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MEASURING BIOLOGICAL ACTIVITY OF BIOPHARMACEUTICALS WITH CELL-BASED ASSAYS:

Method Development and Validation Considerations for cGMP Lot Release and Stability

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Introduction

The biological activity of biopharmaceutical compounds (such as peptides, monoclonal antibodies, growth factors, and cytokines) has a direct bearing on their potency, which is a critical quality attribute and a representation of efficacy. For biopharma companies, measuring a cell's bioactivity is important when:

- Screening compounds to develop the lead candidate;
- Validating potency when applying for regulatory approval;
- Confirming that the biological characteristics of the product remain consistent throughout the development cycle and from lot to lot, or site to site;
- Controlling for a compound's quality prior to product re lease (either for a clinical trial or commercialization);
- Testing for stability; and
- Demonstrating biosimilarity between a biosimilar and the originator compound.

Common methods of measuring biological activity of drug substances and drug products include animal-based biological assays, cell culture-based biological assays (which measure the cell's biochemical or physiological response to the drug), and biochemical assays. Cell-based assays, also called bioassays, performed in a Good Manufacturing Practice (GMP) environment, are the preferred method for measuring the biological activity of a biopharmaceutical because they are sensitive, relatively fast, and cost effective.

The complexity of cells and their many different functions means that there is a wide variety of cell-based assays available to study metabolic pathways at the cellular level. Thus, selecting, developing, validating, and performing the appropriate assay is a highly specialized function that requires considerable experience and expertise in the field, beginning with a deep understanding of cell biology as well as pharmaceutical development.

How Cell-Based Assays Work

The International Conference on Harmonization (ICH) describes cell-based assays as quantitative tests of "the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product." Cell-based assays take advantage of the fact that when a drug is added to a specific cell system, it will demonstrate a "measurable biological response." The type of test used is reflective of the supposed mechanism of action (MOA) of the biologic being studied.

Typically, in the performance of cell-based assays for potency, functional cells are transferred into the wells of a microplate. The pharmaceutical agent under study will be applied to each of the wells according to a dosing protocol. The instruments themselves are not highly specialized, but the application is, and having the right researcher who has been suitably trained is critical. Readouts of certain endpoints (for example florescence, luminescence, electrochemiluminescence, calcium signaling, or ultraviolet light absorbency) will be used to determine the type and degree of response. These readouts will allow for the generation of a dose/response curve.

COMMON TYPES OF CELL-BASED ASSAYS	
Cell Death	G Protein-Coupled Receptor (GPCR)
Cell Differentiation	Phagocytosis
Cell Proliferation	Protein-Protein Interaction
Cell Senescence	Reactive Oxygen Species
Cell Signaling Cascade	Reporter Gene
Cell Transduction	Viral Plaque
Cell Variability	Cytotoxicity

[1] Upsall, Andrew and Galbraith, Daniel N, "Cell-based Potency Assays: Adhering to GMP Standards," *PharmaFocus Asia*. Accessed at: https://www.pharmafocusasia.com/manufacturing/assays-gmp-standards.

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Regulatory Requirements

All cell-based assays must be validated to an ICH standard and meet the GMP requirements of local regulators. Regulatory guidelines pertaining to the use of cell-based assays leave much open to interpretation, but the relevant documents include:

In the U.S.::

- USP<111> General Chapter Design and Analysis of Biological Assays;
- USP <1032> Design and Development of Biological Assays;
- USP <1033> Biological Assay Validation;
- USP <1034> Analysis of Biological Assay;
- Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products. U.S. Food & Drug Administration, January, 2011;
- Guidance for Industry: Bioanalytical Method Validation, U.S. Food & Drug Administration, May, 2001;
- Guidance for Industry Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/ Biological Products. U.S. Food & Drug Administration, August, 1999; and
- Guidance for Industry: Scientific Considerations in Demonstrating Biosimilarity to a Reference Product, April, 2015.

In the EU:

- Guideline on potency testing of cell-based immunotherapy medicinal products for the treatment of cancer, 21 July 2016 EMA/CHMP/BWP/271475/2006 rev.1 ("In principle, the results of a potency assay should provide assurance that the amount of the active ingredient is sufficient to induce a meaningful response and that the amount is consistent, batch to batch."[2]); and
- European Medicines Agency. Guideline on Bioanalytical Method Validation, July, 2009.

Applications of Cell-Based Assays

Cell-based assays for potency, for all their advantages over animal models, are nonetheless hard to control and are time-consuming to develop. The expertise and commitment required demands that they be performed by a specialist, and so leveraging external expertise can facilitate the assay development process.

