PHARMACOKINETIC AND BIOMARKER METHODS DEVELOPMENT REPORT

# Recommendations for Preparing the Required Summary Report

## Introduction

Method validation studies demonstrate that a particular method is appropriate for its intended application. In validating a method, the sources of error are evaluated, and objective operating values are established from the validation studies that control the method performance characteristics and minimize variability. The types of errors covered by validation are: 1) random errors which give rise to a spread of results around the average results. They are defined by the repeatability or reproducibility of the procedures, and 2) systematic errors which cause a bias in the results obtained, such that the average value observed is above or below the true value (sources of inaccuracy). The process of validating a method cannot be separated from the actual development of the method conditions, because the developer will not know whether the method conditions are acceptable until validation studies are performed. Therefore, development and validation are iterative until a method emerges with acceptable efficiency and timeliness.

Not all methods can be validated by conventional means to provide standard measures of the method performance characteristics. Some newer technologies, particularly biomarker assays, have not yet evolved meaningful control parameters, or use samples with very high inter- and intra-subject variability. Additionally, analytical validation as a term in biomarker literature is often confused with measures of the clinical utility of the procedure as a diagnostic test (true positive rates/false positive rates, representative sampling for adequate disease description). These types of validation are not included in this guideline. The recommendations herein are intended primarily for quantitative analytical methods used for specific analytes in a specific biological matrix (blood, urine, saliva, etc.) under certain limitations. A glossary is included as Appendix I for clarity.

All pharmacokinetic and biomarker laboratory techniques, assays, and procedures should be developed and validated according to this manual, and a short draft summary report prepared for NCI, DCP approval.

The degree of validation effort is dependent on the novelty of the technology, sophistication of the analyst and phase of clinical development. Methods that are modifications of already published, validated procedures can be validated by as little as one intra-assay accuracy and precision determination at the new laboratory. In the early stages of drug development, it is usually not necessary to perform all of the various validation studies. Many researchers focus on sensitivity, specificity, linearity, accuracy, and precision studies for drugs in the preclinical through Phase II (preliminary efficacy) stages. The remaining validation studies are performed when the drug reaches the Phase III (efficacy) stage of development and the study agent has a higher probability of becoming a marketed product.

These Methods Development and Validation Reports should be succinct and illustrative, but not exhaustive. Concise summaries and tabulations of descriptive statistics should be used whenever possible. The function of these reports is to provide enough details to facilitate method transfer, prepare a Standard Operating Procedure (SOP), and verify the method development and validation work was performed as expected. DCP does not intend to delay agent development or be burdensome but has a legitimate and compelling need for the development and validation report. The attached Report template (Appendix II) should be modified as needed to provide a full or partial (modification of published procedure) validation but should contain no more than 3 to 5 pages of information.

## Validation Studies

Analytical methods employed for the quantitative determination of analytes in biological samples play a pivotal role in the evaluation and interpretation of pharmacokinetic data. It is essential to employ well-characterized and fully validated analytical methods to yield reliable results which can be satisfactorily interpreted. Specific validation criteria are needed for methods intended for analysis of each analyte. Method validation includes all of the investigations undertaken to demonstrate that a particular method is reliable for the intended application.

In general, the following items can be evaluated for the validation of a quantitative analytical method (see glossary, Appendix I):

* + - * Reference standards
      * Chemical stability of the analyte in the biological matrix during collection, processing, storage and analysis. This includes physical freeze/thaw cycling stability of an analyte.
      * Linearity: The range over which the procedure has been demonstrated to produce an accurate, reproducible, linear response.
      * Specificity/Selectivity: the degree to which the method can quantify the target analyte in the presence of potentially interfering materials, other analytes, or matrices
      * Sensitivity: Limit of detection (LOD), limit of quantitation (LOQ), and 95% confidence interval of standard curve.
      * Accuracy: The closeness of a determined value to the true value.
      * Precision: The variability of replicate determinations.
      * Ruggedness: Variability between operators, instrument, columns, *etc.*
      * System Suitability: start-up parameters and measures of instrument function to indicate the procedure may be successfully performed

The Draft Methods Development and Validation Report must contain all the supporting graphical and computational (with example calculations, if needed) documentation used in method development. A description of how the data will be summarized and how missing data or outlier values will be treated should be provided. Any modification of an analytical method requires re-validation of the procedure and a new Summary Report to be provided.

