

Development of Test Method for Pharmaceutical and BioPharmaceutical Products

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Abstract

Biological products are mostly macromolecular entities that are considerably larger than most chemical products. With the exception of synthetic oligonucleotides or peptides, living cells-complex metabolic factories produce them. The target molecule(s) must be isolated from a biochemical milieu consisting of chemical entities relatively similar to the desired product. As such, it may be difficult to completely eliminate impurities derived from the host system. The purified target may comprise several structurally heterogeneous forms, some or all of which might be active. Compared to traditional chemical drugs, biological materials are highly labile, and unable to tolerate high temperatures or undue chemical or physical stress. Higher-order biological products (e.g., cells and tissues) may have a very short window of viability. It requires numerous complex analytical methods to provide an effective physicochemical profile of biotechnology products.

This paper has presented several factors to consider when selecting the analytical methods to assess the identity, purity, impurities, concentration, potency, stability, and (in some cases) comparability of biotechnology products. Since no single method can provide data on all key product parameters, orthogonal analytical methods should be used to increase confidence in the quality of product. Methods used under good laboratory practice (GLP) or CGMP quality practices must be validated for their intended use. The strategies for qualifying and/or validating biomolecular methods should be based on the type of method, the nature of the product, and the parameter to be evaluated with the data. Laboratories that adopt validated methods (e.g., compendial methods) must experimentally verify the suitable performance of these methods in the user environment. In order to provide a complete product development record, all of these activities must be adequately documented to demonstrate how, when, and by whom they were conducted.

As has been noted, "Some data are worthless; some data are priceless. The conditions and procedures used to find data ultimately determine their value". All decisions regarding the control of the process and the quality of the product are based on data generated by analytical tests. If there are design flaws in the test methods, unrecognized sources of method variation, or a method is chosen that cannot support the specification requirements, the data will inevitably be inadequate, inaccurate, or unreliable. So, while it is certainly critical to understand the process by which a biological or biotechnological product is produced, it is equally vital to understand the methods of analysis that are applied to the product. Otherwise, it will be very difficult to distinguish between those data that are priceless and those that are worthless.

Keywords: Analytical methods; Pharmaceutical products; Bio pharmaceutical products; Validation; System suitability

Introduction

The statement that "The Process Is the Product." was based on the understanding that the main production agent-living organisms-produce large quantities of chemically similar material (e.g., proteins) that must undergo a variety of separation steps that can select the greatest yield of the highest purity of a desired molecular entity from the cellular-derived milieu. The separation processes used to sort out the one or more target proteins from other, often co-purifying, proteins are optimized, scaled, and validated to reliably achieve the same population of molecular entities from each batch of biologically produced material. The nature of these separation processes is such that even subtle changes in some steps can impart significant variations in the resulting population of proteins. It has been shown repeatedly that successfully defining and controlling the process can define and control the product [1] (Table 1).

However, analytical technologies have also been emerging that allow biopharmaceutical materials to be scrutinized in ever more sensitive and specific physicochemical detail. Increasing attention is being given to the tremendous value to be derived from adequate analytical characterization of protein products, and where appropriate,

critical in-process or intermediate materials. It is understood that the majority of biologically derived products are not homogenous biomolecular species: "An inherent degree of structural heterogeneity occurs in proteins due to the biosynthetic processes used by living organisms to produce them. Heterogeneity can also be produced during manufacture and/or storage of the drug substance or drug product" [2].

During early development, in-depth, orthogonal biochemical information gained on the target molecular entity provides a better understanding of the attributes of the product that may contribute to its efficacy. It also allows an assessment of intrinsic product-or process-related impurities that could impact product safety in early

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Received June 23, 2013; Accepted July 02, 2013; Published July 23, 2013

Citation: Shintani H (2013) Development of Test Method for Pharmaceutical and BioPharmaceutical Products. Pharm Anal Acta 4: 258. doi:10.4172/2153-2435.1000258

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S.No	
1	Clearly define the assay's intended use relative to the desired product attribute (e.g., identity, purity, impurities, potency, concentration, stability) and acceptance specification requirements.
2	Understand how the method technology functions to generate data on the parameter of interest. Develop appropriate system suitability measures to assess method performance independently of the test sample performance.
3	Recognize and control potential sources of method and operational variability that can impact the reproducibility of assay procedure. Incorporate system suitability measures to assure the validity of each test run, and to track/trend method performance over time.
4	Confirm that the method will be scientifically sound for its intended use(s) by demonstrating its inherent performance capabilities such as accuracy, precision (intra and inter assay), linearity/range/limit of detection (LOD)/limit of quantitation (LOQ), and specificity (including for product degradants, if stability-indicating).
5	Verify that assay is robust enough under the conditions of expected use to statistically support the specification requirements for the product at each phase of development and commercialization.
6	Assure that all documentation and data from each method's lifecycle events are maintained in archived files which are complete, traceable and retrievable for use in supporting product and method knowledge management over time.

Table 1: Elements for successful analytical method development and implementation.

clinical trials. As development proceeds and the manufacturing process generates additional product batches, analytical characterization can be used to obtain biomolecular profiles of the product and its impurities in order to evaluate process performance: "Since the heterogeneity of these products defines their quality, the degree and profile of this heterogeneity should be characterized to ensure lot-to-lot consistency" [2].

In fact, the term commonly used is "well-characterized biological/biotechnological products". Although later formally designated as "Specified Products" [3] "well-characterized" reflects the important role of rigorous analytics in assuring safe and effective products, as has been demonstrated in numerous case studies and discussions at conferences.

Biomolecular characterization techniques are also used to determine the physicochemical comparability of product batches before and after a process change, to assess the success of process scale-up or scale-down, or following technology transfer of the production to a new manufacturing facility. Citation 2 indicates that "the manufacturer should define the pattern of heterogeneity of the desired product and demonstrate consistency with that of the lots used in pre-clinical and clinical studies. If a consistent pattern of product heterogeneity is demonstrated, an evaluation of the activity, efficacy, and safety (including immunogenicity) of individual forms may not be necessary" [2,4,5]. The pattern of heterogeneity, as well as the degree of comparability, is directly related to the sensitivity and specificity of the analytical methods applied [6]. When structural differences are seen, they are expected to be investigated and a risk assessment is to be performed to determine their potential impact on product quality, safety, and efficacy [7,8].

An emerging class of "generic" biotechnology products, termed biosimilars, subsequent-entry, or follow-on biologics [9] expands application of biomolecular characterization techniques to assess the physicochemical and functional comparability of protein therapeutics. Previously, comparability studies were only applicable within an innovator's own process development and commercialization activities for a given biopharmaceutical product. New regulations now allow consideration of comparability studies between innovator products and subsequent producers of the same biopharmaceutical therapeutic, which could minimize the clinical requirements for the follow-on product. For both innovator and biosimilar products, a major element of the comparability study is the selection of the analytical methods utilized in the analyses.

To be successful, there are many factors that should be considered when selecting, optimizing, and validating analytical test methods, and in using test results to establish appropriate specifications for the product. Choices are made during the development cycle regarding the

types of standard and state-of-the-art technologies that may be suitable for use with the product. Current regulatory guidance documents and several biotechnology industry publications available online give considerable information on the typical analytical methods used with other biological products and current expectations for product characterization, release, and stability testing.

Practical considerations should also be factored into the selection of the methods that will be used for routine quality control (QC) testing of product batches. QC analytical methods must be robust enough to function reliably over time under varying operational conditions. Failure to fully understand the details of the analytical technology and/or failure to define the intended application of the method are prime reasons for methods that end up in QC laboratories unable to reliably perform to (unsupportable) expectations.