The cellular functions that can be measured via cell-based assays include, but are not limited to:

- Cell viability (proliferation and cytotoxicity), which is used to assess products that promote or inhibit cell growth, such as oncology drugs;
- Cell membrane integrity, which relates to protein-protein binding and has been used in the assessment of HIV inhibiting antibodies;
- Programmed cell death (including apoptosis, autophagy and necrosis) – a very active research area in autoimmune drug development;
- Inflammation, which can be measured by cytokine profiling after stimulation of immune cells and is the best measure for products designed to inhibit inflammation;
- Phosphodiesterase (PDE) profiling, an important step in the development of PDE drug candidates such as those that treat cardiovascular diseases, pulmonary hypertension, and inflammatory states. PDE enzymes are also associated with off-target side effects such as heart defects, emesis, and disturbances to vision;
- Cell signaling transduction, which identifies extracellular messenger molecules or ligands as they bind to the receptors on the cell membrane, ultimately affecting intracellular activities and can be applied to signal transduction inhibitors drugs such as Imatinib; and
- Protein-protein interactions (PPI), as they form the backbone of all cellular signaling networks and aberrant PPI contribute to the pathology of several diseases. A classic example of their use is in immunotherapy to prohibit specific signals between cells.

[2]*Guideline on potency testing of cell based immunotherapy medicinal products for the treatment of cancer,* 21 July 2016 EMA/CHMP/BWP/271475/2006 rev.1

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Challenges in the Development and Use of Cell-Based Assays

Developing and using cell-based assays is a challenging undertaking that requires considerable specialized expertise. It takes years of experience, for example, just to understand the biological events and signal transaction pathways that relate to product indications. The levels of complexity in this work are many.

***SELECTING THE PROPER METHOD** TYPICALLY ENTAILS DEVELOPING A HYPOTHESIS AND TESTING DIFFERENT METHODS TO DETECT SIGNAL PRODUCTION*

To begin, one must determine the right kind of cell to use in the assay, selecting from various types of animal and human cells that are commercially available. The type of assay applied must be reflective of the biologic's MOA and the unique response it produces within a given cell type. So, it is important to have as much information as possible about the drug's MOA (although at times, especially in the early development stage of the drug, the information may be very limited). Large molecules are subject to a great deal of structural variations and changes that may or may not have an impact on the product's potency. It is virtually impossible to explore them all, but several quality attributes such as post-translational modifications have been known to affect the product's potency. It is a matter of identifying those that are likely to translate into a clinical benefit along the drug development process. So, selecting the proper method typically entails developing a hypothesis and testing different methods to detect signal production.

The degree of investment that is required to test those different hypotheses will depend on the phase of the clinical development cycle. Generally, the more advanced the stage, the greater the level of detail that will be required to justify the chosen method.
Despite the wide choice in existing assays, some cases require the creation of a customized assay. For products that have multiple MOAs, multiple assays may be needed to demonstrate product potency as well as lot-to-lot comparability sufficiently.

It is imperative that the reference standard included in the assay be well characterized by a wide array of biochemical and biophysical means. If the standard is an internal one, the manufacturing process that produces it also needs to be well defined as to its critical operational parameters, which are known to affect the product's critical quality attributes.

Assays must be validated. Determining the proper acceptance criteria for the validation exercise is difficult because these cells are very sensitive to their environment, and one must understand how they are likely to behave. Normally, cell-based assays have a wide acceptance criteria as compared to other types of assays such as biochemical assays. Typically, these criteria are set on the basis of pre-validation studies. The acceptance range is typically wider in early clinical studies, and progressively tighter in later clinical stages.
Determining the right dose range for the assay is critically important. The rule of thumb is to have the range determined with both the upper and lower concentration limits yielding no further signal change in their successive dilutions, reaching a plateau for baseline and maximum responses, respectively.

Steps in Cell-Based Assessments

The effort and timeline required varies, depending upon the complexity of the compound, but the following steps take a few months on average.

- Determine the compound's MOA, if possible, and establish the reference standard.
- Develop a hypothesis on what signal should be produced/measured in what type of cell.
- Test different method formats to determine which one works best.
- Perform a cell culture to expand and prepare the cells to the desired density and amount.
- Perform the assay.
- Analyze the dose-response curve using various models and calculate potency relative to reference.
- Validate the method in accordance with ICH Q2 expectations

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One must also select the type of readout and use it to optimize the assay conditions that yields a steep response curve within the selected dose range. This is where both science and experience are in play, and is a part of assay development. Applying the Quality-by-Design (QbD) approach using the Design of Experiment (DoE) statistic tool could greatly accelerate identifying the set of optimal conditions. Finally, analyzing the resulting dose-response curve is particularly important as discussed in detail below.

