Jani D, Allinson J, Berisha F et al. Recommendations for use[LP2] and fit-for-purpose validation of biomarker multiplex ligand binding assays in drug development. AAPS J. 18, 1–14 (2016).

5 Hidi, R. Multiplex immunoassays in clinical bioanalysis: the promise and challenges to implementation in clinical development. Life Science Technical Bulletin, August, 2010.

6 Jacqueline MT. Blood cytokines as biomarkers of in vivo toxicity in preclinical safety assessment: considerations for their use. Toxicological Sci. 117(1), 4–16 (2010).

7 Frances N, Bacac M, Bray-French K et al. Novel in vivo and in vitro pharmacokinetic/pharmacodynamic-based human starting dose selection for Glofitamab. J Pharm Sci. 111(4), 1208–1218 (2022).

101 Gyros Protein Technologies. Biomarker monitoring & pharmacodynamics (PD). www.gyrosproteintechnologies.com/immunoassays/biomarker-monitoring-pharmacodynamics-research (Accessed 18 January 2023)

102 Gyros Protein Technologies. Gyrolab® kits & solutions. www.gyrosproteintechnologies.com/immunoassays/products/gyrolab-kits-solutions/#PharmacokineticToxicokinetic (Accessed 18 January 2023)

White Paper

For reprint orders, please contact: reprints@future-science.com

Bioanalysis

Analytical method validation for biomarkers as a drug development tool: points to consider

Yoshiaki Ohtsu[‡,1], Seiji Tanaka[‡,2], Harue Igarashi[3], Masaaki Kakehi[4], Tamiki Mori[5], Takahiro Nakamura[6], Rui Ohashi[7], Hiroyuki Shimizu[8], Yutaka Yasuda[9], Takashige Okayama[10], Hiroyuki Kakuo[10], Hiroyuki Yokoi[11], Mizuki Horiuchi[12], Masataka Katashima[13], Ryosuke Nakamura[14], Kosuke Saito[14] & Yoshiro Saito*[,14]

[1]Kyowa Kirin Co., Ltd. 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka, 411-8731, Japan
[2]ASKA Pharmaceutical Co., Ltd. Muraoka-Higashi 2-chome, Fujisawa-shi, Kanagawa, 251-8555 Japan
[3]GlaxoSmithKline K.K., 1-8-1 Akasaka, Minato-ku, Tokyo, 107-0052, Japan
[4]Takeda Pharmaceutical Company Limited, 26-1, Muraoka-Higashi 2-chome, Fujisawa-shi, Kanagawa, 251-8555, Japan
[5]LSI Medience Corporation, 13-4 Uchikanda 1-chome, Chiyoda-ku, Tokyo, 101-8517, Japan
[6]Shin Nippon Biomedical Laboratories, 2438 Miyanoura, Kagoshima-shi, Kagoshima, 891-1394, Japan
[7]Kyowa Kirin, Inc. 212 Carnegie Center, Suite 400, Princeton, NJ 08540, USA
[8]Mitsubishi Tanabe Pharma Corporation, 2-26-1 Muraoka-Higashi, Fujisawa-shi, Kanagawa, 251-8555, Japan
[9]Toray Research Center, Inc, 10-1 Tebiro 6-chome, Kamakura-shi, Kanagawa, 248-0036, Japan
[10]Taiho Pharmaceutical Co., Ltd. 3 Okubo, Tsukuba-shi, Ibaraki, 300-2611, Japan
[11]Otsuka Pharmaceutical Co., Ltd. 463-10 Kagasuno, Kawauchi-cho, Tokushima-shi, Tokushima, 771-0192, Japan
[12]Sumitomo Dainippon Pharma Co., Ltd. 13-1 Kyobashi 1-Chome, Chuo-ku, Tokyo, 104-8356, Japan
[13]Astellas Pharma Inc. 2-5-1 Nihonbashi-Honcho, Chuo-ku, Tokyo, 103-8411, Japan
[14]National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku, Kawasaki, 210-9501, Japan
*Author for correspondence: Tel.: +81 44 270 6623; yoshiro@nihs.go.jp
‡Authors contributed equally

Biomarkers are an important drug developmental tool. Assessment of quantitative analytical methods of biomarkers is not included in any regulatory documents in Japan. Use of biomarkers in clinical evaluations and supporting the post-marketing evaluation of drug efficacy and/or adverse reactions requires assessment and full validation of analytical methods for these biomarkers. The Biomarker Analytical Method Validation Study Group is a research group in Japan comprising industry and regulatory experts. Group members discussed and prepared this 'points to consider document' covering measurements of endogenous metabolites/peptides/proteins by ligand binding assays and chromatographic methods with or without mass spectrometry. We hope this document contributes to the global harmonization of biomarker assay validation.

First draft submitted: 10 August 2021; Accepted for publication: 3 September 2021; Published online: 14 September 2021

Keywords: assay validation • biomarker • Japan • regulatory submission • study sample analysis • white paper

The use of biomarkers has become important for efficient drug development. Biomarkers that are essential in the use of specific drugs for patient stratification, and dose finding are used as *in vitro* companion diagnostics: The assessment has already been described in Japan in the Notification [1]. In contrast, the assessment (including its concept) of analytical methods for biomarkers as drug-development tools for other purposes, such as clinical study end points, has not been described in any regulatory document. Thus, sponsors validate analytical methods for these biomarkers with reference to the two currently effective guidelines in Japan [2,3], which are relevant to the drugs and normally do not apply to biomarker analytical methods, according to a survey [4].

If the results of biomarker analyses are to be used as part of the end points for clinical evaluations and supporting the post-marketing evaluation of drug efficacy and/or adverse reactions, analytical methods for these biomarkers should be fully assessed and validated. At the same time, it is important that assessments (parameters and acceptance criteria) of analytical methods for biomarkers are based on their intended use or characteristics,

newlands press

including the percentage change in biomarker levels. It can be difficult to uniformly establish acceptance criteria similar to those used for drug bioanalytical methods. Thus, the fit-for-purpose concept [5] is relatively and widely accepted. Moreover, unlike bioanalytical methods for drugs, biomarker analytical methods feature specific issues, including the need for caution due to limited characteristics or availability of reference standards, presence of analytes in matrices and variable concentration of biomarkers among individuals.

The US FDA finalized a revised guidance on bioanalytical method validation for drugs in May 2018 [6]. This guidance, which covers biomarkers, states that the fit-for-purpose concept should be applied to select and specify the necessary parameters and acceptance criteria. Furthermore, incurred sample reanalysis (ISR) should be performed in biomarker analyses. White Papers on the assessment of biomarker analytical methods have been published [4,5,7–9]. In June 2019, the Critical Path Institute (C-Path) in the USA, in collaboration with the FDA researchers, published the final version of a points to consider document on analytical methods for biomarkers [10]. This document pointed out the importance of a parallelism assessment and introduced the concept of allowable Total Analytical Error.

The use of biomarkers as drug-development tools is increasing. In countries other than Japan, biomarker assays have been actively discussed, included in White Papers, and referenced in regulation-related documents. Similar discussions, associated White Papers, and regulatory documents will also be necessary for drug development in Japan. Therefore, the expert members of the Biomarker Analytical Method Validation Study Group (hereafter referred to as Study Group) of the Japan Agency for Medical Research and Development (AMED) on 'Studies of the Acceleration of Global Harmonization for Regulating Safety and Quality Assurance of Pharmaceuticals' had some discussions. As a result, this points to consider document has been compiled and is expected to contribute toward global harmonization of biomarker validation and study sample analysis in drug development.

Scope & basic principle

This points to consider document describes the evidence-based opinion of the Study Group about method validation and study sample analyses necessary to use biomarkers as a drug development tool. This excludes *in vitro* diagnostics, such as *in vitro* companion diagnostics, and laboratory tests for which other guidelines have been issued. This document applies to the measurement of endogenous substances that include low-molecular-weight metabolites, peptides and proteins by ligand binding assays (LBAs) and chromatographic methods, including liquid chromatography (LC) and gas chromatography alone or combined with mass spectrometry (MS). The document also applies to chromatographic methods utilizing antigen-antibody reaction to separate and concentrate an analyte. In principle, this document describes the measurement of a single analyte by a method (i.e., singleplex).

The sections for validation parameters of this document include analytical methods for obtaining data of a relevant biomarker as a surrogate end point or characterization data of a drug (such as pharmacological effects and occurrence of adverse drug reactions) in support of applications for drug marketing authorization. The descriptions included in this document apply to validations of analytical methods and study sample analyses of biomarkers, in which the reliability of the measured concentrations of biomarkers is required for the inclusion of such biomarker data in the Common Technical Document (CTD) that is a part of the application dossiers for pharmaceutical product approval (the Study Group discussions assumed that biomarkers are included in CTD Summaries [Module 2]) [11]. However, for each biomarker method validation during drug development, the necessary validation parameters should be determined by the sponsor and performed based on the fit-for-purpose principle and available experimental materials. This document mainly focuses on biomarker-specific aspects and also the general analytical method validations for biomarkers in consideration of utility for readers.

In addition, the evaluation of analytical methods for purposes other than inclusion in an application dossier is performed at the individual's discretion because required validation parameters and their levels vary among biomarkers and analytical methods. Examples of points of view are shown in the addendum (found at end of this article).

Matrices

Matrices used for method validation include authentic matrices containing endogenous compounds and surrogate matrices (including stripped matrices from which endogenous compounds have been removed). When surrogate matrices, such as phosphate-buffered saline (PBS) or PBS containing bovine serum albumin, and matrices where endogenous substances are depleted by activated charcoal or an affinity column are used, it is recommended to verify the validity of this usage in the course of developing an analytical method through evaluation of parallelism wherever possible. In addition, it should be noted that selectivity, recovery, matrix effect and stability may differ

between an authentic matrix and its surrogate matrix. A validated matrix is used for study sample analysis. When the lot of a matrix is changed, the new one is used after confirming any lot-to-lot differences. When rare matrices are used, evaluation with small numbers of lot or use of surrogate matrices is acceptable.

Full validation

Unless otherwise specified, in principle a validation is performed for the following parameters according to the intended use and characteristics (context of use) of a biomarker. Acceptance criteria for each validation parameter is defined before the start of evaluation and provided in a study plan and other documents.

Reference standards, internal standards & critical reagents

Reference Standard

In the case of a low-molecular-weight analyte, the use of a chemically identical compound is recommended in principle. A stable isotope-labeled analyte may be used as a surrogate reference standard.