Physicochemical Profile of Biotechnological/ Biological Products

Table 2 highlights elements of the structure of proteins and peptides, and the associated physicochemical attributes. To develop a comprehensive profile of a product, multiple aspects of the structure require analysis. In addition, tests to determine the product's concentration and potency are conducted to assess functionality. For clarity, the differences between (1) bioanalytical methods, (2) biomolecular methods, and (3) bioassays should be noted.

Bioanalytical methods

These are test methods used for the quantitative determination of drugs or metabolites in physiological samples (e.g., serum or plasma) derived from animals or humans, which include a milieu of biological components (i.e., proteins, lipids, nucleic acids). For chemical drugs, techniques such as solid phase extraction followed by gas chromatography (GC), liquid chromatography (LC), and/or MS are used to precipitate and remove the biological matrix elements, leaving the chemical analyte in solution for subsequent analysis. For biological drugs, the target entity itself would be precipitated and removed if processed with the same techniques. Therefore, for preclinical and clinical specimens, it requires specific binding techniques such as immunological methods to quantify the biological target in the presence of such a biological matrix.

Bioassays

The bioassays are defined as functional tests used to determine the activity, potency, or biological integrity of a drug product. The World Health Organization/National Institute for Biological Standards and Control (WHO/NIBSC) defines a bioassay as an analytical procedure measuring a biological activity of a test substance based on a specific,

Level of Structural Characterization	Analytical information obtained
Primary Structure	Protein sequence Nucleic acid sequence Amino acid composition Apparent molecular weight Observed molecular mass Post-translational modifications Phosphorylation Glycosylation (monosaccharide composition)
Secondary Structure	Polypeptide chains Peptide fragments Disulfide bond linkages Glycosylation (oligosaccharide structure) Isoforms (e.g. glycoforms)
Tertiary Structure	Receptor binding Epitope recognition Cell modulator release Cell differentiation effect Replication competence Apoptosis

Product + Ligand Conjugate Molar ratios of ligand: product
(e.g. PEG, chemical or Ligand binding sites biological moiety)

Table 2: Physicochemical analysis of biomolecular compound.

functional, biological response of a test system [10]. Most recently, a draft of the new United States Pharmacopeia (USP) chapters on bioassay design and development, biological assay validation, and analysis of biological assays considers the terms bioassay and biological assay to be interchangeable, and defines them as "analysis (as of a drug) to quantify the biological activity/activities of one or more components by determining its capacity for producing an expected biological activity, expressed in terms of units" [11]. Bioassays include *in vitro* methods such as cell culture assays, antiviral assays, infectivity assays, and *in vivo* assays involving animal models [12].

Biomolecular methods

An additional term, biomolecular methods, encompasses the analytical technologies used to perform physicochemical characterization of biological and biotechnological products [13]. Biomolecular methods used for the analysis of protein products include various forms of high-performance liquid chromatography (HPLC), ultra performance liquid chromatography (UPLC), gel electrophoresis, isoelectric focusing, sequencing, various forms of mass spectrometry, amino acid analysis, carbohydrate analysis, peptide mapping, capillary electrophoresis, and so on [14-16].

Analytical Methods used in Production Operations

Every stage of the manufacturing process is supported by established specifications. The International Conference on Harmonization (ICH) defines specifications as "a list of tests, references to analytical procedures, and appropriate acceptance criteria which are numerical limits, ranges, or other criteria for the tests described. It establishes the set of criteria to which a drug substance, drug product, or materials at other stages of its manufacture should conform to be considered acceptable for its intended use. Conformance to specification means that the drug substance and drug product, when tested according to the listed analytical procedures, will meet the acceptance criteria" [2]. Specifications are considered contracts with the regulatory agency whereby the manufacturer agrees to make and test the licensed product as defined in the product submission, and commits to using only those batches of product that pass their given quality control test methods. The total quality of a biotechnology product is recognized to be intrinsically linked to a comprehensive strategy that includes specifications,

thorough product characterization, compliance with current good manufacturing practice (cGMP) operational requirements, validated processes, and validated test methods for assessing product release and stability [2]. It is clear that sound analytical methodology is a central tenet of this quality system.

The biopharmaceutical manufacturing process typically encompasses raw materials going into production, cell culture/fermentation conditions, the purification process, the bulk active product, the formulation of the active product, and the final drug product. In addition, the stability of bulk and final drug product must be assessed. Each of these stages requires samples to be taken and data generated to determine if the materials are suitable for use and/or if they should be processed to the next unit operation [17].

Analytical methods used for the quality control testing of many pharmaceutical raw materials and excipients are typically compendial. These methods have been validated in large-scale collaborative studies. To implement a compendial method in a user laboratory, the method must be verified under conditions of actual use [18]. The verification study should consist of testing the method with the samples in the buffer or placebo matrix to assess potential matrix interference, with limited repeatability to assure reliable performance in the user laboratory [19]. Analytical quality control methods for complex and/or customized raw materials (e.g., cell culture media) are not usually pharmacopeial standard tests. These materials often require methods that assess critical product attributes such as composition, concentration, and suitable function such as growth promotion or enzymatic activity. While the vendor of the material will have some methods in place for product quality testing, they may not address the same parameters as needed by the specific applications used in biotechnology manufacturing processes. In these cases, it will be necessary to develop and validate the necessary raw materials or excipients tests. Where vendor testing is suitable to meet the user requirements, the vendor could be audited to assure the quality of their operations. Then the vendor's certificate of analysis may be accepted as the certification of each batch of material quality. The only test required upon receipt of each batch would then be an identity test method to confirm the correct material was received [20].

To determine their acceptable quality, the bulk and final product must be analyzed for identity, purity, impurities, concentration, and potency [2]. Unless the product is listed in a compendial monograph in which regulatory methods for these product attributes are given, the development of noncompendial methods will be necessary. Many historical biological products, such as plasma fractionation products (i.e., human serum albumin) and vaccine products do have monograph listings that must be followed for product release.

However, most modern biotechnology products are new molecular entities and therefore do not have monograph listings. In the absence of compendial methods, manufacturers of these products must develop and validate their own (noncompendial) analytical methods and product specifications [21]. Manufacturers are also responsible for verifying that analytical methods used for product stability testing are suitably capable of detecting, and as necessary quantifying, degradation products. It should be noted that compendial methods are not necessarily verified to be stability-indicating for the products listed in monographs [22]; the burden is on the user to confirm the appropriate methods for use in stability protocols.

During cell culture and fermentation steps, critical parameters are measured to assure adequate control of the processes. Parameters

such as pH, O₂, and CO₂ levels, glucose, or other sentinel compounds are monitored to confirm they are within required limits. The test methods used to perform these measurements may be simple (e.g., pH or dissolved gas) or more complex (e.g., cell density or target protein concentration). If they are compendial, they may be used with verification, as described previously. If they are noncompendial, they will require validation for routine use under cGMP, as described in the following.

Product fractionation or purification is supported with analytical methods to determine the success of a unit operation and the ability to process the material to the next step. In practice, these in-process methods are typically developed using purified forms of the target product to measure purity, concentration, or potency. Then method performance must be verified with in-process samples, since considerably greater amounts of process-and product-related impurities are present in the in-process samples than in the purified product. Also, buffer components and concentrations may be significantly different in in-process samples. For these reasons, it is usually necessary to document specificity using test buffer blanks in parallel with test samples from process development experiments to determine the effect on test method performance. In these applications, the recovery of target protein is usually calculated as a percent of starting material. When the purification process is finalized, the in-process test methods should be validated prior to use in process validation and full cGMP.