Analyzing the Dose-Response Curve

In the cell-based potency assay, the dose response is premised on the observation of either stimulation or inhibitory effect exerted on signal production by the testing agent at various concentrations.

*TYPICAL DOSE-RESPONSE CURVE **SPANS SEVERAL ORDERS OF DRUG DOSE MAGNITUDE***

The readouts of cell-based assays produce two dose-response curves – one for the reference standard and one for the sample. The curves follow a sigmoidal pattern produced by the asymptotes, or the minimum and maximum signal response plateau. When an unknown compound and a reference standard work by the same biological mechanism, parallel curves result that are often nonlinear, but similar. (See Figure 1.)

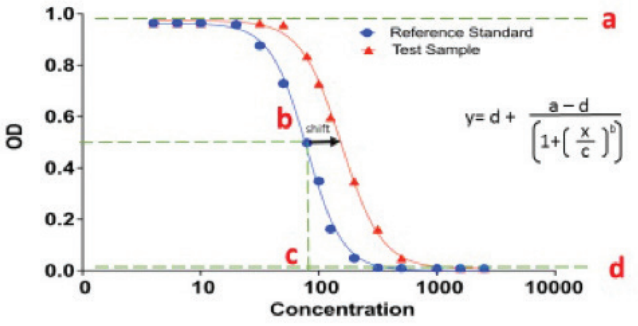
Assessing parallelism is one of the key parameters for evaluating validity of the assay method and should be initiated early during the assay development stage. Based on FDA guidance on bioanalytical method validation, parallelism of diluted study samples should be evaluated with diluted standards to detect any matrix effect.

The relative compound potencies are determined by comparing the curves, which is a statistical process that often requires software such as SoftMax® Pro GxP, Prism, etc. In the past, researchers simply compared the EC50 values of the two curves to calculate the relative potency of the sample and the reference standard. (EC50 value = the concentration of the drug that gives a response halfway between the baseline and the maximum response.) Increasingly, regulators are requesting an analysis of parallelism in addition to a comparison of the EC50 value of the reference and the sample.

A A standard dose-response curve is nonlinearly fitted by four parameters: the baseline response (Bottom), the maximum response (Top), the slope (Hill slope), and the drug concentration that provokes a response halfway between baseline and maximum (EC50). A dose-response curve should be fitted on a log scale of drug concentration rather than on a linear scale. The typical dose-response curve spans several orders of drug dose magnitude. The errors associated with the EC50 parameter follow a Gaussian distribution on a log scale, but not on a linear scale. Therefore, only in the former case are these errors able to be analyzed by the standard statistical analyses. Interested readers may find more information in the book: *Fitting models to biological data using linear and nonlinear regression. A practical guide to curve fitting by Motulsky and Christopoulos.*

RELATIVE POTENCY AND PARALLELISM

A parallelism test is always performed before calculating a relative potency.



4-Parameter logistic nonlinear regression curve

[3]Liszewski, Kathy, "Methods to Develop and Validate Bioassays," *Genetic Engineering & Biotechnology News*, April 15, 2006, Vol 26, No 8.

[4]Wang, Weihong, PhD, "Potency Testing of Biopharmaceutical Products," *American Pharmaceutical Review,* Nov 26, 2014.

[5]Motulsky, H. and Christopoulos, A., "Fitting dose response curves." In H. Motulsky & A. Christopoulos (Eds.), Fitting models to biological data using linear and nonlinear regression. A practical guide to curve fitting (pp.256-265). San Diego, CA: GraphPad Software Inc. (2003).

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Assay Validation Process

According to ICH guideline Q2 (R1), assays must be shown to be suitable for their intended use, fulfilling pre-set criteria. The validation procedure should consider the test's linearity, accuracy, precision, range, specificity, system suitability, and robustness. The process should confirm that the assay is specific to the drug target and locates the right information.

Conclusion

A drug's potency, which is a critical quality attribute, is determined by examining biological activity at the cellular level. Cell-based assays are available to measure this activity. However, selecting, developing, validating, and performing the appropriate assay demands highly-specialized expertise. This work should only be entrusted to GMP laboratories with considerable proven experience. Manufacturers should plan sufficiently for assay development to avoid delays in the development timeline. They should also be prepared to brief their laboratory partner fully on the drug's MOA, since that will help determine the type of assay used. A product development phase-appropriate assay strategy should be devised earlier as a part of the overall product life cycle management. An experienced CRO can augment internal capacity or capability to facilitate the development and the validation of the cell-based potency assay and add value to the drug development process.

Acknowledgement

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