When an analyte is a protein or a high-molecular-weight peptide, a recombinant is often used as a surrogate reference standard. However, it should be noted that the reactivity of an antibody may differ from that of an endogenous substance (an authentic analyte) when the analytical process includes antigen–antibody reactions (including LBAs).

When a surrogate reference standard is used, evaluation of parallelism is recommended to determine any differences in reactivity between the surrogate reference standard and an endogenous substance. The quantification of an endogenous substance using a calibration standard prepared from a reference standard to assess the consistency with literature data may be helpful in determining the degree of differences from the reference standard in reactivity. When two or more surrogate reference standards are available, sponsors are encouraged to investigate differences from an endogenous substance in reactivity during method development.

Internal standards

Internal standards are used in chromatographic methods.

In the case of a low-molecular-weight analyte, the use of stable isotope-labeled internal standards is advisable. In the case of limited availability of stable isotopes, the use of related substances is also acceptable. When related substances are used as internal standards, analytical methods should be developed considering any potential differences in recovery/matrix effect and potential interference with the analyte measurement. If differences in the recovery/matrix effect are detected, it is important to confirm that its ratio between the analyte and the internal standard is consistent.

In the case of peptides, the use of stable isotopes is expected. In contrast, in the case of proteins, it is basically difficult to use stable isotopes. However, when a signature peptide is measured by LC/MS, the use of a stable isotope of the signature peptide may improve the reliability of an analytical method.

Critical reagents

A critical reagent is defined as a reagent that has a direct impact on analytical results. In general, biological matrices used for calibration standards or matrices for quality control (QC) samples, as well as antigens and antibodies in LBAs, are in the category of critical reagents. Evaluation of other reagents during development for each analytical method is recommended to see whether they are critical reagents. As the change of the critical reagent lot may affect analytical results, it is advisable to determine lot-to-lot variability of the critical reagents.

Overall considerations

It is necessary to produce and maintain a record of the source, lot number and storage conditions of reference standards, internal standards and critical reagents. It is advisable to obtain and store any other available important information (such as chemical structure, content/concentration, purity, certificate of analysis and expiry date). Evaluation of these materials before their expiry date allows extension of the expiry date of a certain period. In addition, when reference standards or internal standards are used continuously after the expiry date or when lot and manufacturer is changed, it is recommended to verify the reproducibility of calibration curves and the accuracy/precision of QC samples. For example, regarding calibration standards and QC samples, new lots/products and old lots/products may be alternatively evaluated, or the reproducibility of study samples may be confirmed. Other parameters are also evaluated as needed.

Selectivity

It should be noted that selectivity is evaluated differently between chromatographic methods and LBAs.

If the concentration of an endogenous analyte is sufficiently low and an authentic matrix is used as a calibration standard, the recommendation is that selectivity be evaluated using blank matrices obtained from at least six individual sources for chromatographic methods and from at least ten individual sources for LBAs.

If the concentration of an endogenous analyte is above the lower limit of quantification (LLOQ) and a surrogate matrix is used for a calibration standard, the number of surrogate matrices n = 1 may be acceptable for validation in both chromatographic methods and LBAs if the lot-to-lot variability in the matrix composition is quite small. In the case of a lot change of surrogate matrices, it is advisable that a new lot is used after the comparability between the new lot and the old lot is verified. Moreover, if accuracy is evaluated using an authentic matrix spiked with a reference standard (including a surrogate reference standard), the spike concentration is designated considering the concentration of an endogenous substance.

Biomarker concentrations may vary depending on the target disease. It is recommended to evaluate selectivity in target disease matrices, if the matrices are available as special matrices. When the authentic matrix is plasma or serum, a separate evaluation is recommended for hemolyzed matrices and lipemic matrices.

Specificity

Evaluation of specificity is usually not necessary in chromatographic methods, especially LC/MS, because these methods involve separation of an analyte from interfering substances. In the case of LBAs, it is advisable to evaluate specificity using available substances, including related substances, activated forms and degradation products. In evaluating specificity, it is recommended that concentrations of interfering substances are similar to or higher than their physiological concentrations.

Calibration curve

A calibration range is defined as a range from the LLOQ to the upper limit of quantification (ULOQ). The range is designated considering the potential large inter-individual differences in the concentrations of biomarkers. In the case of LBAs, it is important to have a point above the ULOQ to check for the hook effect, in which suppression of the response can occur due to very high concentrations of an analyte.

When QC samples are prepared by adding a standard solution and the reference standard is a powder, it is advisable to prepare calibration standards and QC samples from separate stock solutions to ensure the accuracy of the overall analysis. When a reference standard is liquid and the homogeneity of solution is assured, calibration standards and QC samples may be prepared from the same stock solution and standard solution within the established stability period. When a reference standard is a powder and experimental results using stock solutions and standard solutions separately prepared are acceptable, subsequent calibration standards and QC samples may be prepared from the same stock solution and standard solution.

It is advisable to use authentic matrices for calibration standards if the concentrations of endogenous substances are sufficiently low (e.g., $\leq$20% of the LLOQ). In the case of LC/MS methods, a calibration curve can be prepared by mixing an authentic matrix with a stable isotope-labeled form of an endogenous substance as a reference standard. In other cases, it is a general practice to use a surrogate matrix to generate a calibration curve. Matrices for QC samples are described in section 'Accuracy and Precision.'

Generation of a calibration curve is recommended for each single analytical run (one series of continuous measurements for chromatographic methods and one plate for LBAs). The number of concentration levels of calibration standards and number of samples per one concentration level are defined in advance for each analytical method in a study protocol or other document, depending on the characteristics or context of use of biomarkers.

When a surrogate matrix or a surrogate reference standard is used for a calibration standard, evaluating parallelism in advance is recommended.

Matrix effect

When an authentic matrix with a low concentration of an analyte (e.g., $\leq$20% of the LLOQ) is available or a stable isotope is used as the reference standard, it is advisable to use the same matrix as the intended study sample to evaluate the matrix effect. When this is difficult to do, evaluation of the matrix effect using a surrogate matrix may be considered.

In the case of LC/MS methods, the recommendation is that samples obtained by spiking blank matrices containing an analyte from at least six lots with an internal standard are evaluated for the matrix effect using the matrix factor or precision.

In the case of LBAs, the matrix effect is evaluated by evaluating selectivity.

Accuracy & precision

Within-run and between-run accuracy and precision are evaluated using QC samples prepared by directly using an authentic matrix, diluting an authentic matrix with a surrogate matrix, spiking an authentic matrix with known concentrations of a reference standard (including a surrogate reference standard), or spiking a surrogate matrix with known concentrations of a reference standard (including a surrogate reference standard), depending on the concentration of a target endogenous substance in a biological sample. However, it is advisable to perform the evaluation using an authentic matrix wherever possible. With either a surrogate matrix or an authentic matrix, the recommendation is to analyze at least three replicates each of at least four concentration levels from different batches. Even when QC samples are prepared with a surrogate matrix, the use of an authentic matrix from at least one lot is advisable to evaluate accuracy and precision.

When an analyte is a low-molecular-weight substance and its reference standard with the same structure is not available, relative accuracy can be allowed. When matrices containing endogenous substances are used to prepare QC samples, concentrations of the endogenous substances in the matrices are analyzed to select a matrix that does not affect the assay or to calculate accuracy using either of the following formulas. The characteristics of biomarkers or the objectives of the evaluation are considered in selecting either equation, and the selected equation is advisable to be used consistently.

$$\text{Accuracy}(\%) = \frac{\left(\text{Concentration of the analyte in sample} - \text{concentration of the endogenous substance}\right)}{\text{Concentration of the spiked reference standard}} \times 100$$

$$\text{Accuracy}(\%) = \frac{\text{Concentration of the analyte in sample}}{\left(\text{Concentration of the endogenous substance} + \text{Concentration of the spiked reference standard}\right)} \times 100$$

The performance standard (PS) and allowable Total Analytical Error concepts proposed by the Critical Path Institute (C-Path) are helpful for establishing the acceptance criteria for accuracy and precision [10].

Parallelism

Evaluation of parallelism is not required for chromatographic methods using authentic matrices involving the chemical structure of a reference standard identical to that of an endogenous substance. However, a parallelism evaluation is advisable in other cases and is recommended especially when using surrogate matrices or surrogate reference standards. For the evaluation of parallelism, at least three serial dilutions of study samples at known concentrations with authentic or surrogate matrices are prepared. Concentration levels to be evaluated is advisable to be considered based on the range of concentrations of the study samples. For any diluted samples, concentrations measured according to the dilution factor are obtained and confirmed. In addition, it is advisable to perform the evaluation using two or more patient samples. For the evaluation of each patient sample, the number of sample analyses to be evaluated is at least n = 1. The need to predefine the acceptance criteria may be low.

In the case where high concentration study samples are not available, possible alternatives include an evaluation of parallelism using low concentration study samples alone or preparation of high concentration samples by adding a reference standard. In the case of chromatographic methods, the addition of an identical substance or its stable isotope is recommended. In the case of LBAs, the addition of recombinants is allowed. In contrast, when analyte concentrations in study samples are lower than those in samples available for the validation, the validation may be performed using a method of diluting high concentration samples with a surrogate matrix and, when low concentration samples are available at a later date, an evaluation using the method shifting from low concentration to higher concentration is acceptable.

Dilution linearity

For the evaluation of dilution linearity, high concentration samples are prepared by spiking a matrix for a calibration curve with a reference standard (including a surrogate reference standard). It is advisable that the highest possible concentration in study samples be used.

Samples are serially diluted with a matrix for a calibration curve up to the dilution factor expected during the study sample analysis to evaluate the accuracy determined from the measured concentration and the nominal concentration of the prepared samples. It is important to determine dilution linearity in advance because concentrations above a calibration range may be detected and analyzed with the dilution of samples during the study sample analysis. Evaluation of dilution linearity is not required when a reference standard (including a surrogate reference standard) is added to prepare high concentration samples for the evaluation of parallelism.

For LBAs, it is important to evaluate whether or not the method can appropriately analyze samples at concentrations exceeding the ULOQ without the influence of the hook effect.

