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# Guidance for Industry

## Bioanalytical Method Validation

### *DRAFT GUIDANCE*

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For questions regarding this draft document contact (CDER) Brian Booth, 301-796-1508 or (CVM) John Kadavil, [John.Kadavil@fda.hhs.gov](mailto:John.Kadavil@fda.hhs.gov)

**U.S. Department of Health and Human Services  
Food and Drug Administration  
Center for Drug Evaluation and Research (CDER)  
Center for Veterinary Medicine (CVM)**

**September 2013  
Biopharmaceutics**

**Revision 1**

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# Guidance for Industry

## Bioanalytical Method Validation

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**U.S. Department of Health and Human Services**  
**Food and Drug Administration**  
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*Contains Nonbinding Recommendations*

*Draft — Not for Implementation*

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**Guidance for Industry<sup>1</sup>**  
**Bioanalytical Method Validation**

This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

**I. INTRODUCTION**

This guidance provides assistance to sponsors of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs), biologic license applications (BLAs), and supplements in developing bioanalytical method validation information used in human clinical pharmacology, bioavailability (BA), and bioequivalence (BE) studies that require pharmacokinetic (PK) or biomarker concentration evaluation. This guidance also applies to bioanalytical methods used for nonclinical pharmacology/toxicology studies. For studies related to the veterinary drug approval process (Investigational New Animal Drug Applications (INADs), New Animal Drug Applications (NADAs), and Abbreviated New Animal Drug Applications (ANADAs)), this guidance may apply to blood and urine BA, BE, and PK studies.

The information in this guidance generally applies to bioanalytical procedures, such as gas chromatography (GC); high-pressure liquid chromatography (LC); combined GC and LC mass spectrometric (MS) procedures, such as LC-MS, LC-MS-MS, GC-MS, and GC-MS-MS; and ligand binding assays (LBAs), and immunological and microbiological procedures that are performed for the quantitative determination of drugs and/or metabolites, and therapeutic proteins in biological matrices, such as blood, serum, plasma, urine, tissue, and skin.

This guidance provides general recommendations for bioanalytical method validation. The recommendations can be modified depending on the specific type of analytical method used.

Originally issued in 2001, this guidance has been revised to reflect advances in science and technology related to validating bioanalytical methods. The guidance is being reissued in draft to enable public review and comment before it is finalized.

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<sup>1</sup> This guidance has been prepared by the Bioanalytical Methods Working Group in the Center for Drug Evaluation and Research (CDER) in cooperation with the Center for Veterinary Medicine (CVM) at the Food and Drug Administration.

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42 FDA's guidance documents, including this guidance, do not establish legally enforceable  
43 responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should  
44 be viewed only as recommendations, unless specific regulatory or statutory requirements are  
45 cited. The use of the word *should* in Agency guidances means that something is suggested or  
46 recommended, but not required.

47

### 48 **II. BACKGROUND**

49

50 This guidance was originally developed based on the deliberations following two workshops:  
51 Analytical Methods Validation: Bioavailability, Bioequivalence, and Pharmacokinetic Studies  
52 (December 3-5, 1990<sup>2</sup>) and Bioanalytical Methods Validation: A Revisit With a Decade of  
53 Progress (January 12-14, 2000<sup>3</sup>). Since publication of the guidance in May 2001, additional  
54 workshops have been held that have helped guide the current revisions to the guidance: the  
55 Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for  
56 Chromatographic and Ligand Binding Assays (May 1-3, 2006<sup>4</sup>) and the AAPS/FDA Workshop  
57 on Incurred Sample Reanalysis (February 2008<sup>5</sup>).

58

59 Selective, sensitive, and validated analytical methods for the quantitative evaluation of drugs and  
60 their metabolites (analytes) and biomarkers are critical for the successful conduct of nonclinical  
61 and/or biopharmaceutics and clinical pharmacology studies. Validating bioanalytical methods  
62 includes performing all of the procedures that demonstrate that a particular method used for  
63 quantitative measurement of analytes in a given biological matrix (e.g., blood, plasma, serum, or  
64 urine) is reliable and reproducible for the intended use. Fundamental parameters for this  
65 validation include the following:

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- Accuracy
- Precision
- Selectivity
- Sensitivity
- Reproducibility
- Stability

Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of a method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method. For pivotal studies that require regulatory action for approval or labeling, such as BE or PK studies, the bioanalytical methods should be fully validated. For exploratory methods used for the sponsor's internal decision making, less validation may be sufficient.

When changes are made to a previously validated method, additional validation may be needed. For example, published methods of analysis are often modified to suit the requirements of the laboratory performing the assay, and during the course of a typical drug development program, a defined bioanalytical method often undergoes many modifications. These modifications should

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<sup>2</sup> Workshop Report: Shah, V.P. et al., *Pharmaceutical Research*: 1992; 9:588-592.

<sup>3</sup> Workshop Report: Shah, V.P. et al., *Pharmaceutical Research*: 2000; 17: 1551-1557

<sup>4</sup> Workshop Report: Viswanathan, C.T., *Pharmaceutical Research*: 2007; 24: 1962-7

<sup>5</sup> Workshop Report: Fast, D., *AAPS Journal*: 2009; 11: 238-241.

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84 be validated to ensure suitable performance of the analytical method. The evolutionary changes  
85 needed to support specific studies call for different levels of validation to demonstrate the  
86 validity of method performance.