An initiative termed Process Analytical Technology [23], which is defined as "a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality. The term 'analytical' in PAT is viewed broadly to include chemical, physical, microbiological, mathematical, and risk analysis conducted in an integrated manner" [21]. The concept marries analytical technology, process development tools (such as statistically designed experiments), mathematical modeling, and risk assessment tools (such as failure modes effects analysis) to define the most effective testing and control scheme to best assure the consistent quality of the manufactured product.

The use of PAT tools is one of many approaches included in the concepts of process design space, as described in ICH Q8 [24]. Establishment of a design space is based on a multidimensional combination and interaction of input variables and process parameters that have been demonstrated to provide assurance of quality. The ability to evaluate process design space is only as good as the data obtained from the experiments conducted on input versus output, i.e., elements that produce a measurable result in the process characteristics. And the data are only as good as the type and capabilities of the analytical methods used to measure results. One element of establishing process design space is to utilize a greater number of analytical techniques to assess the impact of process changes on product characteristics. For example, in addition to methods used to measure the clearance of process-related impurities, methods for assessing product heterogeneity and product modifications (e.g., isoforms, degradants) may also be necessary to generate a meaningful design space.

Methods used for Product Characterization, Release, and Stability Testing

A singular feature of the analysis of biotechnology products is the diversity of analytical technologies necessary to obtain the physiochemical profile. Characterization of a biopharmaceutical product is considered to be the complete description of its physical, chemical, and biological characteristics [25]. A subset of methods used for product characterization can be validated for routine product QC batch release testing. A subset of QC release methods can be suitable for use in product stability protocols. Examples of the types of analytical technologies used in the characterization, release, and stability testing of biotechnology products are shown in Table 3. These exemplify the continuum of molecular complexity among biopharmaceutical products, and illustrate the corresponding set of analytical "tools" applicable to the nature of the product. The methods (Table 3) are procedures applicable to viral-based products such as live attenuated vaccines or gene therapy products using viral vectors [15,16].

Full characterization analysis is typically conducted at key points during the development cycle, and after licensure. The objective of a characterization study is to provide detailed information from a wide array of techniques in order to provide a thorough understanding of

Assay	Purpose
Field flow fractionation multiangle light scattering (FFF-MALS)	Determine particle number and aggregation state
Atomic force microscopy (AFM)	Determine particle number and aggregation state
Transmission electron microscopy (TEM)	Determine particle number
Size exclusion chromatography multi - angle light scattering (SEC-MALS)	Determine particle number and aggregation state
TCID, FFA, plaque, or other assays	Determine proportion of defective particles based on the difference between total particles and infectious particles
Polymerase chain reaction (PCR)	Determine proportion of nucleic acid containing particles
Density gradient centrifugation	Determine proportion of defective particles based on relative densities of particle populations
Analytical ultracentrifugation (AUC)	Determine proportion of defective and aggregated particles based on hydrodynamic properties of particle populations
Capillary electrophoresis (CE)	Determine proportion of defective and aggregated particles based on particle mass and charge
Reversed-phase HPLC (RPHPLC)	Determine proportion of defective and aggregated particles based on hydrophobic interaction properties
Ion-exchange chromatography (IEC)	Determine proportion of defective and aggregated particles based on charge state of the particles
Size exclusion chromatography (SEC)	Determine proportion of defective and aggregated particles based on hydrodynamic sieving properties of particle populations
SDS-PAGE (or equivalent)	Determine composition of proteins contained in preparation based on polypeptide chain sizes
Western blot	Determine composition of immunoreactive proteins contained in preparation
Process residuals (BSA, benzonase, polysorbate, etc.)	Quantify process-related impurities

Table 3: Lot release and stability assays used with live viral vaccines and viral-based gene therapy products.

the expected nature of the material. Characterization establishes the physicochemical attributes the product will, and should, have to support its safety and efficacy. When thorough characterization is performed, some "curious discoveries" have been made on the physicochemical nature of biotechnology products. Errors in translation, incorporation of unusual amino acids, novel cross-links, and amino acid substitutions have all been discovered when state-of-the-art analytical methods such as peptide mapping procedures, mass spectrometry, HPLC, and electrophoretic methods are used to analyze products. Of particular interest in characterization studies is the "fingerprint" of product heterogeneity and product-and process-related impurities of the product. In addition to the major quantitative analyses, qualitative assessment of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) banding patterns, or peptide map and mass spectrophotometric fragment patterns can yield significant information on the capability of the production process to yield material with consistent characteristics. Also, methods that assess higher-order structure are considered essential elements of product characterization and comparability studies [26]. Glycoproteins require characterization of their carbohydrate moieties using a wide variety of analytical techniques [27-29].

Generally, methods used only for drug substance characterization are not validated in accordance with guidelines; however, it is expected that these methods should at a minimum be qualified to ensure they are capable of generating reliable data on the specific material being tested [30].

QC Release Tests

Traditional chemical drugs are typically QC release tested for physical description, physical properties (e.g., pH of solution forms, moisture of solid forms, particle size), identity, assay (drug content and purity), process-related impurities (e.g., solvents), microbial limits, and in some cases, proportion of chiral or polymorphic species [2]. Most of these methods employ spectrophotometric, gravimetric, or chromatographic techniques. Biologically derived pharmaceuticals often require additional immunological, enzymatic, electrophoretic, colorimetric, and cell-based methods for assessing molecular characteristics and complex host-and process-derived impurities. It is expected that no single analytical method will profile all biotechnology product characteristics. Each critical attribute-identity, purity, quality, potency, strength, product-and process-related impurities can be assessed by multiple analytical procedures; each test could yield different results based on differences in method capabilities such as sensitivity and specificity. Table 4 illustrates how a variety of biomolecular methods can be utilized to evaluate specific biotechnology product-and process-related impurities [22].

One class of process-related impurities requires special attention in terms of the analytical technology utilized to produce accurate measurements. Host cell proteins (HCPs) comprise a highly diverse population of process impurities, some of which have molecular properties (e.g., charge, size, polarity) that allow them to co-purify through downstream process steps along with the target biopharmaceutical protein [31]. Moreover, slight variations in process unit operation conditions can alter the population of HCPs that are generated (in the case of upstream cell culture or fermentation) or cleared (in the case of downstream purification steps). The analytical method for measuring HCPs in in-process samples and purified bulk drug substance must be capable of accurately detecting a wide array of proteins produced by the host cells. Currently, the established technology for HCP testing is to use an enzyme-linked immunosorbent

Phenomena	Procedures
Aggregation	SDS-PAGE, SEC-HPLC, light scattering
Deamidation, Oxidation	Peptide map, HPLC, IEF, MS
Proteolytic Cleavage	Peptide map, SDS-PAGE, HPLC, IEF, MS
Amino Acid Substitutions	AAA, Peptide map, MS, protein sequence, CE
Translation Mutations	Peptide map, HPLC, IEF, MS, CE
Host Cell Proteins	SDS-PAGE, Western blot, ELISA
Media Components	SDS-PAGE, Western blot, HPLC, ELISA
Nucleic Acids	DNA hybridization, UV, Protein binding
Affinity Antibodies	SDS-PAGE, Western blot, ELISA
Proteases/Nucleases	HPLC, Western blot, ELISA
Leachates/Extractables	HPLC, MS, GC, gravimetric analysis
Process Residuals	Karl Fisher moisture, GC, ion chromatography

Table 4: Analytical methods for biotechnology product impurities.

assay (ELISA)-like immunoassay. The two most critical reagents in the method are: (1) the mixture of polyclonal antibodies used to immunodetect the HCPs generated by the cell's proteome, and (2) the mixture of proteomic HCPs used for the assay standard curve. In order to successfully validate an HCP ELISA method, the specificity of the anti-HCP polyclonal antibody reagent must be confirmed. That is, it must be demonstrated that the population of polyclonal antibodies present immunorecognizes a majority of the HCP present in the expression system.