Stability

For analytes in authentic matrices, bench top, long-term and freeze–thaw stabilities are considered essential evaluation parameters. In addition, when storage after sample processing is expected in the study sample analysis, it is recommended that stability be evaluated according to the handling method of the study samples (processed sample stability for chromatographic methods). For other evaluation parameters, such as stability in whole blood, the required evaluations of stability are performed depending on the handling method of study samples, as well as the analytical method used and the analyte. Although evaluation of the stability in whole blood during method development is recommended, and measures are implemented in the case of low stability, it should be noted that the evaluation is difficult to perform when serum is used as the matrix. It is recommended to confirm stability using low- and high-concentration samples within an approximate range of the expected concentrations of the study samples. The number of repetitions for evaluations is at least n = 3. The recommendation is that the evaluation is performed for a number larger than the expected number of cycles for freeze-thaw stability and for the period longer than the actually expected period of handling of samples for bench top stability and long-term stability.

For biomarker analysis, it is recommended to evaluate stability using available samples to the extent possible and confirm the stability based on incurred sample stability (ISS) after the start of study sample analysis for validation, since only a limited number of study samples are available at validation stage. In addition, samples spiked with a surrogate reference standard may be used to evaluate stability in the validation of an analytical method before the study samples are available. In such cases, it is important to confirm the stability at low and high concentrations within the concentration range of interest based on ISS after the start of the study sample analysis.

When a matrix of study sample is different from the matrix used for calibration standards or QC samples, the matrix for calibration or QC samples is evaluated for stability for the actual duration of storage. To confirm stability using study samples, it is advisable to use samples shortly after collection wherever possible. Even for samples passed for a certain period after sampling, stability can be evaluated based on a residual ratio relative to the initial analytical value at the start of validation.

It is advisable that stability during sample preparation be performed in the validation process to the extent possible and evaluated with actual conditions to be used in the study sample analysis.

When standard solutions prepared by dissolution and dilution at an analytical laboratory are stored, their stability is evaluated.

Carryover

The recommendation is that carryover is minimized during method development, evaluated during validation, and monitored during study sample analysis. In general, carryover is evaluated by analyzing blank samples with no response after the sample at the ULOQ.

Minimum required dilution

Evaluation of minimum required dilution (MRD) is recommended for LBAs. For biomarker analysis, however, study samples are typically diluted to various extents with an authentic or surrogate matrix with low concentrations of an endogenous substance so that concentrations are within the calibration range. Thus, a specific dilution factor could not be applied to the same analytical method in many cases.

The recommendation is to evaluate the effect of dilution within a possible range of concentrations. In addition, it is advisable that MRD be evaluated during method development and evaluated during the validation. Moreover, if MRD is changed during study sample analysis, partial validation is necessary.

Partial validation

Partial validation is performed when minor changes are made to a fully validated analytical method. The parameters in a partial validation are determined according to the extent and nature of the changes made to the method (following are referred to the Japanese guideline for drugs in biological fluids [2]).

Typical changes subjected to a partial validation are as follows: analytical method transfers between laboratories, changes in analytical instruments, changes in calibration range, changes in sample volume used for analysis, changes in anticoagulants, changes in sample processing procedures or analytical conditions, changes in sample storage conditions, new use of rare matrices, changes of the critical reagent lot and changes in the MRD.

Acceptance criteria used in a partial validation are the same as the predefined acceptance criteria used in the full validation in principle.

Cross validation

Cross validation is conducted, for example, when samples are analyzed in multiple laboratories within a single clinical study or when measured concentrations are compared between analytical methods using different platforms after a full or partial validation. QC samples and study samples are analyzed, and the mean accuracy at each concentration of QC samples and the concentration differences in the measurement of study samples are evaluated.

More specifically, in a cross validation, the recommendation is that the mean accuracy of QC samples (low-, mid- and high-levels) is evaluated by at least three replicates at each level, considering intra- and interlaboratory precision. In addition, for an evaluation using study samples, the recommended sample size is at least 30 samples, depending on the characteristics of an analyte. It is advisable to include samples from many individuals in selecting samples in consideration of a concentration distribution wherever possible. Even a single analytical run of study samples is acceptable. Acceptance criteria for evaluation values are predefined based on the intended use or characteristics of the biomarkers and provided in a study protocol or other document.

Study sample analysis

Study samples are biological specimens subjected to bioanalysis. Study sample analysis should be carried out using a validated analytical method. During analysis, the study samples should be handled under conditions that ensure stability, and analyzed along with calibration standards and QC samples within a confirmed period of stability. In representative clinical studies, such as those using biomarkers as an important evaluation parameter, it is advisable to confirm the reproducibility of an analytical method per matrix by performing ISR. If carryover is a concern, the recommendation is that carryover is evaluated in each analytical run for its effects on the results of the study sample analysis.

Unless otherwise specified, acceptance criteria for the following parameters (see below sections, Calibration curve, QC Samples and Incurred sample reanalysis) are defined according to the intended use and characteristics of the biomarkers before the start of measurement of study samples and provided in a study plan. Depending on the results of an analytical method validation, the analytical method may be required to be checked using subject samples before the study sample analysis.

Calibration curve

A calibration curve is used to determine the concentration of the analyte of interest in the study samples. It is recommended that the calibration curve used in the study sample analysis is generated for each analytical run by using a validated method. The same model as in the validation is used for the regression equation and weighting factors of the calibration curve.

The validity of an analytical method in the study sample analysis should be evaluated by assessing the accuracy or relative accuracy of back-calculated concentrations of calibration standards at each level. If the calibration standard at the LLOQ or ULOQ does not meet the predefined acceptance criteria in the study sample analysis, the next lowest/highest level calibration standard may be used as the LLOQ or ULOQ.

QC samples

The recommendation is that QC samples are analyzed to assess the validity of the analytical method used for the calibration curve and study sample analysis and evaluated in each analytical run. For QC samples that are prepared in authentic matrices, authentic matrices diluted with surrogate matrices, authentic matrices spiked with reference standards (including surrogate reference standards) or surrogate matrices spiked with reference standards (including surrogate reference standards), it is, in principle, recommended that three different concentration levels (low, mid and high) that are within the calibration range, are analyzed in each analytical run. Use of QC samples consisting of authentic matrices is considered wherever possible.

When only QC samples consisting of surrogate matrices spiked with reference standards (including surrogate reference standards) are used, an additional concurrent analysis (not included in the assessment of acceptance criteria) of QC samples prepared by authentic matrices is advisable. This may provide useful information specific to the authentic analyte/matrix by comparison between the analytical runs or studies. In addition, it is advisable to locate the QC samples to sandwich the study samples. The validity of an analytical method in the study sample analysis is confirmed by evaluating the accuracy of the QC samples.

Incurred sample reanalysis

ISR is recommended to be performed for each matrix with samples from representative clinical studies, such as those using biomarkers for an important evaluation to characterize drugs (e.g., using biomarkers as surrogate end points in the late-stage clinical studies).

It is important that ISR is performed within a time window that ensures analyte stability. The recommendation is that, as a guide, approximately 10% of the samples are reanalyzed in cases where the total number of study samples is $\leq$1000, and 10% of the first 1000 samples (i.e., 100) plus approximately 5% of the number of samples exceeding 1000 samples are reanalyzed in cases where the total number of study samples is $>$1000. The samples are advisable to select in consideration of the range of concentrations of biomarkers in study samples. The validity of an analytical method in study sample analysis should be confirmed by evaluating the assay variability.

Points to note

Commercial kits

Commercial kits are sometimes used for the analysis of biomarkers. Commercial kits include kits that authorized as *in vitro* diagnostics or medical devices for clinical laboratory tests and research use-only kits. In principle, commercial kits should be validated at each site.

For clinical laboratory kits, the necessity of validation for their intended use, and validation parameters may be determined on a case-by-case basis, depending on the intended use or characteristics of biomarkers.

For research use-only kits, full validation is conducted without using validation information accompanying the kit. It is advisable to make a decision on use after fully considering whether the relevant kit meets the intended use or characteristics of the relevant biomarker (such as calibration range or specificity). It is acceptable to consider the kit's expiry date determined by the manufacturer. In addition, it is advisable to evaluate a reference standard provided with a research use-only kit using other commercially available products as needed, with careful attention to differences from an endogenous substance of interest. When lot of the kit is changed, the recommendation is to confirm that differences in the measured concentration of an analyte in the same sample between the lots are acceptable. Research use-only kits may become unavailable, and alternative approaches to such kits should be considered.

Reanalysis

It is important that possible reasons for reanalysis of study samples, number of replicates and decision criteria to select the value to be reported should be defined before the start of the reanalysis of study samples.

Reasons for study sample reanalysis include as follows (selected referring to [12]):

- Failure of calibration standards and QC samples to meet the predefined acceptance criteria
- Concentration obtained is above the ULOQ
- Concentration observed is below the revised LLOQ in runs where the lowest calibration standard has been rejected from a calibration curve, resulting in a higher LLOQ compared with other runs
- Improper sample injection or malfunction of equipment

Table 1. Examples on evaluation of analytical methods for purposes other than inclusion in a drug application dossier.

Parameter	Screening assay	Qualified assay
Scope and basic principle	–	–
Matrix	A surrogate matrix is acceptable	A surrogate matrix is acceptable, but evaluation using an authentic matrix is advisable
Full validation	–	–
Reference standards, internal standards, critical reagents		
Reference standards	Required; a surrogate reference standard is acceptable in the case of large molecules (for ligand binding assays: LBAs)	Same as on the left
Internal standards	Its usage is advisable	Same as on the left
Critical Reagents	It is advisable to define critical reagents for each analytical method during method development	In addition to the information on the left, it is advisable to confirm the difference between lots in the case where two or more lots exist
Overall	It is necessary to record and store the source, lot number and storage conditions	Same as on the left. In addition, in the case of lot change, it is advisable to confirm the difference between lots of reference standards, internal standards or critical reagents
Selectivity	Confirmation at n = 1 or more is advisable	Confirmation is advisable
Specificity	It is advisable to confirm the reactivity with an endogenous analyte. In principle, it is not necessary to confirm the reactivity with related substances. It is possible to use a reference standard provided with a kit	It is necessary to confirm the reactivity with an endogenous analyte. It is advisable to confirm the reactivity with related substances. It is advisable to verify a reference standard provided with a kit
Calibration curve	Required	Required
Matrix effect	Not required	Advisable
Accuracy and precision	Confirmation of accuracy/relative accuracy using a surrogate matrix is sufficient. Same as for precision. Fewer concentration levels or analytical runs are allowed	Evaluation using an authentic matrix is advisable, but fewer concentration levels or analytical runs are allowed. Confirmation of accuracy/relative accuracy using a surrogate matrix is acceptable
Parallelism	Not required	Advisable to confirm
Dilution linearity	LC: Not required LBA: Advisable	Advisable to confirm
Stability	Confirmed on a case-by-case basis only for bench top stability	Evaluation of bench top, long-term and freeze-thaw stability is advisable
Carryover	Not required	Advisable to confirm
MRD	It should be determined what MRD is used	Same as on the left
Partial validation	Not required. For LBAs, confirmation is advisable in case of changing MRD or critical reagent lot	Advisable to perform, as described in this document
Cross-validation	Situations requiring cross-validation is less likely to occur normally	Advisable to perform
Study sample analysis		
Calibration curve	Required	Required
QC samples	Advisable to analyze	Required
ISR	Not required	On a case-by-case basis
Points to note		
Commercial kits	Direct use is acceptable	It is advisable to evaluate the integrity of a reference standard
Reanalysis	Advisable to do in case of obviously abnormal values	Same as on the left; in addition, reanalysis is advisable to be performed after the criteria for reanalysis is predefined

Screening assay and Qualified assay are defined in the text.
ISR: incurred sample reanalysis; MRD: Minimum required dilution; QC: Quality control.