87  
88 The following define and characterize the different types and levels of methods validation.

### Full Validation

91  
92 Full validation of bioanalytical methods is important:

- 93
- 94 • During development and implementation of a novel bioanalytical method.
- 95 • For analysis of a new drug entity.
- 96 • For revisions to an existing method that add metabolite quantification

### Partial Validation

97  
98  
99  
100 Partial validations evaluate modifications of already validated bioanalytical methods. Partial  
101 validation can range from as little as one intra-assay accuracy and precision determination to a  
102 nearly full validation. Typical bioanalytical method modifications or changes that fall into this  
103 category include but are not limited to:

- 104
- 105 • Bioanalytical method transfers between laboratories or analysts
- 106 • Change in analytical methodology (e.g., change in detection systems)
- 107 • Change in anticoagulant in harvesting biological fluid (e.g., heparin to EDTA)
- 108 • Change in matrix within species (e.g., human plasma to human urine)
- 109 • Change in sample processing procedures
- 110 • Change in species within matrix (e.g., rat plasma to mouse plasma)
- 111 • Change in relevant concentration range
- 112 • Changes in instruments and/or software platforms
- 113 • Modifications to accommodate limited sample volume (e.g., pediatric study)
- 114 • Rare matrices
- 115 • Selectivity demonstration of an analyte in the presence of concomitant medications

### Cross-Validation

116  
117  
118  
119 Cross-validation is a comparison of validation parameters when two or more bioanalytical  
120 methods are used to generate data within the same study or across different studies. An example  
121 of cross-validation would be a situation in which an original validated bioanalytical method  
122 serves as the *reference*, and the revised bioanalytical method is the *comparator*. The  
123 comparisons should be done both ways.

124  
125 When sample analyses within a single study are conducted at more than one site or more than  
126 one laboratory, cross-validation with spiked matrix standards and subject samples should be  
127 conducted at each site or laboratory to establish inter-laboratory reliability. Cross-validation  
128 should also be considered when data generated using different analytical techniques (e.g., LC-

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129 MS/MS vs. ELISA<sup>6</sup>) in different studies are included in a regulatory submission. All  
130 modifications to an existing method should be assessed to determine the recommended degree of  
131 validation.

132  
133 The analytical laboratory conducting nonclinical pharmacology/toxicology studies for regulatory  
134 submissions should adhere to FDA's Good Laboratory Practices (GLPs) requirements<sup>7</sup> (21 CFR  
135 Part 58). The bioanalytical method for human BA, BE, PK, and drug interaction studies must  
136 meet the criteria specified in 21 CFR 320.29.

137  
138 Analytical laboratories should have written standard operating procedures (SOPs) to ensure a  
139 complete system of quality control and assurance. SOPs should cover all aspects of analysis  
140 from the time the sample is collected and reaches the laboratory until the results of the analysis  
141 are reported. The SOPs also should include record keeping, security and chain of sample custody  
142 (accountability systems that ensure integrity of test articles), sample preparation, and analytical  
143 tools such as methods, reagents, equipment, instrumentation, and procedures for quality control  
144 and verification of results.

145  
146 The following sections discuss in more detail chromatographic methods, ligand binding assays,  
147 incurred sample reanalysis, and other issues that should be considered and how best to document  
148 validation methods.

149

### **150 III. CHROMATOGRAPHIC METHODS**

151

#### **152 A. Reference Standards**

153

154 Analysis of drugs and their metabolites in a biological matrix is performed using calibration  
155 standards and quality control samples (QCs) spiked with reference standards. The purity of the  
156 reference standard used to prepare spiked samples can affect study data. For this reason,  
157 authenticated analytical reference standards of known identity and purity should be used to  
158 prepare solutions of known concentrations. If possible, the reference standard should be identical  
159 to the analyte. When this is not possible, an established chemical form (free base or acid, salt or  
160 ester) of known purity can be used.

161

162 Three types of reference standards are usually used: (1) certified reference standards (e.g., USP  
163 compendial standards), (2) commercially-supplied reference standards obtained from a reputable  
164 commercial source, and/or (3) other materials of documented purity custom-synthesized by an  
165 analytical laboratory or other noncommercial establishment. The source and lot number,  
166 expiration date, certificates of analyses when available, and/or internally or externally generated  
167 evidence of identity and purity should be furnished for each reference and internal standard (IS)  
168 used. If the reference or internal standard expires, stock solutions made with this lot of standard  
169 should not be used unless purity is re-established.

170

#### **171 B. Bioanalytical Method Development and Validation**

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<sup>6</sup> Enzyme linked immunosorbent assay.

<sup>7</sup> For the CVM, all bioequivalence studies are subject to Good Laboratory Practices.

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173 A specific, detailed, written description of the bioanalytical method should be established *a*  
174 *priori*. This can be in the form of a protocol, study plan, report, and/or SOP. Each step in the  
175 method should be investigated to determine the extent to which environmental, matrix, or  
176 procedural variables could affect the estimation of analyte in the matrix from the time of  
177 collection of the samples to the time of analysis.