A suggested template for the Report is provided in Appendix II. It is expected that the analyst has experience with analytical methodology and familiarity with specific study conditions and terminology contained in regulatory and analytical guidances[1-6] to perform the validation studies listed above.

# Recommendations for Pharmacokinetic Analytical Method Development and Validation Draft Summary Report

## Reference standards

Analysis of drugs and their metabolites in a biological matrix is usually carried out using samples prepared with known reference standards (spiked samples, QA samples, concentration standards, calibration standards, etc.). Clearly, the purity and quality of the reference standard used to prepare samples will affect the accuracy of the study. For this reason, an authenticated analytical reference standard of known identity and purity should be used to prepare solutions of known concentrations. If possible, the reference standard should be identical to the analyte. When this is not possible, a different chemical form of the analyte (free base or acid, salt or ester) of known purity may be used and a relative reference standard may be established. A certificate of analysis (COA) for the reference standards should be submitted in the report appendices.

The standard should be stored correctly and used within the established use interval. A reference standard that is not obtained from an official source (i.e., the United States Pharmacopeia) should be of the highest purity that can be obtained by reasonable effort, and it should be thoroughly characterized by appropriate methods to ensure its identity, strength, quality, purity, and potency. The qualitative and quantitative analytical procedures used to characterize a reference standard are expected to be different from, and more extensive than, those used to control the analyte.

## Method Development Summary

The method report should include a title, report author and affiliation(s), statement of purpose, scope of the method, a listing of necessary reagents, test solutions and mixtures, and directions for preparation, storage, and expiration dating of required materials. It should also include a listing of the required instruments, instrumental operating parameters and operating principles. Any health and safety issues should be clearly stated with appropriate precautions stated for the safe use of the method.

## Validation Studies

Each step of the method should be investigated to encompass analyte, matrix, and procedural variables. Whenever possible, the same biological material as that of the intended samples should be used for validation. The usual studies done to examine each of these issues are:

*Linearity*

The concentration range over which the analyte can be accurately determined, based on evaluation of actual standard samples. A standard curve should be prepared consisting of a blank sample (matrix sample without internal standard), a zero sample (matrix sample processed with internal standard), and samples at concentrations covering the expected analyte concentration range, including the Limit of Quantitation (LOQ, see Sensitivity). The regression line generated from this data should contain the calculated point of intersection with the y axis and the coefficient of regression (r2). The range of linearity (in concentration terms) should be stated. The method is valid for quantitation only within the linear concentration range of the standard curve and for the specific analyte in the specific matrix.

The range of linearity is checked by replicate determinations at 5-6 concentrations of the reference standards (prepared in triplicates) including samples at least 20 % below and 20% above the expected target concentration of the specimen samples. A standard curve should be generated for each analytical run for each analyte and the generated curve for that run should be used to calculate the concentration of analyte in unknown samples within that run. At the completion of linearity studies, the appropriate concentration range for the standards should be set for all subsequent studies.

*Specificity/selectivity*

If possible, the specificity of the assay methodology should be established using at least 3 different possibly interfering analytes in six independent sources of the same type of biological matrix. There should be clear separation between the analyte and possible interferences.

*Sensitivity*

The limit of detection (LOD) is the lowest concentration of an analyte that the method can reliably differentiate from background levels. The lower limit of quantitation (LOQ) is the lowest concentration on the standard curve that can be measured with acceptable accuracy, precision and variability. The LOQ is determined by using at least five independent standards and by determining the coefficient of variation (CV) or percent Relative Standard Deviation (%RSD). The LOQ serves as the lowest concentration of the standard curve.

*Accuracy and Precision*

Determination of accuracy (% recovery) and precision can be accomplished by analysis of replicate sets of analyte samples of known concentrations from an equivalent biological matrix, using a minimum of five determinations per concentration. Specific criteria must be set for accuracy and precision over the range of the standard curve. At least three concentrations representing the entire range of the calibration curve should be studied—one near the lower LOQ, one near the center, and one near the upper boundary of the standard curve.