- Diluted study sample is below the LLOQ

It is advisable to document the reanalyzed samples and the initial value, reason for reanalysis, value obtained in the reanalysis, final accepted value and justification for the acceptance considered.

In cases where the first analysis yields a non-reportable result (e.g., concentration above the ULOQ or equipment malfunction), a single reanalysis is considered sufficient. In cases where the obtained value needs to be confirmed, the recommendation is that replicate determinations are performed if sample volume allows.

The safety of trial subjects should take precedence over any other aspect of the trial. Consequently, there may be circumstances when it is necessary to reanalyze specific study samples for the purpose of an investigation.

*Addendum: evaluation of analytical methods for purposes other than inclusion in an application dossier**

For the evaluation of an analytical method for purposes other than inclusion in an application dossier, such as exploration of biomarkers and in-house decision-making, sponsors primarily decide on the required evaluation parameters and their levels for each method of the biomarker, because they vary considerably according to the characteristics and intended use (context of use) of each biomarker. In other words, evaluation parameters and levels should be selected with due consideration by the sponsor uniquely. Examples of the following possible circumstances are listed in the accompanying Table 1. They include:

- Screening assay: This kind of assay assumes the stage of screening multiple biomarker candidates and investigation on the presence or absence of changes in biomarkers, which are evaluated in an exploratory manner.
- Qualified assay: This kind of assay assumes the stage prior to the reliability level of analytical values to be included in a drug application dossier intended by this points-to-consider document. This assay assumes to provide that measured concentrations can be compared between different analytical runs.
- Biomarker assay validation levels where their data are included in the drug application dossier used as a surrogate end point or as data to claim characteristics (such as pharmacological effects or occurrence of adverse drug reactions) of a drug, as described in the section 'Scope and Basic Principle'.

Acknowledgments

The authors thank C Sudo (National Institute of Health Sciences) for secretarial assistance.

Financial & competing interests disclosure

This study was supported in part by grants for Research of Regulatory Sciences on Drugs and Others, and Development of New Drugs, from the Japan Agency for Medical Research and Development. Y Ohtsu and R Ohashi are employees of Kyowa Kirin. S Tanaka is an employee of ASKA Pharmaceutical. H Igarashi was an employee of GlaxoSmithKline K.K. at the time of the research. M Kakehi is an employee of Takeda Pharmaceutical Company Ltd. T Mori is an employee of LSI Medience Corporation. T Nakamura is an employee of Shin Nippon Biomedical Laboratories. H Shimizu is an employee of Mitsubishi Tanabe Pharma Corporation. Y Yasuda is an employee of Toray Research Center. Y Okayama and H Kakuo are employees of Taiho Pharmaceutical. H Yokoi is an employee of Otsuka Pharmaceutical Co. M Horiuchi is an employee of Sumitomo Dainippon Pharma Co. Ltd. M Katashima was an employee of Astellas Pharma Inc. at the time of the research. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Disclaimer

The opinions and perspectives presented in this paper are personal and not the official statement of National Institute of Health Sciences or Ministry of Health, Labour and Welfare of Japan.

References

1. Japan Ministry of Health, Labour and Welfare. Technical Guidance on Development of In Vitro Companion Diagnostics and Corresponding Therapeutic Products, December 26, 2013 (in Japanese). https://www.pmda.go.jp/files/000157570.pdf
2. Japan Ministry of Health, Labour and Welfare. Guideline on Bioanalytical Method Validation in Pharmaceutical Development. July 11, 2013. https://www.pmda.go.jp/files/000206209.pdf
3. Japan Ministry of Health, Labour and Welfare. Guideline on Bioanalytical Method (Ligand Binding Assay) Validation in Pharmaceutical Development. April 1, 2014. https://www.pmda.go.jp/files/000206208.pdf
4. Ohtsu Y, Matsumaru T, Katashima M *et al.* Biomarker assay validation for clinical trials: a questionnaire survey to pharmaceutical companies in Japan. *Bioanalysis* 11(2), 55–60 (2019).
5. Lee JW, Devanarayan V, Barrett YC *et al.* Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm. Res.* 23(2), 312–328 (2006).

6. US FDA. Bioanalytical Method Validation; Guidance for Industry. May 2018. https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf

7. Timmerman P, Herling C, Stoellner D *et al.* European Bioanalysis Forum. European Bioanalysis Forum recommendation on method establishment and bioanalysis of biomarkers in support of drug development. *Bioanalysis* 4(15), 1883–1894 (2012).

8. Hougton R, Gouty D, Allinson J *et al.* Recommendations on biomarker bioanalytical method validation by GCC. *Bioanalysis* 4(20), 2439–2446 (2012).

9. Arnold ME, Booth B, King L, Ray C. Workshop report: Crystal City VI-bioanalytical method validation for biomarkers. *AAPS J.* 18(6), 1366–1372 (2016).

10. Biomarker Assay Collaborative Evidentiary Considerations Writing Group, Critical Path Institute. Points to Consider Document: Scientific and Regulatory Considerations for the Analytical Validation of Assays Used in the Qualification of Biomarkers in Biological Matrices, June 2019. https://c-path.org/wp-content/uploads/2019/06/evidconsid-whitepaper-analyticalsectionv2019.pdf

11. ICH M4. Organisation of the common technical document for the registration of pharmaceuticals for human use, June 15, 2016. https://database.ich.org/sites/default/files/M4_R4__Guideline.pdf

12. ICH M10. Bioanalytical method validation (draft), February 26, 2019. https://database.ich.org/sites/default/files/M10_EWG_Draft_Guideline.pdf

Commentary

For reprint orders, please contact: reprints@future-science.com

Bioanalysis

A guided approach to preclinical bioanalysis of proteins using immunoassays for pharmacokinetic and pharmacodynamic assessments

Padmanabhan Eangoor*,[1]

[1]Pharmacokinetics, Pharmacodynamics & Drug Metabolism, Merck & Co., 33 Avenue Louis Pasteur, Boston, MA 02115, USA

*Author for correspondence: Tel.: +770 356 1860; padmanabhan.eangoor@merck.com

First draft submitted: 14 July 2020; Accepted for publication: 21 July 2020; Published online: 6 August 2020

Keywords: bioanalysis • immunoassay • LBA • method development • PK/PD • preclinical • protein quantitation

Drug discovery is a rapidly transformative process which includes target identification, target validation, lead identification and lead optimization. As projects progress through these stages the bioanalytical needs also change accordingly. Developing suitable bioanalytical methods can provide valuable data on pharmacokinetics and pharmacodynamics (PK/PD) supporting critical efficacy and safety studies at different stages of a project thereby laying a solid foundation to project's progress along the stages. At the early discovery stage, multiple molecules from different modalities are considered simultaneously. There is a wide variety of modalities to choose from depending on the target and its location for example, conventional small molecules, peptides, monoclonal antibodies, antibody-drug conjugates, nanobodies, therapeutic fusion proteins, bispecific biologics and oligonucleotides. The fast pace of discovery in biotech industry along with the rapidly evolving modality landscape is driving the evolution of bioanalytical field accordingly. This evolution can be seen mostly in terms of novel technologies that were developed to shorten the assay run time, multiplex analysis, improve sensitivity, lower sample volume consumption and enable automation among others.

The focus of this article is on preclinical bioanalysis in biotech industry, specifically ligand-binding assays (LBA). For the sake of simplification only LBA for protein therapeutics conducted in sandwich format is evaluated in this communication, in most instances referencing a monoclonal antibody. Though there are multiple advancements in technologies the focus here will be on commercialized and industry-popular techniques. The corresponding references in this article can direct the readers to a much-detailed understanding of the respective topics. Three most popular industry techniques ELISA, electrochemiluminescence (ECL; MSD, NJ, USA) and Gyrolab® (Gyros Protein Technologies; Uppsala, Sweden) are discussed here in detail [1]. Techniques that are not regularly used but considered for special applications are covered briefly: ultrasensitivity (Single Molecule Counting [SMC™])-based Erenna® (Singulex Inc, CA, USA) Single Molecule Array (Simoa™)-based SRX (Quanterix Corporation, MA, USA), immuno-polymerase chain reaction (PCR; Imperacer®, Chimera Biotec GmbH Dortmund, Germany) and MSD S-PLEX (MSD) and multiplexing (Luminex xMAP®, Luminex Corporation; TX, USA), MSD U-Plex and Quanterix (SR-X and SP-X). Method development is a major part of discovery bioanalysis therefore it is discussed as a separate section along with special consideration to tolerance assessments and molecular integrity assessments.

Discovery bioanalysis hinges upon five important parameters: sample volume requirement, dynamic range, assay run time, sensitivity and automation. These parameters are briefly evaluated in this article along with the possible circumstances where they play a major role and guidance to assay platform selection is provided accordingly.

- Sample volume consumption: most serum or plasma samples collected from mice have a volume of approximately 50 µl for nonserial sampling. With an attempt toward three R's (replacement, reduction and refinement) in animal research, multiple microsampling approaches are being explored which further reduces the volume drastically [2]. Additionally, while working with matrices like mice cerebrospinal fluid only 2–5 µl of sample volume will be available. The sample volume obtained should be enough to analyze sample at least twice considering an

newlands press

unexpected loss of samples the first time. Therefore, the techniques that offer low sample volume usage are preferred.