178  
179 Appropriate steps should be taken to ensure the lack of matrix effects throughout the application  
180 of the method, especially if the matrix used for production batches is different from the matrix  
181 used during method validation. Matrix effects on ion suppression or enhancement or on  
182 extraction efficiency should be addressed. A bioanalytical method should be validated for the  
183 intended use or application. All experiments used to make claims or draw conclusions about the  
184 validity of the method should be presented in a report (method validation report), including a  
185 description of validation runs that failed.

186  
187 Measurements for each analyte in the biological matrix should be validated. Method  
188 development and validation for a bioanalytical method should include demonstrations of (1)  
189 selectivity; (2) accuracy, precision, and recovery; (3) the calibration curve; (4) sensitivity; (5)  
190 reproducibility; and (6) stability of analyte in spiked samples.

191  
192 *1. Selectivity*

193  
194 *Selectivity* is the ability of an analytical method to differentiate and quantify the analyte in the  
195 presence of other components in the sample. Evidence should be provided that the substance  
196 quantified is the intended analyte. Analyses of blank samples of the appropriate biological  
197 matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank  
198 sample should be tested for interference, and selectivity should be ensured at the lower limit of  
199 quantification (LLOQ).

200  
201 Potential interfering substances in a biological matrix include endogenous matrix components;  
202 metabolites; decomposition products; and, in the actual study, concomitant medication and other  
203 xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be  
204 tested to ensure that there is no interference.

205  
206 *2. Accuracy, Precision, and Recovery*

207  
208 The *accuracy* of an analytical method describes the closeness of mean test results obtained by  
209 the method to the actual value (concentration) of the analyte. Accuracy is determined by  
210 replicate analysis of samples containing known amounts of the analyte (i.e., QCs). Accuracy  
211 should be measured using a minimum of five determinations per concentration. A minimum of  
212 three concentrations in the range of expected study sample concentrations is recommended. The  
213 mean value should be within 15% of the nominal value except at LLOQ, where it should not  
214 deviate by more than 20%. The deviation of the mean from the nominal value serves as the  
215 measure of accuracy.

216  
217 The *precision* of an analytical method describes the closeness of individual measures of an  
218 analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous



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219 volume of biological matrix. Precision should be measured using a minimum of five  
220 determinations per concentration. A minimum of three concentrations in the range of expected  
221 study sample concentrations is recommended. The precision determined at each concentration  
222 level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it  
223 should not exceed 20% of the CV. Precision is further subdivided into within-run and between-  
224 run precision. *Within-run precision* (intra-batch precision or within-run repeatability) is an  
225 assessment of precision during a single analytical run. *Between-run precision* (inter-batch  
226 precision or between-run repeatability) is an assessment of precision over time and may involve  
227 different analysts, equipment, reagents, and laboratories.

228  
229 Sample concentrations above the upper limit of the standard curve should be diluted. The  
230 accuracy and precision of these diluted samples should be demonstrated in the method  
231 validation.

232  
233 The *recovery* of an analyte in an assay is the detector response obtained from an amount of the  
234 analyte added to and extracted from the biological matrix, compared to the detector response  
235 obtained for the true concentration of the analyte in solvent. Recovery pertains to the extraction  
236 efficiency of an analytical method within the limits of variability. Recovery of the analyte need  
237 not be 100%, but the extent of recovery of an analyte and of the internal standard should be  
238 consistent, precise, and reproducible. Recovery experiments should be performed by comparing  
239 the analytical results for extracted samples at three concentrations (low, medium, and high) with  
240 unextracted standards that represent 100% recovery.

### 241 3. *Calibration Curve*

242  
243  
244 A *calibration (standard) curve* is the relationship between instrument response and known  
245 concentrations of the analyte. The relationship between response and concentration should be  
246 continuous and reproducible. A calibration curve should be generated for each analyte in the  
247 sample. The calibration standards can contain more than one analyte. A calibration curve should  
248 be prepared in the same biological matrix as the samples in the intended study by spiking the  
249 matrix with known concentrations of the analyte. In rare cases, matrices may be difficult to  
250 obtain (e.g., cerebrospinal fluid). In such cases, calibration curves constructed in surrogate  
251 matrices should be justified. Concentrations of standards should be chosen on the basis of the  
252 concentration range expected in a particular study. A calibration curve should consist of a blank  
253 sample (matrix sample processed without analyte or internal standard), a zero sample (matrix  
254 sample processed without analyte but with internal standard), and at least six non-zero samples  
255 (matrix samples processed with analyte and internal standard) covering the expected range,  
256 including LLOQ.

257  
258 Method validation experiments should include a minimum of six runs conducted over several  
259 days, with at least four concentrations (including LLOQ, low, medium, and high) analyzed in  
260 duplicate in each run.

#### 261 a. Lower Limit of Quantification (LLOQ)

262  
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264 The lowest standard on the calibration curve should be accepted as the LLOQ if the  
265 following conditions are met:

- 266
- 267 • The analyte response at the LLOQ should be at least five times the response  
268 compared to blank response.
  - 269 • Analyte peak (response) should be identifiable, discrete, and reproducible, and the  
270 back-calculated concentration should have precision that does not exceed 20% of  
271 the CV and accuracy within 20% of the nominal concentration. The LLOQ should  
272 not be confused with the limit of detection (LOD) and/or the low QC sample.
  - 273 • The LLOQ should be established using at least five samples and determining the  
274 CV and/or appropriate confidence interval should be determined.

