**Fundamentals, Roles & Regulatory requirements of Bioassays in current Pharmaceutical Industries**

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**ABSTRACT**

The physicochemical and structural properties of proteins used as active pharmaceutical ingredients of biopharmaceuticals are determinants to carry out their biological activity. In this regard, the assays intended to evaluate the functionality of biopharmaceuticals provide confirmatory evidence that they contain the appropriate physicochemical properties and structural conformation. The validation of the methodologies used for the assessment of critical quality attributes of biopharmaceuticals is a key requirement for manufacturing under GMP environments.

**INTRODUCTION**

Biopharmaceuticals are heterogeneous molecules manufactured under stringent quality controls intended to ensure their batch-to-batch consistency. Accordingly, regulatory guidelines indicate the relevant characteristics or attributes that need to be evaluated to determine their quality. In this regard, different analytical methodologies are employed for the evaluation of critical quality attributes (CQAs) with respect to identity, structure, heterogeneity, purity, and functionality. The evaluation of functionality in bioassays is a fundamental part of the quality assessment of biopharmaceuticals [[1](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0005)] because it provides confirmation of the appropriateness of other physicochemical and structural CQAs; additionally, functionality assays to properly evaluate its mechanism of action. However, the development, standardization, and implementation of bioassays is a challenging task because they depend on the response of living organisms (i.e. cultured cells), the use of critical reagents, and other uncontrollable sources of variability that affect the system’s performance [[[1]](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0005), [[2]](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0010), [[3]](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0015), [[4]](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0020)].

Bioassays must be capable to reproduce *in vitro* the mechanisms of action by which a biomolecule is capable to achieve its [biological activity](https://www.sciencedirect.com/topics/pharmacology-toxicology-and-pharmaceutical-science/biological-activity) in patients. Additionally, bioassays should incorporate a reliable technique that reveals the interaction between the biomolecule and its target, commonly colorimetric, luminescent or fluorometric signals [[5](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0025)]. Accordingly, the development of bioassays requires a deep understanding of the mechanisms of action of the biomolecules under study; this is important during the design of the assay, because allows defining the critical characteristics to be evaluated based and helps to select the most appropriate analytical approaches for their assessment.

Once the characteristics to evaluate and the approach for assessment have been defined, the experimental conditions of the bioassay must be standardized, and then should be validated to demonstrate that it is suitable for its intended purpose [[[7]](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0035), [[8]](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0040), [[9]](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0045)]. The validation exercise must be focused on the evaluation of characteristics that warranty that the assay is robust under the experimental conditions in which will be performed. In general, the characteristics evaluated during a validation exercise include specificity, accuracy, precision, sensitivity and system suitability [[1](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0005),[7](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0035),[9](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0045),[10](https://www.sciencedirect.com/science/article/pii/S0731708518305363#bib0050)]. However, the validation exercise of bioassays and the stringency to evaluate each characteristic will depend on the nature of each assay, the knowledge gained during its development and standardization, as well as its intended purpose (*e.g.* R&D, Manufacturing, Quality Control, Batch Release, or Bio similarity analysis).

To evaluate whether an assay is fit for use, analysts must clearly specify the purposes for performing the bioassay. Common uses for a bioassay include lot release of drug substance (active pharmaceutical ingredient) and drug product; stability; qualification of Standard sample and other critical reagents; characterization of process intermediates and formulations, contaminants, and degradation products; and support of changes in the product production process.

**FIT FOR USE CONCEPT:**

When assays are used for lot release, a linear-model bioassay may allow sufficient assessment of similarity. For bioassays used to support stability or comparability, to evaluate changes in production processes, or to qualify reference materials, critical reagents, or changes in the assay process, it may be useful to assess similarity using the asymptote of maximum response.

**Significance of biological assays at different levels:**

1. Process development
2. Process characterization
3. Process intermediates effects
4. Product release
5. Stability of samples
6. Qualification of reagents critical to assays
7. Product integrity

In vivo potency assays are bioassays in which a set of dilutions of each of the Standard and Test materials is administered to animals and the dose–response relationships are used to estimate potency. For some animal assays, the end point is simple (e.g., rat body weight gain assay for human growth hormone or rat ovarian weight assay for follicle stimulating hormone), but others require further processing of samples collected from treated animals (e.g., reticulocyte count for erythropoietin, steroidogenesis for gonadotropins, neutrophil count for granulocyte colony stimulating factor, or antibody titer after administration of vaccines). With the advent of cell lines specific for the putative physiological mechanism of action (MOA), the use of animals for the measurement of potency has greatly diminished (*Ref* *USP chapter 1032*).