- Dynamic range: it is beneficial for an assay to operate with a broad dynamic range (at least 3–4 logs). For PK analysis this allows high minimum required dilution to minimize matrix effects along with providing the required sensitivity for low dose samples. Similarly, for PD, it allows parallelism to be tested in a broader range which thereby allows high sample dilution for abundant biomarkers. For biomarker measurements the dilution can additionally help minimize the matrix difference between samples and buffer based standard curve (a surrogate matrix) which leads to more accurate data generation.
- Assay run time: assay run time is the time needed to perform an assay while analyzing samples for PK/PD assessment and considering an assay was previously developed for that application. This also assumes that the reagents have been labeled, buffers have been prepared and a suitable instrument has been prepped for the assay. In a non-GLP preclinical bioanalytical laboratory, the time is of the essence and the end goal in most cases is to keep the assay run time short while providing reliable and reproducible data. This increases the efficiency of the laboratory and allows the department to support multiple programs simultaneously. The assay run time can range between 1 and 5 h (with an additional overnight capture step for some techniques) to analyze a plate of 96 samples depending on the technique used.
- Sensitivity: most PK assays for traditional monoclonal antibodies require a LLOQ in the range of low ng/ml. Some highly potent PK molecules (requiring low dose) and low abundance (or downregulated) biomarkers may need higher sensitivity. A platform selection should be made for these applications on a case-by-case basis. Moreover, sensitivity is highly reagent dependent, therefore obtaining highly specific and high affinity reagents can greatly increase the sensitivity of an assay [3].
- Automation: the automation platforms can reduce the assay run time for analysts at bench and increase the efficiency of the bioanalytical group. In a preclinical setup full automation of a platform can sometimes be beneficial in cases where a method is developed and routine sample analysis is expected. For method development, method optimization and method qualifications for matrix or species changes, flexibility to modify assays on the platforms is required along with automation. In addition to assay automation, preparation of standards and quality controls can be automated which can save time, lower the usage of precious reference material and reduce manual pipetting errors. While some platforms like Gyrolab allow assay automation, platforms like Hamilton and HP D300 allow automation in transferring liquids or preparing standards and QCs.

ELISA

A traditional ELISA has been the most commonly used technique for decades for protein quantification measurements, therefore it is one of the most reliable techniques. Even today most of the commercial assay kits for biomarkers are ELISA based. Most of the comparisons of novel technologies are made with ELISA considering this technique as a gold standard. However, this technique has drawbacks, for example long assay run time of approximately 4–5 h, large sample volume consumption (for an assay volume of 100 µl), low sensitivity with LLOQ in most cases close to low-mid ng/ml and a short dynamic range of 2–3 logs. This technique is still attractive for some applications in preclinical bioanalysis like monoclonal antibody PK in serum. The platform also provides flexibility to evaluate different assay components in the early stages of method development. Recent advancements have encouraged bioanalysts to pursue other techniques.

Electrochemiluminescence (MSD)

MSD's ECL technology is widely popular in the industry for bioanalysis. It offers the advantages of ELISA like flexibility in assay development and additionally excels in other areas like smaller sample volume requirement (assay volume of 25–30 µl) better sensitivity (low-mid pg/ml) and broad dynamic range (4–5 logs). The assay run time is toward the high end of approximately 3–4 h but considering the other advantages this platform offers, assay time can be considered a reasonable compromise. This platform allows running multiple 96-well plates simultaneously, therefore requiring the same amount of time for one or multiple plates which can be advantageous when analyzing hundreds of samples. This platform is not automated therefore it requires analyst's attention at every step of the assay. Additionally, the platform provides laboratory information management system integration and US FDA 21 Code of Federal Regulations (CFR) part 11 compliance allowing assays to be easily transferred to GLP environment.

Gyrolab

Gyrolab is 'beads on a compact disc (CD)' platform and is automated. Its microfluidic system along with centrifugal operation allows working with small volumes efficiently, so most analyses conducted in duplicates require sample volume less than 5 µl [4]. The unused samples can be rerun from the same plate. Each CD can analyze 112 samples (on a 20 or 200 nl CD) or 96 samples (on a 1000 nl CD) and one CD can be analyzed in an hour. An excellent feature of Gyrolab is that CD selection can be made to adjust dynamic ranges toward lower or higher concentrations. The basic xPlore™ instrument allows analyzing one CD at a time unattended while the xPand™ instruments allows up to 5 CDs (1 h/CD run time). The dynamic range in most cases is 3–4 logs. The sensitivity of this platform is comparable to that of MSD's ECL platform (low-mid pg/ml). Using this platform is highly advantageous in terms of short assay time, elimination of laborious manual pipetting and attention at every step of the assay. This makes the platform very attractive for both method development and sample analysis. Additionally, the platform provides laboratory information management system integration and FDA 21 CFR part 11 compliance allowing assays to be easily transferred to GLP environment. The technique is undoubtedly very efficient for regular preclinical PK/PD analysis of large molecules. One possible drawback with Gyros is that if an assay was developed on a plate-based format, the same reagents can give a suboptimal performance when transferred to Gyros and may need significant optimization for the best performance [5]. This is due to the extremely low incubation time on Gyros which require high association rate capture reagents which is not needed for plate-based assay where incubation time offsets this need [6]. Therefore, if using Gyros, it is beneficial to screen the reagents and then check the performance on the same platform.

Ultrasensitive techniques

Certain projects and matrix analyses may need ultrasensitive assays with fg/ml–low pg/ml level sensitivity. Some of those situations are encountered while working with low abundance biomarkers, PK analysis of potent molecules and handling analytes that are highly susceptible to matrix effects where excess dilution can lower sensitivity on some of the assay platforms. Currently available platforms for ultrasensitivity that allow in-house method development include Erenna from Singulex utilizing single molecule counting, Simoa based digital ELISA from Quanterix on SR-X platform and immuno-PCR-based Imperacer from Chimera Biotec GmbH. Detailed reviews of these three ultrasensitive techniques along with less popular techniques have been published previously [3,7]. All three techniques require either a vendor-provided or a special instrument for analysis. All three techniques allow users to develop homebrew assays. Immuno-PCR can also be developed independent of vendor provided reagents or instruments when a real-time quantitative PCR (qPCR) instrument (thermal cycler fitted with a fluorimeter) is available [8,9]. Some of the unconventional aspects which might need special attention in using these techniques are magnetic bead conjugation for Simoa, DNA conjugation to the detection reagent and contamination-free operation for immuno-PCR. Another ultrasensitive platform is MSD's S-PLEX which provides fg/ml level sensitivity using its proprietary TURBO-BOOST antibody label and TURBO-TAG reagent. Currently, the S-PLEX assay reagents are not available for purchase and the assay can only be developed at MSD. Subsequently, this platform will be an attractive option when the reagents are available to develop homebrew assays.

Multiplexing techniques

Multiplexing the assays can save time and precious samples for analysis. However, the multiplexed assays have a limitation that they have been strictly optimized to work in the specified range and matrix. Different analytes react differently to different matrices and dilutions, so a deviation from the optimized parameters for one analyte can force a re-optimization of the multiplexed assay. In preclinical bioanalysis, multiple therapeutic molecules, multiple variants of a therapeutic molecule, multiple combinations of therapeutic molecules and multiple biomarkers are explored. So, a flexibility in assays is very critical and multiplexing may not be very helpful on a regular basis. Under special circumstances where sample volume is limited, multiplexing can be explored. Some of the platforms that allow homebrew multiplexing are: MSD's U-PLEX platform which can multiplex up to ten analytes using its multi-array based, spatially separated, ECL detection, Luminex's xMAP which can analyze 50–500 analytes utilizing uniquely dyed beads and depending on the instrument used (MAGPIX, LUMINEX 200 or FLEXMAP 3D), Quanterix SR-X can multiplex up to six analytes using six unique beads and Quanterix SP-X can multiplex up to ten analytes using its planar array based spatially separated chemiluminescence imaging technology. Among these, MSD allows a straightforward assay conversion from its standard gold singleplex platform to its U-PLEX platform [10] and Quanterix's SR-X and SP-X platforms can provide ultrasensitivity while multiplexing.

Method development

A discovery bioanalytical group is the first place a molecule's bioanalytical assay is developed and this is the stage which consumes the most time before the assay is ready for sample analysis. While developing methods for molecules, it is important to have an end goal in mind. The major points to consider before beginning method development are knowing what matrices are analyzed, sample volume available and sensitivity needed. In addition, it is important to know what the expected matrix concentrations is based on the reference studies and what is the assay range needed to analyze samples with multiple dosing levels. At the very early stages, most methods are fit-for-purpose and then more rigor is added as the molecule progresses along the project. The minimal requirement for method development is to screen for optimally working reagents, set an minimum required dilution (for PK) or parallelism range (for PD; standards prepared in a surrogate matrix), define assay range and test for accuracy and precision. When unclear, it is good to refer to internal guidance documents and published literature for an optimal assay development [11–14]. The selection of assay platforms must be made based on the end goal and ease of developing the assay on a platform. Additionally, under some circumstances assay tolerance and analyte's molecular integrity needs to be tested, so the assay should be developed accordingly.

Tolerance assessments

For programs in which large molecule therapeutics target the soluble ligands in circulation, the major challenge with bioanalytical assay development is to establish tolerant assays for the target and the therapeutic. A therapeutic dosed to target a soluble ligand can result in multiple molecular species in matrices: free ligand, bound ligand, free drug and bound drug. Depending on the stage and the program's need, the team may be interested in free, bound or total (free + bound) target and drug, therefore multiple bioanalytical assays will be needed to quantify these analytes [15]. Finding the right reagent pair for each of these assays and having a good understanding of binding kinetics and equilibrium is crucial for optimal assay performance. It is also noteworthy, while measuring the bound form of target or drug, reagents can interfere with the bound component (either a complete epitope blocking or steric hinderance) thereby affecting the measured concentrations. Conducting tolerance assessments can help select the right reagents. Moreover, epitope mapping via surface plasmon resonance (SPR)-based platforms can allow an informed decision making in selecting a reagent pair. SPR based experiments can be invaluable while screening for reagents with noncompeting epitopes especially in the selection of drug or target tolerant reagents [6].