275

276 b. Upper Limit of Quantification (ULOQ)

277

278 The highest standard will define the ULOQ of an analytical method.

- 279
- 280 • Analyte peak (response) should be reproducible and the back-calculated  
281 concentration should have precision that does not exceed 15% of the CV and  
282 accuracy within 15% of the nominal concentration

283

284 c. Calibration Curve/Standard Curve/Concentration-Response

- 285
- 286 • The simplest model that adequately describes the concentration-response  
287 relationship should be used. Selection of weighting and use of a complex  
288 regression equation should be justified. Standards/calibrators should not deviate  
289 by more than 15% of nominal concentrations, except at LLOQ where the  
290 standard/calibrator should not deviate by more than 20%.
  - 291 • The acceptance criterion for the standard curve is that at least 75% of non-zero  
292 standards should meet the above criteria, including the LLOQ. Excluding an  
293 individual standard should not change the model used. Exclusion of calibrators  
294 for reasons other than failing to meet acceptance criteria and assignable causes is  
295 discouraged.

296

297 d. Quality Control Samples (QCs)

- 298
- 299 • At least three concentrations of QCs in duplicate should be incorporated into each  
300 run as follows: one within three times the LLOQ (low QC), one in the midrange  
301 (middle QC), and one approaching the high end (high QC) of the range of the  
302 expected study concentrations.
  - 303 • The QCs provide the basis of accepting or rejecting the run. At least 67% (e.g., at  
304 least four out of six) of the QCs concentration results should be within 15% of  
305 their respective nominal (theoretical) values. At least 50% of QCs at each level  
306 should be within 15% of their nominal concentrations. A confidence interval

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307 approach yielding comparable accuracy and precision in the run is an appropriate  
308 alternative.

309 • The minimum number of QCs should be at least 5% of the number of unknown  
310 samples or six total QCs, whichever is greater.

311 • It is recommended that calibration standards and QCs be prepared from separate  
312 stock solutions. However, standards and QCs can be prepared from the same  
313 spiking stock solution, provided the stability and accuracy of the stock solution  
314 have been verified. A single source of blank matrix may also be used, provided  
315 absence of matrix effects on extraction recovery and detection has been verified.  
316 At least one demonstration of precision and accuracy of calibrators and QCs  
317 prepared from separate stock solutions is expected.

318  
319 Acceptance/rejection criteria for spiked, matrix-based calibration standards and QCs should be  
320 based on the nominal (theoretical) concentration of analytes.

### 321 4. *Sensitivity*

322  
323 *Sensitivity* is defined as the lowest analyte concentration that can be measured with acceptable  
324 accuracy and precision (i.e., LLOQ).

### 325 326 5. *Reproducibility*

327  
328 *Reproducibility* of the method is assessed by replicate measurements using the assay, including  
329 quality controls and possibly incurred samples. Reinjection reproducibility should be evaluated  
330 to determine if an analytical run could be reanalyzed in the case of instrument interruptions.

### 331 332 6. *Stability*

333  
334 The chemical stability of an analyte in a given matrix under specific conditions for given time  
335 intervals is assessed in several ways. Pre-study stability evaluations should cover the expected  
336 sample handling and storage conditions during the conduct of the study, including conditions at  
337 the clinical site, during shipment, and at all other secondary sites.

338  
339 Drug stability in a biological fluid is a function of the storage conditions, the physicochemical  
340 properties of the drug, the matrix, and the container system. The stability of an analyte in a  
341 particular matrix and container system is relevant only to that matrix and container system and  
342 should not be extrapolated to other matrices and container systems.

343  
344 Stability testing should evaluate the stability of the analytes during sample collection and  
345 handling, after long-term (frozen at the intended storage temperature) and short-term (bench top,  
346 room temperature) storage, and after freeze and thaw cycles and the analytical process.  
347 Conditions used in stability experiments should reflect situations likely to be encountered during  
348 actual sample handling and analysis. If, during sample analysis for a study, storage conditions  
349 changed and/or exceeded the sample storage conditions evaluated during method validation,  
350 stability should be established under these new conditions.  
351

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352

353 The procedure should also include an evaluation of analyte stability in stock solution.

354 All stability determinations should use a set of samples prepared from a freshly made stock  
355 solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock  
356 solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at  
357 known concentrations. Stability samples should be compared to freshly made calibrators and/or  
358 freshly made QCs. At least three replicates at each of the low and high concentrations should be  
359 assessed. Stability sample results should be within 15% of nominal concentrations.

360

361 a. Freeze and Thaw Stability

362

363 During freeze/thaw stability evaluations, the freezing and thawing of stability samples  
364 should mimic the intended sample handling conditions to be used during sample analysis.  
365 Stability should be assessed for a minimum of three freeze-thaw cycles.

366

367 b. Bench-Top Stability

368

369 Bench top stability experiments should be designed and conducted to cover the laboratory  
370 handling conditions that are expected for study samples.

371

372 c. Long-Term Stability

373

374 The storage time in a long-term stability evaluation should equal or exceed the time  
375 between the date of first sample collection and the date of last sample analysis.

376

377 d. Stock Solution Stability

378

379 The stability of stock solutions of drug and internal standard should be evaluated. When  
380 the stock solution exists in a different state (solution vs. solid) or in a different buffer  
381 composition (generally the case for macromolecules) from the certified reference  
382 standard, the stability data on this stock solution should be generated to justify the  
383 duration of stock solution storage stability.