*System Suitability*

Quality-control (QC) samples are matrix spiked with analyte. QC samples in duplicate at three concentrations (one near the LOQ, one in midrange, and one approaching the high end of the range) should be incorporated into each run. Results of QC samples should be within the validated performance values to provide a basis for accepting or rejecting the run. Typically, there should be 2 or more suitability parameters.

*Stability studies*

Stability studies should use analyte in the specific matrix of the assay method. Variability of the matrix due to its physical state and the influence of freeze-thaw cycles on analyte and matrix integrity should be examined. The analyte and matrix should be demonstrated to be chemically and physically stable for a period of time equal to the initial sampling to the final analytical data output.

## Biomarkers Methods Development and Validation

For biomarker assays in general, the recommendations above (when possible) should be followed. Though many types of assays exist, only immunohistochemistry is covered by this Guidance. Immunohistochemistry is used to characterize a wide range of biomarkers. This technique is not linear; it saturates at higher intensities of staining and is accentuated at the lower intensities of staining. A semi-quantitative index has been developed [7] incorporating both the staining intensity of individual cells and the percent of cells staining at each intensity.

The following parameters are essential to ensure the acceptability of the results reported from this technique:

* stability of the antigen in the biological sample (*e.g*., tissue biopsy) under study storage conditions,
* sensitivity of the assay,
* specificity of the antibody, and
* intra- and inter-assay reproducibility.

## Recommendations for Immunohistochemical Methods Validation

*Procedure*

A detailed description and protocol of analytical method and scoring methodology (standard operating procedure) should be outlined, including a statement of the method operation principles and a listing of necessary reagents, test solutions and mixtures with directions for their preparation, storage conditions and usable shelf life specified. It should also include a listing of the required instruments and instrumental parameters, which contains sufficient information to allow repetition of the method by a qualified investigator. If a detailed method has been published previously, it is sufficient to cite the publication and summarize the key operational principles (e.g., source and dilution of antibody used, conditions for the assay such as overnight binding to primary antibody, use of automatic stainer, type of chromogen, etc.).

Stability of the antigen in the biological sample is determined under identical study storage conditions (*e.g*., temperature, duration) and sample conditions (*e.g*., frozen tissue biopsy, paraffin-embedded tissue).

Sensitivity of the assay should be determined using at least three sequential antibody dilutions. Specificity of the assay should be determined by using at least two different antibodies to the same antigen, if feasible. It is also important to perform the assay in the absence of antibody in order to determine nonspecific staining. Intra-assay reproducibility should be determined by staining different serial sections from the same biological sample and/or by showing that different antibodies to the same antigen give similar patterns and intensities of staining.

Inter-assay reproducibility should be determined by staining the same tissue sections for the same antibodies during multiple different assay runs on different days. Several parameters can influence reproducibility, but variability in antigen expression within the tissue can be minimized by using adjacent sections. Day-to-day variability due to differences in staining procedure and conditions can be minimized by using an automated stainer. Variability introduced by the evaluator can be minimized by averaging the scores of two or more observers.

## REFERENCES

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# PHARMACOKINETIC AND BIOMARKER METHODS DEVELOPMENT REPORT:

## APPENDIX 1. GLOSSARY

**Accuracy:** The degree of closeness of the determined value to the nominal or known true value under prescribed conditions. This is sometimes termed *trueness*.

**Analyte:** A specific chemical moiety being measured, which can be intact drug, biomolecule or its derivative, metabolite, and/or degradation product in a biologic matrix.

**Analytical run (or batch):** A complete set of analytical and study samples with appropriate number of standards and QCs for their validation. Several runs (or batches) may be completed in one day, or one run (or batch) may take several days to complete.

**Biological marker (biomarker):**  A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

**Biological matrix:** A discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples are blood, serum, plasma, urine, feces, saliva, sputum, and various discrete tissues.

**Blank:** A sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method.

**Calibration standard:** A biological matrix to which a known amount of analyte has been added or *spiked*. Calibration standards are used to construct calibration curves from which the concentrations of analytes in QCs and in unknown study samples are determined.

**Internal standard:** Test compound(s) (e.g. structurally similar analog, stable labeled compound) added to both calibration standards and samples at known and constant concentration to facilitate quantification of the target analyte(s).

**Known valid biomarker:**  A biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is widespread agreement in the medical or scientific community about the physiologic, toxicologic, pharmacologic, or clinical significance of the results.