Molecular integrity

Some of the novel modalities like therapeutic fusion proteins, bispecific and multispecific biologics offer higher selectivity to targets and half-life extension but also pose complex problems like proteolytic degradation, subunit clipping, deamidation or oxidation *in vivo* which can affect safety and efficacy [16]. Characterizing the different molecular species and quantifying them can tremendously help engineer stable constructs. Immunoassays alone will not be enough in such cases. Other orthogonal bioanalytical methods like Western blots, LC–high-resolution mass spectrometry (intact LC–MS or top-down LC–MS) and hybrid LBA/LC–MS (signature peptide based immunoaffinity capture LC–MS/MS or bottom-up LC–MS) will be needed to answer all the questions related to molecular integrity. Immunoassay is the most sensitive platform among the three platforms and can assess the conformational and, to some extent, the functional integrity of the ligands in the molecule via differential assays [17]. Intact LC–MS can provide comprehensive information about the relative abundance of the different molecular species present in matrices, but it suffers from poor sensitivity. Recently, efforts were made to improve the sensitivity for monoclonal antibody quantitation via intact LC–MS [18]. However, their applicability to less stable molecules like fusion proteins is yet to be tested. Peptide LC–MS can specifically quantify multiple peptides from different regions of the molecule but cannot inform on the protein conformation. So, a combination of these techniques will be needed based on the project needs.

Summary

In summary, most of the discovery PK/PD immunoassays can be addressed on MSD or Gyrolab platform. Gyrolab provides additional advantages like short run time, small sample volume requirement and automation which makes it attractive for preclinical bioanalysis. Regardless of the platform, it is highly recommended to screen the reagents and test assay performance on the same platform as the final desired assay. However, ultrasensitive and multiplexing platforms are special application platforms and do not normally allow high throughput screening of reagents. Therefore, it is advantageous to screen the reagents on ELISA, MSD or SPR-based platforms initially

and then optimize the methods on specialty platforms. While working on soluble target programs, it is beneficial to utilize SPR from the very beginning of the method development to overcome tolerance issues. Assay platform selection should be made with an end goal in mind. While it is ideal to achieve perfection with assay parameters, one should be realistic in terms of assay limitations and project needs, therefore be willing to compromise with sample volume usage or assay run time to meet the needs. Good communication with the project team can help prioritize efforts, address needs and meet timelines. When the goals of the project are expected to change, it is also important to keep the assay format flexible enough to allow an easy transfer between the platforms. Each platform differs most uniquely in terms of their detection reagents (Sulfo-tag, horseradish peroxidase [HRP] or other enzymes), so biotinylation of the reagents can allow a seamless transfer between these platforms when used with their respective streptavidin-tagged reagents. In some platforms that require biotinylation of capture regents (Gyrolab) these biotinylated reagents can serve as capture. Whether analyzing free versus bound fractions in soluble target programs or analyzing molecular integrity of complex molecules the versatility of the immunoassays should be exploited and the assay platforms should be considered accordingly. While the field of immunoassay is evolving rapidly, orthogonal bioanalytical platforms like LC–MS is also evolving in parallel, which sometimes can complement immunoassays. Therefore, a good understanding of the orthogonal platforms and timely direction of the teams to those platforms can lead to the success of those projects.

Acknowledgments

The author gives thanks to C Fandozzi, K Bateman, V Pucci, S Zhang, HW Chong and N Chavan for reviewing the article and providing feedback.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

References

1. Dudal S, Baltrukonis D, Crisino R *et al.* Assay formats: recommendation for best practices and harmonization from the Global Bioanalysis Consortium Harmonization Team. *AAPS J.* 16, 194–205 (2014).
2. Patel SR, Bryan P, Spooner N, Timmerman P, Wickremsinhe E. Microsampling for quantitative bioanalysis, an industry update: output from an AAPS/EBF survey. *Bioanalysis* 11(7), 619–628 (2019).
3. Fischer SK, Joyce A, Spengler M *et al.* Emerging technologies to increase ligand binding assay sensitivity. *AAPS J.* 17(1), 93–101 (2015).
4. Roman J, Qiu J, Dornadula G, Hamuro L, Bakhtiar R, Verch T. Application of miniaturized immunoassays to discovery pharmacokinetic bioanalysis. *J. Pharmacol. Toxicol. Methods* 63(3), 227–235 (2011).
5. Leary BA, Lawrence-Henderson R, Mallozzi C *et al.* Bioanalytical platform comparison using a generic human IgG PK assay format. *J. Immunol. Methods* 397(1–2), 28–36 (2013).
6. Duo J, Bruno J, Kozhich A *et al.* Surface plasmon resonance as a tool for ligand-binding assay reagent characterization in bioanalysis of biotherapeutics. *Bioanalysis* 10(8), 559–576 (2018).
7. Spengler M, Adler M, Niemeyer CM. Highly sensitive ligand-binding assays in pre-clinical and clinical applications: immuno-PCR and other emerging techniques. *Analyst* 140(18), 6175–6194 (2015).
8. Lind K, Kubista M. Development and evaluation of three real-time immuno-PCR assemblages for quantification of PSA. *J. Immunol. Methods* 304(1–2), 107–116 (2005).
9. Attallah C, Rodríguez MC, Lozano V, Etcheverrigaray M, Oggero M. Design and validation of an immuno-PCR assay for IFN-α2b quantification in human plasma. *Bioanalysis* 11(23), 2175–2188 (2019).
10. Woodbury N, Bald E, Geist B, Yang T-Y. Application of multiplexed pharmacokinetic immunoassay to quantify *in vivo* drug forms and coadministered biologics. *Bioanalysis* 11(24), 2251–2268 (2019).
11. Stevenson LF, Purushothama S. Parallelism: considerations for the development, validation and implementation of PK and biomarker ligand-binding assays. *Bioanalysis* 6(2), 185–198 (2014).
12. Lee JW, Devanarayan V, Barrett YC *et al.* Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm. Res.* 23(2), 312–328 (2006).
13. Stevenson L, Kelley M, Gorovits B *et al.* Large molecule specific assay operation: recommendation for best practices and harmonization from the Global Bioanalysis Consortium Harmonization Team. *AAPS J.* 16(1), 83–88 (2014).

14. Desilva B, Smith W, Weiner R *et al.* Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm. Res.* 20(11), 1885–1900 (2003).

15. Lee JW, Kelley M, King LE *et al.* Bioanalytical approaches to quantify 'total' and 'free' therapeutic antibodies and their targets: technical challenges and PK/PD applications over the course of drug development. *AAPS J.* 13(1), 99–110 (2011).

16. Fandozzi C, Evans C, Wilson A *et al.* 2019 White paper on recent issues in bioanalysis: chromatographic assays (part 1 – innovation in small molecules and oligonucleotides & mass spectrometric method development strategies for large molecule bioanalysis). *Bioanalysis* 11(22), 2029–2048 (2019).

17. Pearson JT, Rock DA. Bioanalytical approaches to assess the proteolytic stability of therapeutic fusion proteins. *Bioanalysis* 7(23), 3035–3051 (2015).

18. Vasicek LA, Zhu X, Spellman DS, Bateman KP. Direct quantitation of therapeutic antibodies for pharmacokinetic studies using immuno-purification and intact mass analysis. *Bioanalysis* 11(3), 203–213 (2019).

White Paper

For reprint orders, please contact: reprints@future-science.com

Bioanalysis

Biomarker context-of-use: how organizational design can impact the implementation of the appropriate biomarker assay strategy

Kyra J Cowan[1], Michaela Golob[2], Joanne Goodman[3], Anna Laurén[4], Lene Andersen[5], Philip De Decker[6], Lien Dejager[7], Marianne Scheel Fjording[8], Peter Groenen[9], Renaud Jasnowski[10], Nicole Justies[11], Matti Kimberg[‡,12], Ulrich Kunz[13], James Lawrence[14], Mario Richter[15], Laetitia Sordé[16], Radboud van Trigt[17], Laurent Vermet[18], Alessandra Vitaliti[19], Michael Wright[20] & Philip Timmerman*[,21]

[1]Merck KGaA, New Biological Entities Drug Metabolism & Pharmacokinetics, Darmstadt, 64293, Germany
[2]Nuvisan GmbH, Bioanalysis, Neu-Ulm, 89231, Germany
[3]Integrated Bioanalysis, Clinical Pharmacology & Safety Sciences, BioPharmaceuticals R&D, AstraZeneca, Cambridge, CB21 6GH, UK
[4]Novo Nordisk A/S, Non-clinical & Clinical Assay Sciences, Global Discovery & Development Sciences, Global Drug Discovery, Maløv, DK-2760, Denmark
[5]Lundbeck A/S, Experimental Medicine & Clinical Development, Valby, DK-2500, Denmark representing Orphazyme A/S, Clinical Development, Copenhagen N, DK-2200, Denmark
[6]Argenx BV, Bioanalytics, Zwijnaarde, 9052, Belgium
[7]UCB Pharma, Development Science, Translational Biomarkers & Bioanalysis, Chemin du Foriest, Braine-l'Alleud, B-1420, Belgium
[8]BioAgilytix Labs, Lademannbogen 10, Hamburg, 22339, Germany
[9]Idorsia Pharmaceuticals Ltd., Translational Biomarkers, Allschwil, 4123, Switzerland
[10]Active Biomarkers, 60F Avenue Rockefeller, Lyon, 69008, France
[11]Roche Innovation Center Basel, Roche Pharmaceutical Research & Early Development, Basel, 4070, Switzerland
[12]Synexa Life Sciences BV, Leiden, 2333 CS, The Netherlands
[13]Boehringer Ingelheim Pharma GmbH & Co. KG, Translational Medicine & Clinical Pharmacology, Biberach an der Riss, 88397, Germany
[14]F-star Therapeutics, Cambridge, CB22 3AT, UK
[15]AbbVie Deutschland GmbH & Co KG, DMPK-BA, Knollstrasse, Ludwigshafen, 67061, Germany
[16]Sobi AG, Bioanalytical Sciences, Plan-les-Ouates, 1228, Switzerland
[17]ICON Bioanalytical Laboratories, Assen, 9407 TK, The Netherlands
[18]Sanofi Research & Development, Translational Medicine & Early Development, Biomarkers & Clinical Bioanalysis, Montpellier, 34080, France
[19]Novartis Institutes for Biomedical Research, Novartis Pharma AG, Basel, 4056, Switzerland
[20]GlaxoSmithKline, Bioanalysis Immunogenicity & Biomarkers, Stevenage, SG1 2NY, UK
[21]European Bioanalysis Forum, Brussels, 1000, Belgium
*Author for correspondence: Tel.: +32 479 910 132; chair@e-b-f.eu
‡Deceased. Matti Kimberg (co-author) passed away during the publication of this article.

Since 2011, the European Bioanalysis Forum has been discussing the topic of context-of-use for biomarker assays, in support of a cross-industry implementation of its principles. The discussions have led to the acknowledgement of the challenges that we face as an industry in implementing these principles. In addition to scientific recommendations, the European Bioanalysis Forum has addressed these challenges by providing recommendations on organizational design, and what works in both sponsor and contract research organizations, to support and enable context-of-use across biomarker strategies. Here, we highlight the key considerations for organizational design to help ensure that biomarker assays are characterized and validated according to the right context-of-use, to ensure that the right decisions based on the biomarker data can be made during drug development.