384

385 e. Processed Sample Stability

386

387 The stability of processed samples, including the resident time in the autosampler, should  
388 be determined.

389

### **C. Validated Method: Use, Data Analysis, and Reporting**

391

392 This section describes the expectations for the use of a validated bioanalytical method for routine  
393 drug analysis.

394

- 395 • System suitability: If system suitability is assessed, a specific SOP should be used.  
396 Apparatus conditioning and instrument performance should be determined using spiked

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- 397 samples independent of the study calibrators, QCs, or study samples. Data should be  
398 maintained with the study records.
- 399 • Calibration curves and QCs should be included in all analytical runs.  
400 An analytical run should consist of QCs, calibration standards, and one or more batches  
401 of processed samples. A batch may consist of all of the processed unknown samples of  
402 one or more subjects in a study and QCs. If the bioanalytical method necessitates  
403 separation of the overall analytical run into distinct processing batches (e.g., capacity  
404 limit of 96-well plates or solid phase extraction manifold, extraction by multiple  
405 analysts), each distinct processing batch should process at least duplicates QCs at all QC  
406 levels (e.g., low, middle, high) along with the study samples. In such cases, acceptance  
407 criteria should be established for the analytical run as a whole as well as the distinct  
408 processing batches.
  - 409 • The calibration (standard) curve should cover the expected study sample concentration  
410 range.
  - 411 • Accuracy and precision as outlined in section III.B.2. should be provided for both the  
412 inter-run and intra-run experiments and tabulated for all runs (passed and failed).
  - 413 • Concentrations in unknown samples should not be extrapolated below the LLOQ or  
414 above the ULOQ of the standard curve. Instead, the standard curve should be extended  
415 and revalidated, or samples with higher concentration should be diluted and reanalyzed.  
416 Concentrations below the LLOQ should be reported as zeros.  
417 Any required sample dilutions should use like matrix (e.g. human to human).
  - 418 • Assays of all samples of an analyte in a biological matrix should be completed within the  
419 time period for which stability data are available.
  - 420 • Response Function: Typically, the same curve fitting, weighting, and goodness of fit  
421 determined during pre-study validation should be used for the calibration curve within the  
422 study. Response function should be determined by appropriate statistical tests based on  
423 the actual standard points during each run in the validation. Changes in the response  
424 function relationship between pre-study validation and routine run validation indicate  
425 potential problems. Internal standard response should be monitored for drift. An SOP  
426 should be developed *a priori* to address issues related to variability of the IS response.
  - 427 • The QCs should be used to accept or reject the run. Runs should be rejected if the  
428 calibration standards or QCs fall outside the acceptance criteria stated above (III.B.2).
  - 429 • QCs should be interspersed with study samples during processing and analysis. The  
430 minimum number of QCs to ensure proper control of the assay should be at least 5% of  
431 the number of unknown samples or a total of six QCs, whichever is greater.
  - 432 • If the study sample concentrations are clustered in a narrow range of the standard curve,  
433 additional QCs should be added to cover the sample range. Accuracy and precision of  
434 the additional QCs should be validated before continuing with the analysis. If the partial  
435 validation is acceptable, samples that have already been analyzed do not require re-  
436 analysis.
  - 437 • All study samples from a subject should be analyzed in a single run.

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- 438 • Carryover should be assessed and monitored during analysis. If carryover occurs, it  
439 should be mitigated or reduced.
- 440 • Incurred sample reanalysis (ISR) should be performed (See Section V. Incurred Sample  
441 Reanalysis).
- 442 • Repeat Analysis: It is important to establish an SOP or guideline for repeat analysis and  
443 acceptance criteria. This SOP or guideline should explain the reasons for repeating  
444 sample analysis. Reasons for repeat analyses could include samples outside of the assay  
445 range, sample processing errors, equipment failure, and poor chromatography. Reassays  
446 should be done in triplicate if sample volume allows. The rationale, approach, and all  
447 data for the repeat analysis and reporting should be clearly documented.
- 448 • Samples involving multiple analytes should not be rejected based on the data from one  
449 analyte failing the acceptance criteria.
- 450 • The data from rejected runs should be documented but need not be reported; however, the  
451 fact that a run was rejected and the reason for failure should be reported.
- 452 • If a unique or disproportionately high concentration of a metabolite is discovered in  
453 human studies, a fully validated assay may need to be developed for the metabolite,  
454 depending upon its activity (refer to the FDA guidance for industry *Safety Testing of*  
455 *Drug Metabolites*).
- 456 • Reported method validation data and the determination of accuracy and precision should  
457 include all outliers; however, calculations of accuracy and precision excluding values that  
458 are determined as outliers should also be reported.
- 459 • Sample Data Reintegration: An SOP or guideline for sample data reintegration should be  
460 established *a priori*. This SOP or guideline should define the criteria for reintegration and  
461 how the reintegration is to be performed. The rationale for the reintegration should be  
462 clearly described and documented. Audit trails should be maintained. Original and  
463 reintegration data should be reported.

464

### **IV. LIGAND BINDING ASSAYS**

466

467 Many of the bioanalytical validation parameters and principles discussed above are also  
468 applicable to microbiological and ligand-binding assays (LBA). These types of assays have a  
469 variety of design configurations that possess some unique characteristics that should be  
470 considered during method validation.