Typically, the polyclonal anti-HCP antibodies are produced by immunizing animals with a mixture of HCPs (from the biopharmaceutical product's host expression system), then collecting the hyperimmune serum which contains anti-HCP antibodies [31]. Then, to verify that the antibodies produced are adequately specific for the HCP population, a Western blot study is performed. The HCP mixture is first separated on 2-D gels to resolve the population of proteins by charge and size. One gel is then stained to reveal the proteins present in the HCP mixture. A parallel 2-D gel of the HCP mixture is electroblotted to a membrane, which is then probed with the anti-HCP antibody preparation, then reacted to visualize the immunobinding reactions. The two images should correspond as closely as possible; every HCP protein spot that is present in the stained gel should yield a corresponding immunoreactive spot in the anti-HCP Western blot. Due to differences in inherent antigenicity among the proteins from the host cell proteome, as well as differences in the affinity/avidity of the polyclonal antibody species generated, and technical challenges in efficiently separating, electroblotting, and immunoreacting hundreds of different proteins simultaneously, it is not always possible to see 100% correlation of spots between the stained gel and Western blot.

However, there must be a very high degree of correlation in order for these reagents to be accurate enough for use in an ELISA-like method. Otherwise the signal produced in the ELISA binding reactions will only represent a subset of the possible HCPs in the test samples as well as in the HCP standard curve, and the values produced (typically in units of ppm) will underestimate the level of HCPs present. Until the specificity of the polyclonal antibodies for the HCP population is confirmed, other performance parameters of the ELISA method, such as accuracy, linearity, limit of quantitation, and limit of detection, cannot be validated. There is commercially available HCP ELISA test kits for several of the most common biopharmaceutical expression systems, such as *E. coli* and Chinese hamster ovary (CHO). With some biopharmaceutical products, 2-D gels and blots will demonstrate that the immunoreagents in these kits are adequately specific and sensitive for the user's expression system, and the ELISA method that utilizes them can be validated for its intended use. In other applications,

2-D gels and blots reveal that the commercial immunoreagents are not adequately specific, recognizing only 3/4 (or less) of the HCP population in the user's host expression system. In such cases, the user will have to develop a process-specific HCP mixture and anti-HCP polyclonal antibodies for their expression system's proteome. Then, if the specificity is confirmed by 2-D gels and blots, the reagents can be used to develop and validate a custom HCP ELISA.

For biotechnology product QC release testing, it is expected that orthogonal analytical methods will be used for key product attributes [25]. "Orthogonal" refers to methods that exploit different chemical or physical mechanisms for analysis. For example, size-exclusion HPLC (SEC-HPLC) and reversed-phase HPLC (RP-HPLC) are based on two different separation mechanisms. Similarly, ion exchange (IEX)-HPLC and capillary isoelectric focusing (cIEF) are "orthogonal" methods. Typically, for well-characterized biotechnology products, parameters such as purity and identity are supported with a minimum of two orthogonal QC release methods'.

Stability Tests

For biopharmaceutical products, it is recognized that "the evaluation of stability may necessitate complex analytical methodologies. Appropriate physiochemical, biochemical, and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be a part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies" [32]. Methods used in the stability protocol should detect significant changes in the quality of the product (i.e., purity, potency) with a focus on the ability of the methods to determine product degradation. There are several well-known physical and chemical degradation pathways for proteins, such as aggregation, fragmentation, oxidation, deamidation, and so on [33]. However, the specific degradation pathways and kinetics (which are rarely linear) for a given protein will be unique to its primary, secondary, tertiary, and (for multimeric proteins) quaternary structure. While some pathways can be expected, such as aggregation caused by agitation of protein solutions, it cannot be assumed that any given protein will-or will not-experience multiple types over time or under conditions of stress. The analytical methods being of degradation used to assess product stability must be proven to be capable of detecting all possible degradants that could be formed if the product begins to degrade. Otherwise, there will be no data to prove the product is stable; changes could be occurring, but the methods are analytically "blind" to them.

The best way to demonstrate whether or not a test method is truly capable of detecting or quantifying product degradation is to conduct a forced degradation study. Forced degradation studies provide critical information on the inherent stability of the product, its degradation pathways, and confirm the capabilities and suitability of the analytical methods to be used in stability testing. This study on a single batch is not considered a part of the normal stability protocol. As shown in Table 5, it should stress the drug substance in several physical and chemical experiments, including various pH solutions, in the presence of oxygen and light, and at elevated temperature and humidity increments. For biotechnology products in solution, it should include agitation stress. For products stored frozen, multiple freeze-thaw cycles should be examined. It is recognized that stress conditions may create product degradants not formed under normal storage, shipping, and handling conditions, but the force-degraded product preparations are considered to be important as reagents used to challenge the potential stability-indicating analytical methods [25].

S.No	Chemical and Physical Treatments to Promote
1	Aggregation
2	Fragmentation
3	Precipitation
4	Dephosphorylation
5	Deamidation
6	Deglycosylation
7	Hydrolysis
8	Oxidation
9	Disulfide bond exchange
10	Ligand release

Table 5: Purposeful (forced) degradation of biotechnology products.

Whenever significant qualitative or quantitative changes indicative of product degradation are detected during long-term, accelerated, or stress studies, consideration should be given to the potential hazards and to the need for characterization and quantitation of degradants [32]. The goal is to ensure product safety by clearly understanding the physiochemical nature and potential adverse impact of a product's inherent degradation pathway. This is especially true for biopharmaceutical products, where degradants could create new epitopes that could trigger a neo-antigenic immune response in patients, such as heat-treating product preparations to achieve viral inactivation [34].

Ideally, initial stress studies should be done early in product development to allow selection of the stability-indicating methods for real-time stability studies [25]. Experiments are designed to cover all potential degradation pathways of a given product, allowing the degradation conditions to proceed and removing sentinel samples at designated points and subjecting them to analysis using orthogonal analytical methods [35,36]. Accelerated and stress stability experiments can provide valuable information on the degradation pathways and kinetics of degradation [37]. But unlike chemical pharmaceutical products, expiration dates and retest periods for biological/biotechnological products cannot be determined from extrapolation of short-term results, but can only be established through real-time stability studies [32].

Product Potency Assays

When discussing release and stability tests for biotechnology products, one frequently heard comment is: "Why can't a potency assay suffice as evidence of product quality and stability? Is it the ultimate proof of acceptable product performance?" It is true that biotechnology products may be intrinsically heterogeneous, and the specifications may include designated impurities [2], but it is necessary to assure continued product safety and efficacy by monitoring and maintaining the degree of heterogeneity and impurities to the levels demonstrated in preclinical and clinical trials. Ideally, the potency assay should be linked to the product's expected mechanism of action [2]. For some proteins, such as monoclonal antibodies, there may be various classes of function [38].