First draft submitted: 8 June 2022; Accepted for publication: 13 July 2022; Published online: 29 July 2022

Keywords: assays • biomarkers • context of use

newlands press

The European Bioanalysis Forum (EBF) has been playing a leading role in discussions with pharmaceutical and biotechnology companies, academia, health authority representatives and contract research organizations (CROs) on biomarker assay validation strategies and the principles of context-of-use (CoU) for many years, which has led to multiple contributions in international forums. These discussions and publications have included the recommendations from the 2012 and 2020 papers [1,2], workshops [3–5] and open symposia [6–8] on biomarker (BM) assay CoU. The goal of all of these interactions has been to focus on the scientific risk of applying Bioanalytical method validation (BMV) assay validation criteria related to pharmacokinetics (PK) for biomarker assays and to find a common understanding of CoU across organizations involved in biomarker bioanalysis, and a solution for cross-industry implementation of the CoU principles. The learnings from these cross-industry interactions have further shown that there remain fundamental challenges in the mindset and also in organizational design disfavoring and even preventing the implementation of CoU. As a result, the EBF biomarker team, involving many members from the EBF community, is regrouped to share and discuss how organizational design related to biomarker research (or the lack thereof) supports or in fact hampers the implementation of the CoU principles for biomarker assay validation and identify potential approaches that could be supportive of CoU implementation for companies.

CoU for biomarker assays is a detailed definition of the purpose of a biomarker assay for each analyte being investigated. This must be understood and agreed upon by all stakeholders, and documented, for example in method summaries, validation plans, and validation reports. It can be as simple as a few sentences, which define the biomarker to be assessed and why. Some of the key components to consider for a CoU statement include understanding the biology or pharmacological effect of the biomarker, including understanding the expected biological variability and why the data is needed. For example, what scientific or safety decisions could be taken based on the biomarker measurement. It is then possible to consider the options from a bioanalytical perspective, as the CoU statement supports the selection of the biomarker assay, and the corresponding assay validation strategy. Therefore, it is not a simple statement such as "to quantify the analyte", and ultimately, the CoU will help ensure the appropriate collection and interpretation of data to serve patients.

In our 2020 recommendation paper [2], we re-emphasize the following for CoU implementation to be successful:

1. Communication between all stakeholders is key and must be sustained. This can be a major challenge throughout the industry, across all kinds of company settings, given organizational structures and individual or group perceptions;
2. Stakeholder mapping is critical. Understanding the complexity of a matrix environment and knowing the stakeholders and involving them is essential;
3. Internal stakeholders may include molecule program leads, project managers, safety representatives, pharmacologists, modelers, biomarker leads, and other members of a matrixed work environment or, even more challenging, from across different regions;
4. External stakeholders may include business development representatives, project managers and scientists at CROs as well as vendors of analytical solutions, equipment and reagents;
5. All of this may require some high level, appropriate training of stakeholders to gain common ground across a variety of functions and different scientific expertise. This in turn will help ensure that the team has the right assay implemented for meaningful results, and subsequent appropriate interpretation of the data to support the right decisions.

Documenting the CoU is critical not only to ensure the right assays are set up and used, but also because institutional knowledge may change over time, with incoming new team members, or with career changes as team members leave. In addition, the purpose of the biomarker results may change from one study to the next, or the types of decisions being made based on the biomarker assay results may vary, and this should be communicated before an analyte is measured. Fundamentally, without an agreed upon CoU, there is a risk of implementing the wrong assay, with inappropriate characterization and validation of the assay, and consequently a misinterpretation of the biomarker data. This in turn can lead to incorrect decisions based on that data, all of which could ultimately negatively impact patients. Thus, an agreement across the teams on the purpose of each biomarker assay, and documentation of that CoU for each analyte, are both essential. Biomarker assay selection and characterization should be an iterative process, with modifications driven by the CoU, with data generated during assay development potentially also having an influence.

We as an industry have begun to recognize that the bioanalytical scientist must take ownership and accountability to communicate with their stakeholders and provide adequate cross-functional education. We have echoed [2], along with other papers [9], that a BM assay is distinctly not a PK assay, and thus should not be characterized as one, due to the different scientific and analytical challenges encountered [2]. The default application of PK approaches and criteria as per BMV is wrong. We recognize that CoU as a principle is a game-changer for many bioanalytical laboratories, and this remains a major challenge to fully understand and subsequently implement. The EBF decided to reach out to stakeholders within and outside of the bioanalytical community to understand cross-functional implementation of CoU principles for biomarker assays, and to understand what stimulates or hinders approaching biomarker assay validation to start from the principles of CoU. We heard common statements such as:

"*I believe that we, as assay developers, understand and apply this principle to our development and degree of validation efforts, but I don't think the rest of the world is aligned to this.*"
"*Often I feel that people cite CoU without understanding what boundaries are defined.*"
"*I guess I know what it is but not sure I fully understand what it involves.*"

And from a 2021 community poll, we still found these challenges remain: the wish for "proper guidance", to understand what is expected for the various purposes; convincing stakeholders of applying the CoU process and receiving the correct feedback from stakeholders; the tendency to think in terms of broad categories (exploratory, and primary and secondary end point); and the expectation is that an off the shelf commercial kit or prior validated method will meet the requirements of the biomarker measurement, regardless of changes in its CoU. And all the meanwhile in an environment of potential scope creep by our industry, by our community in the lab and ultimately potentially by regulatory authorities in cross-industry discussions, through the stretching of the BMV guidance for PK assays onto BM assays.

EBF biomarker team

During 2021, the EBF biomarker team re-focused their discussion to take a deeper dive into aforementioned challenges, and to discuss what could be done about them. And most importantly, how do we as bioanalytical scientists keep the momentum going to reach a common agreement across the industry what CoU really is, and how to effectively implement CoU principles [5]. The biomarker team members involved in these discussions were representatives from across the industry, including the pharmaceutical industry, mid-sized and small biotech companies and CROs. Our focus was on how to change the way of thinking and how to effectively drive the topic of CoU, internally to our organizations as well as externally. The team viewed the goal as not a repackaging of the Jean Lee or subsequent fit-for-purpose (FFP) papers [16,17], but rather a deeper dive into the strategies that support CoU implementation. The intent is to engage with the health authorities and avoid inappropriate (new) draft guidance, or the continued inappropriate implementation of BMV guidance/guidelines on biomarker assays in general [10–15], and to work together to build clarity and alignment across the industry and health authorities more intensely.

Observations by the EBF team on organizational design: sponsor perspective

We found that the following does not work in sponsor organizations toward supporting the implementation of CoU principles for all biomarker assays:

1. The absence of a biomarker strategy, particularly after lead optimization during the drug discovery phase;
2. The lack of biomarker assay expertise or relying on PK assay experts without further training;
3. Siloed operational teams, or complex team organizations, so that the input from the bioanalytical experts and their involvement in the teams is lost;
4. Fractioned responsibilities across functions without a single biomarker lead with oversight across all biomarker deliverables;
5. Applying the wrong regulations (like the BMV guidance or PK standard operating procedures (SOPs)) and check boxes;
6. A lack of scientific rationale or discussion around the biomarker analyte, which would directly influence what assay format, technology, and assay acceptance criteria would be used for any particular analyte, and therefore the team being beholden to the BMV because it provides a familiar framework for assay validation.

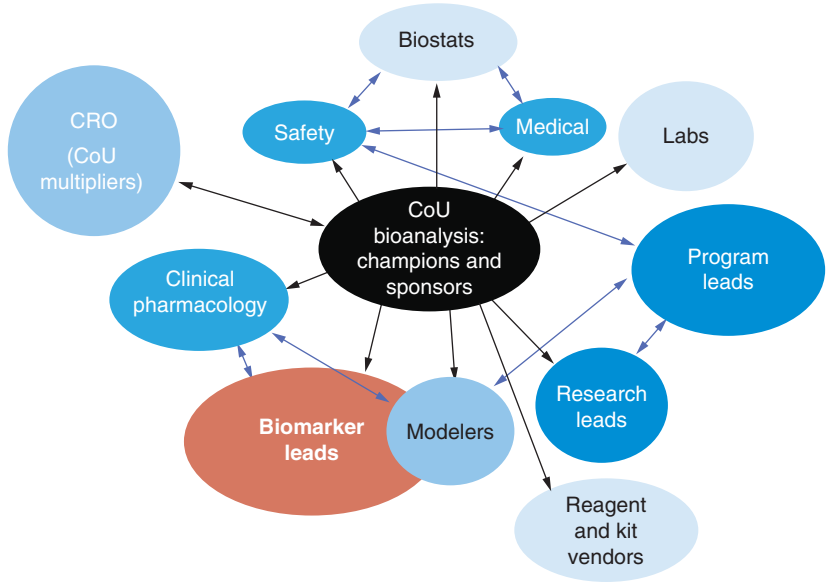

Figure 1. Stakeholder management chart. How can we ensure the principle of CoU is implemented? What is missing in the communication? How can we best educate/train? How can we make sure we are relaying the right message? How do we achieve a sense of urgency for CoU? How do we ensure consistent buy-in? Knowing the key stakeholders and understanding how to influence them to invest in CoU is critical.

We found that the following key approaches do work for pharmaceutical and biotech organizations:

1. Ensuring there is a clearly written documented biomarker strategy, and an integrated biomarker approach available to all team members, including the bioanalytical group. As part of this integration, the team members must ensure that the biology of each analyte to be measured is discussed and understood.
2. If possible, having a clearly defined, centralized biomarker group (or lead) that is accountable for the biomarker assays, operational topics, and the overarching analytical biomarker strategy expertise and corresponding responsibilities and working closely with the stakeholder:
   a) This team (lead) would ideally have an overarching view on the CoU for each biomarker, and all corresponding biomarker activities, including the assays, samples, data analysis, and documentation of the CoU;
   b) Ideally, there would also be an operational separation between decision-making biomarker assay development and characterization from the rest of the bioanalytical team responsible for regulated (PK and immunogenicity) assays.
3. If a centralized, biomarker group is not organizationally possible:
   a) Ensuring a close collaboration between bioanalytical and biomarker lead organizations if they are separate functions. Co-location of these groups or individual experts would be preferred;
   b) Close collaboration with all stakeholders to implement BM strategy;
   c) Training of laboratory staff to understand the CoU principles and fit-for-purpose assay qualifications;
   d) This would still require a high functioning matrix work environment with clear roles and responsibilities across the teams and close collaborations across departments.
4. Implementation and documentation of the CoU for each biomarker analyte measured, which would include said documentation in (or equivalent to) method summaries, in validation plans and reports, in assay specification documents, and in an online "living document" for the biomarker assay strategy. Templates for this are suggested.
5. Inclusion of the bioanalytical scientist responsible for the implementation of the CoU for any biomarker, and therefore the biomarker assay for each analyte used, in the clinical (and as needed, preclinical) protocol reviews (Figure 1).