471

#### **A. Key Reagents**

473

474 Key reagents, such as reference standards, antibodies, tracers, and matrices should be  
475 characterized appropriately and stored under defined conditions.

476

477 Assay reoptimization or validation may be important when there are changes in key reagents.  
478 For example:

479

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480 Labeled analytes (tracers)

- 481 • Binding should be reoptimized.
- 482 • Performance should be verified with standard curve and QCs.

483

484 Antibodies

- 485 • Key cross-reactivities should be checked.
- 486 • Tracer experiments above should be repeated.

487

488 Matrices

- 489 • Tracer experiments above should be repeated.

490

### **B. Bioanalytical Method Development and Validation**

492

493 A specific, detailed, written description of the bioanalytical method should be established *a*  
494 *priori*. This can be in the form of a protocol, study plan, report, and/or SOP. Each step in the  
495 method should be investigated to determine the extent to which environmental, matrix, or  
496 procedural variables can affect the estimation of analyte in the matrix from the time of collection  
497 of the samples to the time of analysis.

498

499 It may be important to consider the variability of the matrix. Appropriate steps should be taken to  
500 ensure the lack of matrix effects throughout the application of the method, especially if the  
501 nature of the matrix changes from the matrix used during method validation. A bioanalytical  
502 method should be validated for the intended use or application. All experiments used to make  
503 claims or draw conclusions about the validity of the method should be presented in a report  
504 (method validation report).

505

506 Measurements for each analyte in the biological matrix should be validated. Method  
507 development and validation for a bioanalytical method should include demonstrations of (1)  
508 selectivity, (2) accuracy, precision, recovery, (3) the calibration curve, (4) sensitivity, (5)  
509 reproducibility, and (6) stability of analyte in spiked samples.

510

#### *I. Selectivity*

512

513 As with chromatographic methods (described in Section III), LBAs should be shown to be  
514 selective for the analyte. The following recommendations for dealing with two selectivity issues  
515 should be considered:

516

##### a. Interference from Substances Physiochemically Similar to the Analyte

518

- 519 • Cross-reactivity of metabolites, concomitant medications, and their significant  
520 metabolites, or endogenous compounds should be evaluated individually and in  
521 combination with the analyte of interest.
- 522 • When possible, the LBA should be compared with a validated reference method  
523 (such as LC-MS) using incurred samples and predetermined criteria to assess the  
524 accuracy of the LBA method.

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### 526 b. Matrix Effects

527

528

529

Matrix effects should be evaluated. For example:

530

531

532

533

534

535

- The calibration curve in biological fluids should be compared with calibrators in buffer to detect matrix effects using at least ten sources of blank matrix.
- Parallelism of diluted study samples should be evaluated with diluted standards to detect matrix effects.
- Nonspecific binding should be determined.

536

### 2. Accuracy, Precision and Recovery

537

538 *Accuracy* is determined by replicate analysis of samples containing known amounts of the

539 analyte (QCs). Accuracy should be measured using a minimum of five determinations per

540 concentration. A minimum of three concentrations in the range of expected study sample

541 concentrations is recommended. The mean value should be within 20% of the actual value

542 except at LLOQ, where it should not deviate by more than 25%.

543

544 The *precision* should be measured using a minimum of five determinations per concentration. A

545 minimum of three concentrations in the range of expected study sample concentrations is

546 recommended. The precision determined at each concentration level should not exceed 20% of

547 the CV except for the LLOQ, where it should not exceed 25% of the CV. Precision is further

548 subdivided into *within-run* and *between-run* precision. *Within-run* (also known as intra-batch

549 precision or repeatability) is an assessment of the precision during a single analytical run.

550 *Between-run* precision (also known as interbatch precision or repeatability), is a measurement of

551 the precision with time, and may involve different analysts, equipment, reagents, and

552 laboratories.

553

554 Samples with concentrations over the ULOQ should be diluted with the same matrix as used for

555 the study samples, and accuracy and precision should be demonstrated.

556

557 For LBAs that employ sample extraction, the *recovery* of an analyte is the measured

558 concentration relative to the known amount added to the matrix. Recovery experiments should be

559 performed for extracted samples at three concentrations.

560

### 3. Calibration Curve

561

562 Most LBA calibration (standard) curves are inherently nonlinear and, in general, more

563 concentration points may be recommended to define the fit over the standard curve range than

564 for chromatographic assays. In addition to their nonlinear characteristics, the response-error

565 relationship for immunoassay standard curves is a variable function of the mean response

566 (heteroscedasticity). For these reasons, the standard curve should consist of a minimum of six,

567 duplicate non-zero calibrator concentrations covering the entire range including LLOQ and

568 excluding blanks (either single or replicate). The concentration-response relationship is most

569 often fitted to a 4- or 5-parameter logistic model, although other models may be used with

570 suitable validation. Calibrators should be prepared in the same matrix as the study samples. If an

571



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572 alternate matrix is used, proper justification should be provided. A calibration curve should be  
573 generated for each analyte in the sample.

574  
575 Method validation experiments should include a minimum of six runs conducted over several  
576 days, with at least six concentrations (including LLOQ, low, medium, and high) analyzed in  
577 duplicate in each run.