When choosing a quality control testing scheme, the question to ask is: "What are the parameters that best demonstrate manufacturing consistency?" These will likely encompass more than just the parameters that are known to impact clinical efficacy, because the first objective of quality control testing is to assure continued product safety. For biotechnology products, "since the degree of heterogeneity defines their quality, the degree and profile of this heterogeneity should be characterized to ensure lot to lot consistency" [2]. Product potency

assays alone are not capable of measuring product heterogeneity (i.e., the type and ratio of product-related substances), product-related impurities (e.g., product degradants), and process-related impurities. However, to be suitable for the intended use of assuring product quality and stability, the potency assay should be capable of measuring the difference between "good" and "bad" lots of product based on the quality of the active specie(s) at time of release and over time with handling and storage. To that end, the potency assay should be sensitive to some degree of product degradation, demonstrating a decrease in potency with increased degradation. But because potency assays (and even immunoassays) are based on the measurement of a defined target activity, they are often insensitive to varying levels of impurities unless they interfere with the specific reaction being detected. Typically, potency assays are used to measure the loss of intact product activity rather than the increase of individual degradants. Also, because potency assays are often less precise than physiochemical methods, it may take a higher degree of product degradation to be accurately and reliably measured by potency than by methods such as chromatography or electrophoresis. Some biopharmaceutical products have more than one functional domain, such as monoclonal antibodies with an epitope recognition moiety (e.g., the Fab region) and a cell binding moiety (e.g., the Fe region). When multiple functional domains are a part of the product's critical characteristics, it is necessary to assure that both (or all) of the domains meet physical and functional specifications for quality control. The nature of the procedure(s) utilized for product potency testing range from (relatively) simple ligand binding or enzymatic procedures through cell-based assays to *in vivo* animal assays.

Comparability Assessment

There are several points at which it is advantageous to assess the comparability of product batches. At its simplest, a comparability study can be thought of as side-by-side characterization of test materials. That is, test samples from Batch A and Batch B are assayed. While it is possible, it is less desirable to compare test results from samples assayed independently (i.e., on different days, months, or even years), because the effect of intrinsic test method variability can confound the ability to accurately assess the similarities or differences. This is especially true when qualitative "fingerprint" methods are used, because slight variations in method performance can significantly change subtle, but critical, product profile elements. Therefore, the best case for comparability is made when product samples are run together using validated methods. Then, even if the test methods are not fully validated for robustness over time, all of the experimental bias should at least be skewed in the same direction for all samples, allowing more confidence in the interpretation of results.

One key point for comparability assessment is to link safety and efficacy data from all phases of product development to the physiochemical characteristics of the preclinical and clinical lots. To demonstrate developmental continuity of product characteristics relative to the preclinical and clinical experiences, Phase I/II drug substance and drug product batches should be compared to the batches used for Phase III to show consistency of manufacturing quality, purity, and potency among them [2]. The most effective means by which to make these pre-BLA (Biologic License Application) comparisons is to prospectively plan to retain samples of each preclinical and clinical batch of drug substance and drug product at each phase of development, starting with the lots used for toxicology studies. While long term stability will not be assured, as these samples are stored before formal stability studies are conducted, the effects of degradation on the

comparability of lots can be experimentally confirmed. However, if these samples are not retained, the determination of comparability will have to be based on test data collected at different points in time. Any variability in the performance of the method, including small method changes made for optimization or validation at each phase, will make the evaluation considerably more difficult.

If the characteristics of the lots, particularly in terms of impurities, change dramatically as the process is optimized, it could call into question the applicability of early toxicology and safety test results. That is, product- or process-related impurities should not be different from, or greater than, those seen in the product batches used in safety studies conducted in early development. If they are, the sponsor may have to repeat some of these earlier studies to assure that all new or elevated impurities have been experimentally tested for patient safety. Similarly, in drug substance and drug product stability testing, when degradation products result in heterogeneity patterns that differ from those observed in preclinical and clinical development, the significance of these alterations should be evaluated to ensure the continued safety and efficacy of the product [2].

After licensure, continuous improvements in the process, or technology transfer to other manufacturing facilities, can be assessed with FDA-approved comparability protocols. A comparability protocol is a well-defined, detailed, written plan for assessing the effect of specific chemistry, manufacturing, and control (CMC) changes on the identity, strength, quality, purity, or potency of a specific drug product as these factors relate to the safety and effectiveness of the product.

A comparability protocol describes the changes that are covered under the protocol and specifies the tests and studies that will be performed, including analytical procedures that will be used, and the acceptance criteria that will be achieved to demonstrate that the specified CMC changes do not adversely affect the product (drug substance, drug product, intermediate, or in-process material [3,4].

Development, Qualification, and Validation of a Product-Specific Noncompendial Analytical Method

The objective of method development is to deliver a procedure that is capable of performing reliably to measure a defined attribute of a test sample. Individual firms may use different terminology for some activities (i.e., method optimization, qualification, or validation), or divide parts of the strategy among different operational groups (e.g., analytical development and quality control). The method development strategy can be outlined in terms of questions to be asked. The answers to these questions (and the data to support them) should be captured in writing in method development reports. Method development reports serve as the historical scientific record of the rationale and justification for how the method was selected and why it is considered suitable for use. Well-written, comprehensive development reports provide valuable background information to future users of the method. They support the technical content of the CMC sections of product regulatory filings [39].

In addition to method development reports, it is important to document the entire life cycle of each analytical test method, from selection and development through method qualification and (where applicable) validation. But there are other significant events in the life cycle of an analytical procedure: method technology transfers (pre- and post-qualification or validation), method re-optimization, method re-qualification/re-validation, method comparability (i.e., comparing the performance capabilities of two different procedures),

and method bridging (i.e., replacing an old test method with a new test method). Each of these events should be supported with a prospective experimental plan outlining the objectives of the exercise and defining what would constitute a successful outcome. For example, the general requirement for replacing an old analytical method with a new one is that the new procedure must be at least as sensitive and specific for the intended use as the old procedure [4]. After the exercise, there should be complete documentation of the experiments performed, the test materials used in the study, and all results (including raw data). Documentation should also include copies of the method standard operating procedure (SOP) versions in place at the time of the study, as well as the method SOP versions generated as a result of the study (e.g., updated with new instructions after re-validation, or the method SOP from the new testing site). As a part of product knowledge management [40], these method documentation packages should be available for reference throughout the entire life cycle of the procedure.

The ICH Common Technical Document, the "generic" international regulatory dossier template for CMC quality details, indicates that a summary of the history of the analytical methods used in batch release testing and stability protocols during the pre-clinical and clinical development phases should be provided in the product commercial application [41,42]. In addition, citation 40, Pharmaceutical Quality System, describes the life-cycle approach to product information whereby early phase activities, such as method selection and development, are incorporated into a total knowledge management paradigm that remains with the product through commercialization and beyond. Likewise, in the 21st Century Initiatives for cGMP, the FDA acknowledges the key role of product development information in assuring that quality was built into the product design, which depends heavily on the appropriate analytical methods for characterization, comparability, and QC release and stability testing. For all of these reasons, method development reports will likely be of increased interest to regulatory reviewers when they are evaluating the analytical information in product license applications and annual reports.

Outline of Test Method Development Strategy

Test purpose

Identify the attribute of test samples that require measurement (i.e., identity, purity, potency, concentration, or other attributes, e.g., moisture).

To do this effectively, consider what statement, or claim, about the product will be made based upon the test results, such as:

1. The A_{280} nm protein concentration of the product batch is 5.6 mg/ml.
2. The purity of product batch is 98.6% by SEC-HPLC.
3. The product bands co-migrated with the reference standard on SDS-PAGE.
4. The product amino acid sequence corresponds to that of curecancerin.
5. No single impurity exceeded the limits of quantitation by SEC-HPLC.
6. Potency of the batch was 1450 mU/mg with the chromogenic assay.
7. No degradants were detectable by RP-HPLC after 9 months at 5°C.

Establishing the method's intended use is arguably the most critical component of test method development and validation activities [17]. Perhaps because of the wide array of analytical techniques required for

the complete analysis of biotechnology and biological products, it is often difficult to dissect the individual intended use for each type of method. But without clearly defining the method's intended use from the very beginning, the entire process of method development and validation could yield the metaphorical situation.