The EBF biomarker strategy team has proposed a simple cross-functional team structure, that could easily support and ensure the consistent implementation of CoU principles (Figure 2). Here, we depict a translational

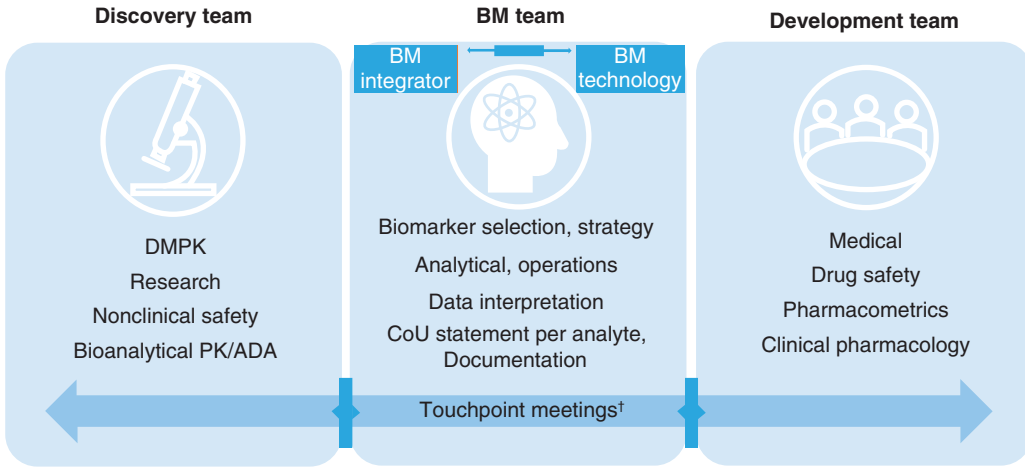

Figure 2. Schematic of the suggested cross-functional team structure, to support consistent implementation of CoU principles.
†Touchpoint meetings with BM lead from BM team would need to involve all relevant functions.

biomarker team of representative key experts. The team lead would ensure that a BM "integrator", who ensures the biomarker strategy is discussed, and a BM technology lead, who ensures the appropriate BM platforms and technologies are discussed, are a part of this team. This cross-functional BM team would work closely with members of the "discovery" and "development" teams and meet regularly through "touchpoint meetings", to ensure the BM strategy meets the goals of the drug development team in general.

The CRO perspective

The CRO perspective was also discussed by the EBF biomarker team, recognizing that CROs are a key stakeholder in implementing CoU principles and considering all the challenges that come with this. Once again, communication is key and is often the biggest issue when trying to ensure CoU principles are being followed. Optimally in a sponsor to CRO relationship, there would be an explanation of the CoU for the biomarker, to inform the decision on the biomarker assay to be used for that particular CoU, which is then followed by the selection of the analytical technology to answer the specific question. Only then would the fit-for-purpose validation parameters be defined, as well as acceptance criteria for the assay to support the analysis of any study samples. Unfortunately, the reality is usually that the initial discussion does not occur – the team found through their discussions that normally, if the CoU statement exists for any biomarker, that is not always shared with the CRO, let alone openly debated, and this often can be related to either IP concerns, or trust issues, both combined, or just simply omitted and not requested during outsourcing process. The sponsor also often suggests or recommends a commercially available kit and expects a "full validation" according to BMV principles "to be on the safe side", rather than a fit-for-purpose approach. This then removes the opportunity to select the appropriate technology to address a specific question. And in the rare instances when a CoU statement is disclosed to the CRO, it is often not truly a CoU statement. Typically, it is an oversimplified purpose statement like "exploratory", "I got this list of biomarkers to measure", or "the CoU will be shared before sample analysis". Also, the challenge in getting a CoU statement from a sponsor can be due to challenges in contracting. Operationally, a CRO can often get locked into an initial cost proposal and subsequent contract, so that having the CoU discussion ultimately becomes "out-of-scope". Alternatively, the operational team is not aware of or does not request the CoU. And finally, every client is different, and it is often a challenge to have specific business processes in place when the sponsor does not adhere to them, and to generate business it's often needed to keep the contracting process as simple as possible.

Ideally, the sponsor to CRO dynamic over biomarker assay CoU implementation should be optimized according to at least these following principles:

1. Getting the request for a proposal or service estimate;
2. Discussion with the CRO on CoU for the biomarker from the sponsor, ideally through a questionnaire;

3. Selection of a possible method, which is then discussed and agreed upon by the relevant people, specifically the bioanalytical scientists (sometimes the lab head or the principal investigator) at the CRO responsible for the validity of the biomarker data, and the sponsor stakeholder (oftentimes the clinical or biomarker team leader) accountable for the usefulness of the biomarker results for the sponsor team;
4. Proposal and agreement of fit-for purpose validation parameters, so that the biomarker assay for that particular analyte can be "fully validated" for that particular CoU;
5. Preparation of proposal/service estimate for the client.

The sponsor and bioanalytical scientist at the CRO both need to insist on a clearly written rationale for any requested analysis. A written statement on the purpose of the biomarker data must be documented, at least in the validation and sample analysis reports. Only when the CoU is clear will the sponsor and CRO be confident that the data will fit the purpose.

Conclusion

CoU is critical for patients, but it remains as a challenge to properly implement. The omission of CoU for biomarker assays is dangerous: the wrong CoU can lead to inappropriate assay acceptance criteria, poor use of resources and time, wrong decisions and ultimately failed drug development. In the regulated bioanalytical community, we must take ownership for implementing CoU principles and influence our stakeholders to do the same. As a community of scientists, we are responsible for the validity of the biomarker data, which includes ensuring that we are implementing CoU principles properly, that the CoU information is accurate and practical to implement, and understanding how that information directly affects what will be done in the lab. Analytical decisions need to be driven by science, not following a framework or categories, as the diversity and complexity of biomarker assays are wide. A framework may stifle the crucial conversations that are needed for defining the assay purpose. In addition, CoU must be re-evaluated as the purpose of the biomarker data changes and will dictate assay characterization and much later validation. In addition to previous EBF recommendations on CoU, we recognize the importance of how an organization is built around promoting and supporting CoU as a steppingstone to its successful implementation. The suggestions discussed in this manuscript can help organizations change the mindset toward CoU and show how it can be implemented through thoughtful organizational design. Going forward, the EBF will continue to support the bioanalytical and drug development community in the implementation of CoU principles for biomarker assays and connect with all stakeholders as necessary, as we believe it will improve the quality and value of the biomarker data in support of efficacious and safe drugs for patients.

Disclaimer

The views and conclusion presented in this paper are those of the European Bioanalysis Forum and do not necessarily reflect the representative affiliation or company's position on the subject.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

References

1. Timmerman P, Golob M, Goodman J *et al.* Toward decision-based acceptance criteria for bioanalytical method validation: a proposal for discussion from the European bioanalysis forum. *Bioanalysis* 10(16), 1255–1259 (2018).
2. Goodman J, Cowan K, Golob M *et al.* Update to the European bioanalysis forum recommendation on biomarkers assays; bringing context of use into practice. *Bioanalysis* 12(20), 1427–1437 (2020).
3. EBF Focus workshop: biomarker assay validation – bringing context of use into practice. https://e-b-f.eu/fw201909-slides/GoogleScholar
4. EBF Focus workshop biomarkers in pharma R&D – a roadmap from context of use to using the data. https://e-b-f.eu/fw202001-slides/GoogleScholar
5. Timmerman P, Barfield M, Cowan K *et al.* Recommendations and discussion points on immunogenicity, biomarkers, automation/technology and protein-MS from the 2021 European Bioanalysis Forum Focus Workshops. *Bioanalysis* 13(19), 1459–1465 (2021).

6. EBF Open Symposium 2019: imagine! – a New Bioanalytical Earthrise. https://e-b-f.eu/bcn2019-slides/GoogleScholar
7. EBF Open Symposium 2020: cyberspace: staying connected. https://e-b-f.eu/bcn2020-slides/GoogleScholar
8. EBF Open Symposium 2021: science – our universal language. https://e-b-f.eu/bcnnov2021-slides/GoogleScholar
9. Stevenson LF. Evolving our thinking on biomarker assay validation: are we ready for the next leap? *Bioanalysis* 11(7), 572–573 (2019).
10. Guideline on bioanalytical method validation. European Medicines Agency (2011). https://www.ema.europa.eu/en/bioanalytical-method-validation
11. US FDA. Guidance for industry: bioanalytical method validation (2018). https://www.fda.gov/downloads/drugs/guidances/ucm070107.Pdf
12. Guideline on bioanalytical method validation in pharmaceutical development. Ministry of Health, Labour and Welfare (2013). www.nihs.go.jp/drug/BMV/index.html
13. Guideline on bioanalytical method (ligand binding assay) validation in pharmaceutical development. Ministry of Health, Labour and Welfare (2014). www.nihs.go.jp/drug/BMV/index.html
14. ANVISA RDC0027/2012. http://portal.anvisa.gov.br/documents/33880/2568070/rdc0027_17_05_2012.pdf/c6edeb56-200d-4482-8a19-99fa11c33fd3
15. China Food and Drug Administration. Guidance for bioanalytical method validation. www.drugfuture.com/Pharmacopoeia/CP2015-4/363-368.pdf
16. Lee JW, Weiner RS, Sailstad JM *et al.* Method validation and measurement of biomarkers in nonclinical and clinical samples in drug development. A conference report. *Pharm. Res.* 22, 499–511 (2005).
17. Lee JW, Devanarayan V, Barrett YC *et al.* Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm. Res.* 23(2), 312–28 (2006).

Bioanalysis ZONE

Contact us

Editorial department
editor@bioanalysis-zone.com

Business Development and Support
advertising@future-science-group.com

This supplement is brought to you by Bioanalysis Zone in association with:

GYROS PROTEIN Technologies