**Drawbacks for In-vivo assay**

Cost, low throughput, ethics, and other practical issues argue against the use of animal bioassays and restrict the use of animals for industrial purposes.

**Regulatory Requirement**

Regulatory agencies have encouraged the responsible limitation of animal use whenever possible (see The Interagency Coordinating Committee on the Validation of Alternative Methods, Mission, Vision, and Strategic Priorities; February 2004).

When in vitro activity is not strongly associated with in vivo activity (e.g., EPO), the combination of in vitro cell-based assay and a suitable physicochemical method (e.g., IEF, glycan analysis) may substitute for in vivo assays.

However, a need for in vivo assays may remain when in vitro assays cannot detect differences that may affect in vivo activity.

Cells or tissues from human or animal donors can be cultured in the laboratory and used to assess the activity of a Test article. In the case of cytokines, the majority of assays use cells from the hematopoietic system and subsets of hematopoietic cells from peripheral blood such as peripheral blood mononuclear cells or peripheral blood lymphocytes. For proteins that act on solid tissues, such as growth factors and hormones, specific tissue on which they act can be removed from animals, dissociated, and cultured for a limited period either as adherent or semi-adherent cells. Although an ex vivo assay system has the advantage of similarity to the natural milieu, it may also suffer from substantial donor-to-donor variability, as well as challenging availability of appropriate cells.

Bioassays that involve live tissues or cells from an animal (e.g., rat hepatocyte glucagon method) require process management similar to that of in vivo assays to minimize assay variability and bias. The level of effort to manage bias (e.g., randomization) should be appropriate for the purpose of the assay. Additional factors that may affect assay results include time of day, weight or maturity of animal, anaesthetic used, buffer components/reagents, incubation bath temperature and position, and cell viability.

Cell-based bioassay design should reflect knowledge of the factors that influence response of cells to the Test agent. Response variability is often reflected in parameters such as slope, EC50 of the dose–response curve, or the ratio of maximum to minimum response. Even though the adoption of relative potency methodology alleviates the effect of these parameters on potency estimates, such response variability could result in significant effects on system suitability, precision, and accuracy. Therefore sources of variability relevant to a given bioassay method should be identified, and control strategies to minimize their effects should be implemented.

The development of a cell-based bioassay begins with the selection or generation of a cell substrate. The cell substrate is usually the most critical reagent in a cell-based assay. To ensure an adequate and consistent supply of cells for product testing, the cell line(s) used in the assay should be banked. Several considerations arise in developing the assay cell banks.

**Selection and identification of cell substrate or cell line**

The source of the cell line, whether generated by the product manufacturer or acquired from a collaborator, academic institution, or culture collection, must have documentation that details the cell line’s history from origin to banking and supports its application for commercial use. The origin, generation, and propagation of the cell line through to the point at which the cell line is selected for use must be documented and described in detail sufficient to permit recreation of a similar cell line if necessary. Information pertaining to the cell line should be documented during assay development and before banking. This information may include but is not limited to identity (e.g., isoenzyme, phenotypic markers, genetic analysis); morphology (e.g., archived photographic images); purity (e.g., mycoplasma, bacteria, fungus and virus testing); cryopreservation; thaw and culture conditions (e.g., media components, thaw temperature and method, methods of propagation, seeding densities, harvest conditions); thaw viability (immediately after being frozen and after time in storage); growth characteristics (e.g., cell doubling times); and functional stability (e.g., ploidy)

**STATISTICAL ASPECTS OF BIOASSAY FUNDAMENTALS**

The statistical elements of bioassay development include consideration of data type and the bioassay model, along with statistical criteria for assessing and ensuring the quality of bioassay results. These form the framework for the bioassay system that will be used to estimate the potency of a Test article.