For quantitative methods, there is a correlation between the target specifications of the intended use and the capability required of the method to support those specifications [43]. For example, for a specification of 90-110% of a target concentration, the corresponding level of validated test method precision should be less than 5% relative standard deviation (RSD) to reliably achieve accurate results using a minimum of test method replicates (i.e., $n = 3$). Or, turning this around, if the test method can only achieve a validated precision of 10% RSD, the specifications may only be supported for 80-120% unless the number of test replicates is greatly increased. The requirements for method precision are inversely related to accuracy/recovery; that is, a lower recovery requires a more precise method to support the same claims than a higher recovery method would require. There are published standard probability curves (operating characteristic curves) that link test method precision capabilities and the number of samples needed to support the desired level of confidence in accuracy [44].

Certain biomolecular techniques can provide information on more than one attribute of the product, such as identity and purity, or identity and potency. If there are multiple intended uses for a single method, these must be investigated individually to assure the method can support each one adequately. For example, if SDS-PAGE is intended to support claims for purity and identity, the test method procedure should be designed to allow results to be evaluated individually for each attribute. Samples and reference standards might need to be in adjacent lanes on the gels to support the identity claim if co-migration of bands is the acceptance criteria. Sample concentration might not be critical as long as the test sample and reference standard were loaded in equivalent amounts and all bands are visible on the gel. In order to obtain purity data, lane order may be flexible but sample loading concentration critical to assure densitometric scanning values will fall in the linear range of the test method. If an ELISA test method is intended to support claims for identity and potency, the primary antibodies must be demonstrated to be specific for the product to claim immunoidentity, whereas if potency alone were the test method claim, intrinsic background cross-reactivity might be acceptable in the presence of the proper internal controls.

Test procedure

Assess the nature of the method technology (immunoreactivity, biochemical activity, quantitation of mass, resolution of polypeptides/impurities, etc.).

If necessary, refer to literature(s) to understand exactly how the method-and importantly, the instruments used-technically function. Each technology has limitations that can significantly impact the ability of the test to meet its intended use. Edman protein sequencing and mass spectrometry can be very specific for product identity via molecular mass and/or sequence data, but neither is routinely used in a quantitative manner because the nature of the technologies renders absolute quantitation hard to achieve. SDS-PAGE with Coomassie stain and quantitative image analysis can be developed and validated for purity and impurity determination for many biotechnology products. By comparison, SDS-PAGE using silver staining methods is highly sensitive to low levels of protein and is very useful in product comparability studies, but it is very difficult to reproducibly analyze

silver-stained gels using scanning densitometry to establish meaningful quantitative specifications.

If the nature of the technique is well understood and the method's intended use is clearly defined, designing the appropriate development and validation strategy should become a logical exercise to examine those aspects of the technology that are likely to affect the performance of the method for the parameter of interest. The emerging risk-based assessment strategies described in citation 44 could be of considerable utility in defining and prioritizing method performance parameters for a given intended use. One such exercise would be to map out the elements of the procedure, the instruments, the samples, and the reagents with a cause-and-effect diagram to identify the relationships among key elements and to uncover potential sources of variability [45]. Then experiments can be designed to assess the impact of these variables on the performance of the test method. For quantitative methods, statistically designed experimental (DOE) tools can be highly valuable in assessing the effect of multiple variables simultaneously [1].

Specific reagent

Identify any reagents or materials that could be critical to the reliable, robust performance of the method (e.g., antibodies, enzymes, substrates, cofactors, commercial kit materials, internal calibration standards, types of cuvettes, specific microtiter plates).

Many biomolecular methods require the use of critical reagents, such as antibodies in immunoassays or cell lines in cell-based assays, which are biologically derived components. Even common materials such as plastics and glass can interact with protein products or the method's biological reagents, and the lot-to-lot or vendor-to-vendor differences in composition can affect successful test method performance. Table 6 lists different types of methods and components that can contribute to variability in method performance. Any of these items can impart a significant degree of variation in test methods unless strategies are in place to prospectively address them [46]. Investigate the impact on test method performance of different lots, or the range of handling conditions. Develop an experimental study plan to bridge old lots of critical reagents to new ones before using the new lots in the test method. Include this study plan in the method SOP to assure future users will recognize when and how to perform reagent bridging with the test method.

Reference standard

Determine the appropriate product-specific reference material(s) for the intended use(s) of the method. Check for international standards if it is an existing product. Organizations such as the International Organization for Standardization (ISO) and the National Institute for Biological Standards and Controls (NIBSC) maintain certified reference standards for many currently licensed biological products, and some biotechnology products. For some biologics, the FDA Center for Biologics Evaluation and Research (CBER) has designated reference standards. Reference standards from a certification agency should only be used as primary standards against which in-house working standards are regularly qualified.

Note, however, that international standards for biological products are usually only certified for the calibration of potency or activity, and possibly molecular identity. They may be unsuitable for use in product assays for purity or impurities. In many cases, each biopharmaceutical firm, based on the nature of their purification process and their formulations, must generate their own purity and impurity reference standards. When the product is a new biomolecular entity for

Biomolecular Assay Type	Potential Batch to Batch Variability
Colorimetric	Unique buffer components Chromogenic reagent Commercial kit active components
Enzymatic	Unique buffer components Substrates Enzymes Cofactors Detection reagents Commercial kit active components
Chromatographic	Unique buffer components Labile mobile phase solvents Chromatography column resin Derivatization or conjugation reagents
Electrophoretic	Unique electrode buffers Gel matrix reagents Sample treatment reagents Staining reagents Commercial kit components
Immunological	Primary antibodies Secondary antibodies Conjugated antibodies Blocking reagents Detection reagents Commercial kit active components Plastic cuvettes or micro titer plates
Ligand Binding	Unique buffer components Target receptor Target ligand Detection reagents Commercial kit active components Plastic cuvettes or microtiter plates
Cell Based Bioassay	Cell seed stock (homogeneity and viability) Cell culture (passage number and density) Media components Growth factors Antimicrobial agents Harvest reagents (e.g., trypsin) Cell reactants (e.g., induction compounds) Plastic flasks or plates

Table 6: Different types of methods and components that can contribute to variability in performance of an analytical method.

which there are no preexisting reference standards, the innovator is responsible for establishing their own reference standards for critical product attributes, including (in some cases) key product degradants [2].

System suitability

Establish appropriate system suitability controls based on the technology of the method and its intended use.

These are to be included with each run to show the run was valid. Each analytical test method should incorporate relevant system suitability measures to allow the analyst to verify that the test system is performing to expectations at the time of use [46]. Some system suitability measures are simple (i.e., calibration of a pH meter with standard solutions, or the level of precision among replicates), and others are more complex (i.e., the use of designated reference materials in structural or functional tests).

The inherent value of system suitability is that it provides a mechanism to assess the performance characteristics of the test method at the time and in the location of each use.

It has been noted that defining and utilizing appropriate system suitability measures, particularly for nonchromatographic analytical test methods, is one of the most misunderstood aspects of method development and validation [47]. System suitability consists of two

parts: 1) the material(s) used in the assessment, and 2) the specifications associated with that material's performance (validity criteria). Typical system suitability measures for nonchromatographic methods are in the form of calibrators or controls. For quantitative methods, accuracy and precision is usually confirmed through the use of calibration standards or by the preparation of standard curves. For qualitative methods, positive and negative controls often serve as system suitability measures. In most test methods, a product-specific reference standard is included in the procedure; additional validity criteria can be designed for its use.

Each system suitability measure should have established criteria by which to determine if the materials pass or fail their performance in the test method. These are known as validity criteria; they are the specifications used to determine if a method is acceptable each time it is performed. For product-specific reference standards, validity criteria can include elution time and peak profile in chromatographic tests, migration distance and banding pattern in gel-based assays, immunoidentity in ELISAs and Western blots, concentration value in protein determination assays, activity values in potency assays, peptide fragment pattern in peptide mapping assays, and composition or fingerprint pattern in test methods for post-translational modifications.