578

579 a. Lower Limit of Quantification (LLOQ)

580

- 581 • The lowest concentration on the calibration curve should be the LLOQ if the  
582 following conditions are met:
- 583 • Analyte peak (response) should be identifiable, discrete, and reproducible and  
584 back-calculated concentration should have precision that does not exceed 25%  
585 CV and accuracy within 25% of the nominal concentration. The LLOQ should not  
586 be confused with the LOD and/or the low QCs.
- 587 • The LLOQ should be established using at least five samples and determining  
588 coefficient of variation and/or appropriate confidence intervals.

589

590 b. Upper Limit of Quantification (ULOQ)

591

592 The highest standard will define the ULOQ of an analytical method.

593

- 594 • Analyte response should be reproducible and the back-calculated concentration  
595 should have precision that does not exceed 20% CV and accuracy within 20% of  
596 the nominal concentration.

597

598 c. Calibration Curve/Standard Curve/Concentration-Response

599

- 600 • The simplest model that adequately describes the concentration-response  
601 relationship should be used. Selection of weighting and use of a complex  
602 regression equation should be justified. The standard calibrator concentrations  
603 should be within 25% of the nominal concentration at LLOQ and within 20% of  
604 the nominal concentration at all other concentrations.
- 605 • The acceptance criterion for the standard curve is that at least 75% of non-zero  
606 standards should meet the above criteria, including the LLOQ. Excluding an  
607 individual standard should not change the model used. Exclusion of calibrators for  
608 reasons other than failing to meet acceptance criteria and assignable causes is  
609 discouraged.
- 610 • Total error (accuracy and precision) should not exceed 30%. Values falling  
611 outside these limits should be discarded, provided they do not change the  
612 established model.

613

614 d. Quality Control Samples (QCs)

615

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- 616
- 617
- 618
- 619
- At least three concentrations of QCs in duplicate should be incorporated into each run as follows: one within three times the LLOQ (low QC), one in the midrange (middle QC), and one approaching the high end (high QC) of the range of the expected study sample concentrations.
- 620
- 621
- 622
- 623
- 624
- 625
- The results of the QCs provide the basis of accepting or rejecting the run. At least 67% (e.g., at least four out of six) of the QC concentration results should be within 20% of their respective nominal (theoretical) values. At least 50% of QCs at each level should be within 20% of their nominal concentrations. A confidence interval approach yielding comparable accuracy and precision in the run is an appropriate alternative.
- 626
- 627
- The minimum number of QCs should be at least 5% of the number of unknown samples or six total QCs, whichever is greater.
- 628
- 629
- 630
- 631
- 632
- 633
- 634
- It is recommended that calibration standards and QCs be prepared from separate stock solutions. However, standards and QCs can be prepared from the same spiking stock solution, provided the stability and accuracy of the stock solution have been verified. A single source of blank matrix may also be used, provided absence of matrix effects on extraction recovery and detection has been verified. At least one demonstration of precision and accuracy of calibrators and QCs prepared from separate stock solutions is expected.

635

636 Acceptance/rejection criteria for spiked, matrix-based calibration standards and QCs should be

637 based on the nominal (theoretical) concentration of analytes.

638

### *4. Sensitivity*

640

641 *Sensitivity* is defined as the lowest analyte concentration that can be measured with acceptable

642 accuracy and precision.

643

### *5. Reproducibility*

644

645

646 *Reproducibility* of the method is assessed by replicate measurements using the assay, including

647 quality controls and possibly incurred samples. Reinjection reproducibility should be evaluated

648 to determine if an analytical run could be reanalyzed in the case of instrument interruptions.

649

### *6. Stability*

650

651

652 The chemical stability of an analyte in a given matrix under specific conditions for given time

653 intervals is assessed in several ways. Pre-study stability evaluations should cover the expected

654 sample handling and storage conditions during the conduct of the study, including conditions at

655 the clinical site, during shipment, and at all other secondary sites.

656

657 Stability samples should be compared to freshly made calibrators and/or freshly made QCs. At

658 least three replicates at each of the low and high concentrations should be assessed. Assessments

659 of analyte stability should be conducted in the same matrix as that of the study samples. All

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660 stability determinations should use samples prepared from a freshly made stock solution.  
661 Conditions used in stability experiments should reflect situations likely to be encountered during  
662 actual sample handling and analysis (e.g., long-term, bench top, and room temperature storage;  
663 and freeze-thaw cycles). If, during sample analysis for a study, storage conditions changed  
664 and/or exceed the sample storage conditions evaluated during method validation, stability should  
665 be established under the new conditions. Stock solution stability also should be assessed.  
666 Stability sample results should be within 15% of nominal concentrations.

667  
668 a. Freeze and Thaw Stability

669  
670 During freeze/thaw stability evaluations, the freezing and thawing of stability samples  
671 should mimic the intended sample handling conditions to be used during sample analysis.  
672 Stability should be assessed for a minimum of three freeze-thaw cycles.

673  
674 b. Bench-Top Stability

675  
676 Bench top stability experiments should be designed and conducted to cover the laboratory  
677 handling conditions that are expected for study samples.

678  
679 c. Long-Term Stability

680  
681 The storage time in a long-term stability evaluation should equal or exceed the time  
682 between the date of first sample collection and the date of last sample analysis.