There are fundamentally two bioassay data types:

**Quantitative and quantal (categorical)**

**Quantitative data** can be either continuous (e.g., collected from an instrument), counts (e.g., plaque-forming units), or discrete (e.g., endpoint dilution titres).

**Quantal data** are typically dichotomous, e.g., survival in an animal model that uses challenge with a pathogen to measure the protection afforded by a Test article or positivity in a plate-based infectivity assay those results in destruction of a cell monolayer following administration of an infectious agent.

Quantitative data can be transformed to quantal data by selecting a threshold that statistically distinguishes a positive response from a negative response. Such a threshold can be calculated from data acquired from a negative control, e.g., adding (or subtracting) a measure of uncertainty, such as two times the standard deviation of negative control responses, to the negative control average. Analysts should be cautious about transforming quantitative data to quantal data because this results in a loss of information that may affect bioassay measurement (*Ref USP Chapter 1032*).

**BIOANALYTICAL METHOD VALIDATION GUIDING PRINCIPLE:**

The purpose of bioanalytical method development is to define the design, operating conditions, limitations, and suitability of the method for its intended purpose and to ensure that the method is optimized for validation.

Method development involves optimizing the procedures and conditions involved with extracting and detecting the analyte. Method development includes the optimization of the following bioanalytical parameters to ensure that the method is suitable for validation:

• Reference standards

• Critical reagents

• Calibration curve

• Quality control samples (QCs)

• Selectivity and specificity

• Sensitivity

• Accuracy

• Precision

• Recovery

• Stability of the analyte in the matrix

Bioanalytical method development does not require extensive record keeping or notation. Bioanalytical method validation proves that the optimized method is suited to the analysis of the study samples (*Ref may 2018 biopharmaceuticals 05/24/18*).

The sponsor should:

* Conduct a full validation of any new bioanalytical method for the analysis of a new drug entity, its metabolite(s), or biomarkers.
* Conduct a full validation for any revisions to an existing validated method that adds a metabolite or an additional analyte.
* Establish a detailed, written description (e.g., protocol, study plan, and/or standard operating procedure (SOP)) for the bioanalytical method before initiating validation. The description should identify procedures that control critical parameters in the method (e.g., environmental, matrix, procedural variables) from the time of collection of the samples to the time of analysis to minimize their effects on the measurement of the analyte in the matrix.
* Document and report (in the method validation report) all experiments used to make claims or draw conclusions about the validity of the method.
* Validate the measurement of each analyte in the biological matrix.

**BIOANALYTICAL PARAMETER THAT PLAYS CRITICAL ROLES IN METHOD VALIDATION OF BIOLOGICAL ASSAYS:**

**REFERENCE STANDARDS**

The purity of reference standards used to prepare calibrators and QCs can affect the study data. Therefore, the sponsor should use authenticated analytical reference standards with known identities and purities to prepare solutions of known concentrations. The sponsor should provide the certificates of analyses (CoA), including the source, lot number, and expiration date (with the exception of United States Pharmacopeia (USP) standards) for commercially available reference standards. For internally or externally generated reference standards that do not have a CoA, the sponsor should provide evidence of the standard’s identity and purity in addition to the source and the lot number. When using expired reference standards, the sponsor should provide an updated CoA or re-establish the identity and purity of the standard (*Ref may 2018 biopharmaceuticals 05/24/18*).

**CRITICAL REAGENTS**

Assay validation is important when there are changes to the critical reagents, such as lot-to-lot changes or switches to another make of reagent. For example, if there are changes to the labelled analytes, detector reagents, or antibodies.

The sponsor should:

• Evaluate binding to specific sites and re-optimize assays if required

• Verify performance with a standard curve and QCs preparations

• Evaluate cross-reactivity with other reagents in same assay

* Performed the bridging study between the reagents.

**CALIBRATION CURVE**

When the method is validated, the calibration curve should be continuous and reproducible. The sponsor should prepare the calibration standards in the same biological matrix as the samples in the intended study. Study samples may contain more than one analyte. The sponsor should generate a calibration curve for each analyte in the sample. When surrogate matrices are necessary, the sponsor should justify and validate the calibration curves.