For biomolecular methods, it can be highly valuable to utilize non product-related test materials as additional system suitability measures. Test method reference materials, sometimes referred to as surrogate or generic test method standards, can be selected and validated as a part of development of each method. To choose the right type of surrogate test method standard, consider the physiochemical nature of the material relative to that of the product and the test method application. For example, if the biotechnology product is an IgG molecule and the test method is SEC-HPLC, a purified commercial IgG might serve as a system suitability control for column performance. If the biotechnology product is a glycoprotein and the test method is for monosaccharide composition, purified bovine fetuin may be included as a system suitability control for accuracy and precision.

Many analytical laboratories have used surrogate test method standards for a variety of biomolecular test methods. Bovine serum albumin (BSA) standard reference material from the National Institute of Standards and Technologies is often used as a system suitability control for the accuracy and precision of amino acid analysis composition and concentration [48]. A new biomolecular surrogate test method standards are currently under development [49]. Three synthetic peptides have recently been prepared for potential use as system suitability measures in methods such as mass spectrometry, capillary electrophoresis, amino acid sequencing, amino acid analysis, and HPLC. The USP is in the process of preparing and certifying glycoprotein surrogate test method standards for use in a wide variety of biomolecular methods. Also, a test method reference material was recently established by the Adenovirus Reference Material Working Group (ARWMG) for system suitability use in the analysis of adenoviral gene therapy vectors, and is now available from American Type Culture Collection (ATCC VR-1516, [50]). To best assure the quality and integrity of surrogate test method standard materials, whenever possible use material that is certified for specified physiochemical properties, such as reference materials. If test method system suitability surrogate standards are obtained from a noncertified source, document the Certificates of Analysis from the vendor for each batch of material.

Demonstrating that system suitability passes its validity criteria confirms that the test method run is valid, and that the resulting sample data can be confidently evaluated.

When investigating an out-of-specification event or an unexpected result, it is important to first systematically evaluate the method's system suitability measures. When any part of system suitability fails its validity criteria, the method test run should be critically reviewed to determine and correct the assignable cause. It is considered unacceptable to use the results from invalid test runs in decisions made during cGMP product manufacturing; however, invalidating test data requires a clear justification that is supported by technical evidence [51]. Building sound system suitability measures into the test method procedure often provides the empirical evidence necessary to quickly and conclusively isolate the source of the assignable cause.

Performance of the Procedure

Write a draft SOP describing the steps required to perform the method

Include details on sample preparation, preparation of standards and controls, and system suitability measures. If instruments are used, refer to the appropriate instrument SOPs in the method SOP, or give specific instructions on instrument operation in the method SOP. Specify the number of replicates for each sample/standard, and show exactly how to calculate the results, and define how results are to be reported (i.e., significant digits).

When developing and validating analytical methods, it is important to understand the implication of using significant digits in defining reportable values and setting specifications. An all-too-common mistake is to allow extra digits to be reported in the test results, or worse, to incorporate unnecessary digits into the product specification values. The number of significant digits that can be accurately reported is related to the level of sensitivity. The method must be sensitive enough to measure differences that are one decimal place beyond the specification.

Also, the effect of rounding on the outcome of reportable results should be recognized when using additional significant digits. For example, in order to pass a specification of 7.0 to 8.0, assay results of 6.95 to 8.45 are acceptable. However, to pass specifications of 7.00 to 8.00, assay results of 6.995 to 8.004 are at least necessary. To use these results, the analytical method must be capable of generating accurate and precise values to the thousandth decimal place. The acceptable procedures for rounding values to achieve the desired number of significant digits in reportable assay values have been defined. It may be useful to review practical examples of utilizing suitable significant digits in relation to the capabilities of analytical methods [52].

Even the wording of the method SOP can impact the ability of different analysts to comparably reproduce the procedure. Initiators who are very familiar with the method sometimes leave out subtle details that can impact test method performance. The SOP instruction: "Vortex the sample" can range operationally from gentle rotation to vigorous agitation, yielding dramatically different outcomes. Also, experienced scientists may inadvertently omit an instruction if it is assumed to be common to the technique. For example, after heating samples for SDS-PAGE, it is common practice to subject the sample to brief microcentrifugation to pool the solution droplets created in the sample vial. Failure to note this "common practice" in the SOP (and failure to specify the time and speed of centrifugation) can propagate sample-handling inconsistencies that may lead to test method problems. The SOP should be as complete and detailed as necessary to allow the method steps to be performed the same way by any analyst, including the data reduction steps. It is a good idea to allow a less-

experienced analyst to run through the draft SOPs independently to see if they can complete the procedure solely based on the instructions written in the document. If not, the SOP should be revised until it provides adequate, unambiguous instructions. The best approach is always to clearly write the method procedure to be validated, and then run the validated method procedure exactly as it is written.

Method Appropriateness

Consider all potential variations in test samples (concentrations, buffers, formulation constituents) that will be included in the intended use of the method.

Run the method per SOP using representative product test materials. Include analysis of buffer or formulation solutions to assess matrix effects. If necessary, optimize test method parameters to achieve suitable preliminary performance. Edit the draft SOP to reflect any changes resulting from optimization experiments.

Most analytical methods for biotechnology products are developed and optimized using samples of the drug substance, since it is often the most suitable material for these studies. But if the method is ultimately intended for use with test samples taken from in-process, conjugation, or formulation steps, it will have to be assessed for performance with those specific types of samples. As shown in Table 7, there are several compounds that can be used in the formulation of biotechnology products [53-56].

Many conjugate reagents or formulation excipients interfere with the analytical methods developed for bulk product. For example, the presence of amino acids can affect compositional analysis, protein concentration assays, and sequencing results. Solubilizers such as Tween^R can interfere with colorimetric methods at higher concentrations. Sugars can precipitate during HPLC runs if the mobile phase becomes too polar. For both formulated and in-process samples, the concentration of drug substance may be very low ($\mu\text{g/ml}$), falling below the range of test method linearity where poor accuracy and precision may yield unreliable data. Also, unpredictable interactions between drug substance and excipients may occur. In certain cases, excipient degradation may require its own evaluation and stability testing. For these reasons, analytical methods that were validated for bulk substance may require revalidation for intermediates, conjugates, or formulated product.

Initial Performance Capabilities of the Test Method

Conduct a test method qualification or characterization study to systematically investigate the performance ranges of the method for the designated types of samples.

Prior to using a test method, studies should be conducted to investigate the working ranges of the test method for the parameters

S.No	
1	Osmotic agents (salts)
2	Chelators (EDTA, citrate)
3	Cations
4	Sugars (mannose, maltose, dextrose)
5	Amino acids (arginine, glycine, glutamic acid)
6	Redox agents (ascorbate, reducing sugars)
7	Solubilizers (Tween, Deoxycholate)
8	Stabilizers (albumin, lipids)
9	Solvents (aqueous, nonaqueous)

Table 7: Typical formulation candidates for proteins and peptides.

that could affect the intended use. These studies are sometimes called method qualification or characterization studies. Methods that are used only for product or process characterization and comparability studies should be qualified, but do not require full validation [30]. Methods that are used for the quality control and stability testing of Phase 1 clinical trial intermediates, drug substance, or drug product must be demonstrated to be scientifically sound (i.e., qualified), or validated using a limited experimental design prior to conducting the required late-phase full test method validation exercise [41].