**Limit of detection (LOD):** The lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise. Typically, the LOD is three times the mean peak noise level.

**Limit of quantification (LLOQ):** The lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. If not determined experimentally, the quantitation limit is often calculated as the analyte concentration that gives a Signal/Noise ratio of ten.

**Matrix effect:** The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.

**Method:** A comprehensive description of all procedures used in sample analysis.

**Precision:** The closeness of agreement (*degree of scatter*) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

**Probable valid biomarker:**  A biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is a scientific framework or body of evidence that appears to elucidate the physiologic, toxicologic, pharmacologic, or clinical significance of the test results.

**Processed:** The final extract (prior to instrumental analysis) of a sample that has been subjected to various manipulations (e.g., extraction, dilution, concentration).

**Quality control sample (QC):** A spiked sample used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

**Quantification range:** The range of concentration, including upper and lower limits and LLOQ, which can be reliably and reproducibly quantified with accuracy and precision through the use of a concentration-response relationship.

**Recovery:** The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.

**Reproducibility:** The precision between two laboratories. It also represents precision of the method under the same operating conditions over a short period of time.

**Sample:** A generic term encompassing controls, blanks, unknowns, and processed samples, as described below:

**Selectivity:** The ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present. These could include metabolites, impurities, degradants, or matrix components.

**Stability:** The chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

**Standard curve:** The relationship between the experimental response value and the analytical concentration (also called a calibration curve).

**System suitability:** Determination of instrument performance (e.g., sensitivity and chromatographic retention) by analysis of a reference standard prior to running the analytical batch.

**Unknown:** A biological sample that is the subject of the analysis.

**Valid biomarker:**  A biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is an established scientific framework or body of evidence that elucidates the physiologic, toxicologic, pharmacologic, or clinical significance of the test results.  The classification of biomarkers is context specific.  Likewise, validation of a biomarker is context-specific and the criteria for validation will vary with the intended use of the biomarker.  The clinical utility (e.g., predict toxicity, effectiveness or dosing) and use of epidemiology/population data (e.g., strength of genotype-phenotype associations) are examples of approaches that can be used to determine the specific context and the necessary criteria for validation.

**Validation:**

* Full validation: Establishment of all validation parameters to apply to sample analysis for the bioanalytical method for each analyte.
* Partial validation: Modification of validated bioanalytical methods that do not necessarily call for full revalidation.
* Cross-validation: Comparison of validation parameters of two bioanalytical methods.

# PHARMACOKINETIC AND BIOMARKER METHODS DEVELOPMENT REPORT:

## APPENDIX II. Suggested Report Template

1. Title:

Ex: “Single Laboratory Validation of the Determination of [Analyte] in [Matrix] by [Nature of Determination]” Author, Laboratory Affiliation, Other participants

2. Method Summary, Applicability of Method, Scope and Limitations

3. Analytes

Common and chemical name

CAS Registry Number or Merck Index Number

Matrices used

In presence of:

In absence of:

Safety statements and precautions applicable to product

4. Preparation of sample (following sections as applicable)

Extraction

Purification

Separation

Measurement

Alternatives

Interferences

5. Reagents

Reference standards, identity, source, purity,

Calibration standard solutions,

Preparation, storage, stability

Solvents (special requirements)

Buffers

6. Apparatus (equipment usually present in a laboratory need not be listed); provide source, internet address, and catalog numbers of special items)

Analytical equipment

Operating conditions

System suitability conditions and acceptance criteria for equipment

Expected suitability parameters

Temperature controlled equipment

Separation equipment (centrifuges, filters)

7. Calibration Range, number and distribution of standards, replicates.

8. Procedure

List all steps of method, including any homogenization of the test sample.

Critical points

Stopping points

9. Calculation Formulae, symbols, significant figures, example calculations, data treatment, outliers, out of specification results and their investigation.

10. Controls

11. Results of Validation

Analytes measured and properties utilized

Matrices tested

Reference standard, source, identity, purity

Performance data

Recovery of control material

Repeatability (by replication of entire procedure on same test sample)

Linearity

Limit of detection

Limit of quantitation

12. Stability data of analyte in biological matrix, analytical solutions and biological matrix alone. At least 3 freeze/thaw cycles

Appendices (Chromatograms, definitions, glossary, etc.)