**QUALITY CONTROL SAMPLES**

Quality control samples are used to assess the precision and accuracy of an assay and the stability of the samples. Sponsors should prepare QCs in the same matrix as the study samples to be assessed with the validated method. Freshly prepared QCs are recommended for precision and accuracy analyses during method development, as stability data are generally not available at this time.

During method validation, QCs evaluate the performance of a method and the stability of an analyte. Performance QCs (50%, 100%, 150% & 200%) are included in validation runs to determine the precision and accuracy of the method. Stability QCs evaluate the stability of an analyte under various stress conditions (*Ref may 2018 biopharmaceuticals 05/24/18*).

The sponsor should prepare any calibration standards and QCs from separate stock solutions. However, if the sponsor can demonstrate the precision and accuracy in one validation run using calibrators and QCs prepared from separate stock solutions, then the sponsor can use calibrators and QCs prepared from the same stock solution in subsequent runs. The sponsor should make up calibrators and QCs in lots of blank matrix that is free of interference or matrix effects.

**SELECTIVITY AND SPECIFICITY**

During validation, the sponsor should confirm that the assay is free of potential interfering substances including endogenous matrix components, metabolites, anticipated concomitant medications, etc. If the study sample contains more than one analyte and the analytes are intended to be quantified by different methods, the sponsor should test each method for interference from the other analyte.

The sponsor should analyse blank samples of the appropriate biological matrix (e.g. plasma) from at least six individual preparations. The sponsor should ensure that there are no matrix effects throughout the application of the method.

For LBAs (ligand Binding Assays), it is important to investigate any interference originating from structurally or physiologically similar analyses (i.e., exogenous interference) or matrix effects (i.e., endogenous interference). Investigating exogenous interference involves determining the cross-reactivity of molecules that could potentially interfere with the binding interaction, including molecules structurally related to the drug, any metabolites, concomitant medications (and their significant metabolites), or endogenous matrix components. The sponsor should evaluate each factor individually and in combination with the analyte of interest to determine its ability to cause interference.

**SENSITIVITY**

The method should be developed and validated such that it will be able to meet the requirements necessary for the intended study samples. The LLOQ evaluation can be done separately or as part of the precision and accuracy assessment for the calibration range.

**ACCURACY, PRECISION, AND RECOVERY**

Method validation experiments for estimating accuracy and precision should include a minimum of three (for Cell Culture) and six (for Ligand Binding Assays) independent runs (i.e., accuracy and precision (Accuracy & Precision, A & P runs) runs) conducted over several days. Each A & P run should include a calibration curve and multiple QC concentrations that are analysed in replicates. The sponsor should determine the accuracy and precision of the method based on the performance of the QC in the A & P runs. The sponsor should use freshly prepared calibrators and QCs in all A & P runs. Use of freshly prepared QCs in all A & P runs is preferred; however, if this is not possible, the sponsor should use freshly prepared QCs in one or more A & P runs

**STABILITY**

During validation, stability evaluations should cover the expected sample conditions before receipt at the analytical site (e.g., at the clinical site, during shipment, and at all other secondary sites) as well as during receipt and analysis at the analytical site. Validation of drug stability in a biological fluid is a function of the storage conditions, the physicochemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems.

All stability determinations (see list below) should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix.

**DILUTION EFFECTS**

If the method measures diluted samples, the integrity of the dilution should be monitored during validation by diluting QC samples above the ULOQ with like matrix to bring to within quantitation range, and the accuracy and precision of these diluted QCs should be demonstrated. Dilutions used during the validation should mimic the expected dilutions in the study. The prozone effect should be demonstrated in LBAs.

**CONCLUSION:**

Based on different approaches it can be concluded that biological assays play significant roles in industries, academics, and research institutes at various levels. Development and validation of biologicals assays are critical to measure the quality parameters of commercial drugs or products. During development, we need to consider the critical parameters of method development to assess the method variability and to overcome the method problems or errors. Method Validation of biological assays is critical to providing a high competent and robust method for industrial usage purposes. Method validation defines the selectivity, accuracy, precision, stability, and robustness of the method, which enhance the reliability of the method in its regular usage (*Ref may 2018 biopharmaceuticals 05/24/18*).

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