In most cases, experiments are conducted to assess all of the parameters typically included in a validation study for the type of method such as linearity, accuracy, precision, specificity, and so on. From these experiments are derived the initial performance capabilities of the test method. These results should be compared to the intended application of the test method. If it is seen that method performance is not meeting the requirements of use, optimization experiments are usually conducted until (or unless) the method becomes acceptable. If the method cannot be optimized to meet the initial intended use, either the method will have to be replaced or the acceptance specifications for method performance will have to be reassessed.

The nature and impact of test method variability, particularly with quantitative methods such as those used for purity and potency determinations, should be clearly understood for each method prior to finalizing product acceptance specifications for that method. In some cases, an analytical method can demonstrate such inherent variability that it will have to be eliminated from consideration for use with the product, and replaced with a technique that can perform appropriately. If not, there will be a statistically predictable percent probability that a given test result will not fall within the product specification range simply due to test method variability [57].

In most cases, the nature and source of test method variability can be identified via a rigorous test method development approach, with attention to even deceptively simple parameters like test sample preparation and reference standard stability. However, some sources of variation may not be detected until the test method has been used over a long period of time. Tracking and trending the performance of a new method is a valuable tool to monitor the ongoing reliability of the method for its intended use. Tracking and trending system suitability results independent of the sample results can provide a simple (but powerful) mechanism to distinguish product variability from test method variability. This information can be used to rapidly focus troubleshooting investigations to isolate and correct the root cause of change.

Validation Method to Meet its Intended Use

Design the validation protocol using sound scientific judgment in alignment with current regulatory expectations.

There are several guidance documents on the current regulatory expectations for test method validation. For QC test methods to be used in cGMP applications, validation studies should follow the guidance in current ICH guidelines. In addition, there are many excellent historical and current articles on test method validation, many with specific examples of validation strategies and protocols, with extensive citations.

For those with limited experience, it is strongly recommended that each of these references be thoroughly reviewed to obtain a comprehensive understanding of the core requirements and different approaches possible when validating a test method. However, it should be noted that most are based on traditional chromatographic

methodology. BioPharmaceutical products require a broad range of methods utilizing widely different technologies. For these methods, the principle of a validation study remains the same (i.e., to demonstrate suitability for its intended use), but the experimental design to meet each parameter can differ considerably, based upon the nature of the technology. Currently, there are only a few specific publications on the validation of techniques used specifically for the quality control testing of biotechnology products [17,33,48,58-60]. Table 8 lists the required method validation parameters given by the ICH. The parameters must be selected appropriate to the test method intended use. The specific experiments to be conducted to achieve validation of each parameter will be based on the nature of the test method. Most of the references discussed have detailed experimental designs for chromatographic assays; these experiments should be adapted for use with other, nonchromatographic biomolecular assays for biotechnology products as needed to assure the test method will meet its intended use.

It should be noted that there are two special applications of test method validation that are distinct from the test method validation applications. Compendial methods are intended for application in any analytical laboratory, extensive collaborative studies are needed to verify the repeatability and robustness of the method in order to establish global performance specifications. Also, validation of an analytical test method for quality control applications should not be confused with the validation of test methods applied to the detection or quantitation of biological markers in clinical samples. In addition to the parameters such as linearity, accuracy, precision, specificity, and robustness, these biomarker assays also require patient studies that correlate the activity measured *in vitro* with the *in vivo* intended application (such as the designated clinical disease or condition [61]). If the method is intended to monitor product stability, it should be verified for this capability.

Procedure to Fail the Method Validation to Meet Performance Expectations

Thoroughly investigate the root cause of the failure(s) and determine what corrective actions would prevent the same problem from occurring again.

If the assignable cause for the failure of the method to meet performance requirements is identified, implement the appropriate corrective action and repeat the affected validation run(s). If the

assignable cause was related to analyst error, it would be wise to immediately address how to prevent propagating the same error in future runs of the validated test method. Sometimes the SOP simply requires enhanced clarity, such as more specific instructions or a more logical organization of steps. These document adjustments can usually be justified during validation if they do not change the method. Note that regulatory bodies indicating laboratory error should be relatively rare [51], so management should be alert to chronic method performance problems that are related solely to laboratory operations. Laboratory variability can usually be minimized with attention to issues such as clearly written SOPs, well-maintained instruments, a meaningful training program, and adequate staffing to prevent chronically hurried, and thereby inadvertently careless, analyst performance. Regardless of how thoroughly a test method has been validated, if it is not implemented in an adequately controlled laboratory environment, it will not be able to perform reliably. On the other hand, even sound laboratory operations may not be able to compensate for poorly written method SOPs or nonrobust analytical methods.

If the assignable cause for failure is not identifiable, the test method should not be considered validated. In this case, the test method should be remanded back to the method development process for further assessment of its actual suitability for the intended use(s). Experience shows that unresolved analytical test method problems that arise during method validation usually continue throughout the life cycle of test method use [52].

Re-Validation of a Validated Test Method

When something changes that could impact its continued suitability for the intended use.

Some firms have a policy of reviewing and re-validating test methods with established frequency (e.g., every 2 years). Routinely reviewing test method SOPs against laboratory practices and test records have the advantage of maintaining a strong connection between "creeping" performance habits and the actual written steps, and can catch disconnects relatively quickly. However, periodic re-validation studies when nothing has changed might be more rigorous than most facilities can operationally support. A more effective approach is to look at method re-validation strategies from a risk-based perspective. In other words, when changes do occur, what is the level of risk that the change will impact the state of validation of the test method?

Analytical Assay Procedure	Identification	Testing for Impurities		
		Quantitative	Limit	Content
Characteristics				
Potency	-	+	-	+
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Intermediate	-		+ ¹	
Precision				
Specificity ²	+	+	+	+
Detection Limit	-	- ³	+	-
Quantitation Limit	-	+	-	+
Linearity	-	+	-	+
Range	-	+	-	+

- Signifies that this characteristic is not normally evaluated

+ Signifies that this characteristic is normally evaluated

¹In cases where reproducibility has been performed, intermediate precision is not needed

²Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)

³May be needed in some cases

Table 8: Validation requirements from ICH validation of analytical procedure.

S.No	
1	Changes to the product that could affect method performance (e.g., formulation excipients, product concentration)
2	Changes in critical assay reagents that cannot meet prior specifications (e.g., gel or blot materials, enzymes)
3	Changes in instrumentation that cannot perform equivalent procedures (e.g., hydrolysis systems)
4	Changes in the procedure to improve robustness of the method (e.g., new qualification procedures for critical reagents, re-optimized sample preparation steps)
5	Changes in the product specifications that go beyond the capabilities of the test method (e.g., specification on quantifying impurities drops from 0.5% to 0.1%; assay limit of quantitation is 0.25%)

Table 9: Potential triggers for method revalidation.

Table 9 lists five categories of change that should trigger a review of the test method and possibly require a measure of re-validation: 1) changes to the product that could affect method performance (e.g., formulation excipients, product concentration), 2) changes in critical assay reagents that cannot meet prior performance requirements (e.g., gel reagents, enzymes, antibodies), 3) changes in instrumentation that cannot meet prior performance settings (e.g., automated amino acid hydrolysis systems), 4) changes in the procedure to improve robustness of the method (e.g., adding new sample preparation steps), or 5) changes in the product specifications that are beyond the test method capabilities. In most cases, the re-validation may be limited to a set of studies that bridge the old part of the procedure to the new part of the procedure. In cases where method performance capabilities are significantly affected and, as a result, will require changes to method specifications, a complete re-validation may be necessary. Regardless of the extent of re-validation needed, it is imperative to confirm that all analysts are sufficiently trained on the new procedure to assure successful results. It is a useful management tool to monitor trends in method performance following re-validation to spot problems and make the appropriate corrections quickly.

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