683  
684 d. Stock Solution Stability

685  
686 The stability of stock solutions of drug should be evaluated. When the stock solution  
687 exists in a different state (solutions vs. solid) or in a different buffer composition  
688 (generally the case for macromolecules) from the certified reference standard, the  
689 stability data on this stock solution should be generated to justify the duration of stock  
690 solution storage stability.

691  
692 e. Processed Sample Stability

693  
694 The stability of processed samples, including the time until completion of analysis,  
695 should be determined.

696  
697 **C. Validated Method: Use, Data Analysis, and Reporting**

698  
699 This section describes the expectations for the use of a validated bioanalytical method for routine  
700 drug analysis.

- 701
- 702 • Standard curves and QCs should be included in all analytical runs.
  - 703 • The calibration (standard) curve should cover the expected study sample concentration
  - 704 range.

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- 705 • Accuracy and precision as outlined in Section IV.B.2 should be provided for both the  
706 inter-run and intra-run experiments and tabulated for all runs (passed and failed).
- 707 • Concentrations in unknown samples should not be extrapolated below the LLOQ or  
708 above the ULOQ of the standard curve. Instead, the standard curve should be extended  
709 and revalidated, or samples with higher concentrations should be diluted and reanalyzed.  
710 Concentrations below the LLOQ should be reported as zeros. Any required sample  
711 dilutions should use like matrix (e.g., human to human).
- 712 • Assays of all samples of an analyte in a biological matrix should be completed within the  
713 time period for which stability has been demonstrated.
- 714 • Response Function: Typically, the same curve fitting, weighting, and goodness of fit  
715 determined during pre-study validation should be used for the standard curve within the  
716 study. Response function is determined by appropriate statistical tests based on the actual  
717 standard points during each run in the validation. Any changes in the response function  
718 relationship between pre-study validation and routine run validation indicate potential  
719 problems. An SOP should be developed *a priori* to address such issues.
- 720 • The QCs should be used to accept or reject the run. Runs should be rejected if the  
721 calibration standards or QCs fall outside the acceptance criteria stated above.
- 722 • QCs should be interspersed with study samples during processing and analysis. The  
723 minimum number of QCs to ensure proper control of the assay should be at least 5% of  
724 the number of unknown samples or a total of six QCs, whichever is greater.
- 725 • If the study sample concentrations are clustered in a narrow range of the standard curve,  
726 additional QCs should be added in the sample range. Accuracy and precision of the  
727 additional QCs should be validated before continuing with the analysis. If the partial  
728 validation is acceptable, samples that have already been analyzed do not require re-  
729 analysis.
- 730 • All study samples from a subject should be analyzed in a single run.
- 731 • Carryover should be assessed and monitored during analysis. If carryover occurs, it  
732 should be mitigated or reduced.
- 733 • Incurred sample reanalysis (ISR) should be performed (See Section V. Incurred Sample  
734 Reanalysis).
- 735 • Repeat Analysis: It is important to establish an SOP or guideline for repeat analysis and  
736 acceptance criteria. This SOP or guideline should explain the reasons for repeating  
737 sample analysis. Reasons for repeat analyses could include samples outside of the assay  
738 range, sample processing errors, and equipment failure. The rationale, approach, and all  
739 data for the repeat analysis and reporting should be clearly documented.
- 740 • Samples involving multiple analytes should not be rejected based on the data from one  
741 analyte failing the acceptance criteria.
- 742 • The data from rejected runs should be documented, but need not be reported; however,  
743 the fact that a run was rejected and the reason for failure should be reported.

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- 744 • If a unique or disproportionately high concentration of a metabolite is discovered in  
745 human studies, a fully validated assay may need to be developed for the metabolite  
746 depending on its activity (see guidance for industry *Safety Testing of Drug Metabolites*).
- 747 • Reported method validation data and the determination of accuracy and precision should  
748 include all outliers; however, calculations of accuracy and precision, excluding values  
749 that are determined as outliers, should also be reported.

### **V. INCURRED SAMPLE REANALYSIS**

750  
751 Incurred sample reanalysis (ISR) is a necessary component of bioanalytical method validation  
752 and is intended to verify the reliability of the reported subject sample analyte concentrations.  
753 ISR is conducted by repeating the analysis of a subset of subject samples from a given study in  
754 separate runs on different days to critically support the precision and accuracy measurements  
755 established with spiked QCs; the original and repeat analysis is conducted using the same  
756 bioanalytical method procedures. ISR samples should be compared to freshly prepared  
757 calibrators. ISR is expected for all in vivo human BE studies and all pivotal PK or  
758 pharmacodynamic (PD) studies. For nonclinical safety studies, the performing laboratory should  
759 conduct ISR at least once for each method and species.  
760

761  
762 For regulatory submissions containing only a few studies, it may be advantageous to incorporate  
763 ISR into the method development and validation stage by conducting a pilot study prior to the  
764 pivotal study. This approach allows for the remediation of methodological issues prior to  
765 conduct of the pivotal study. For applications with a greater number of pivotal PK or PD studies,  
766 ISR should be monitored in a larger number and variety of studies.  
767

768 Standard operating procedures should be established and followed to address the following  
769 points:  
770

- 771 • The total number of ISR samples should be 7% of the study sample size.
- 772 • In selecting samples for reanalysis, adequate coverage of the PK profile in its entirety  
773 should be provided and should include assessments around C<sub>max</sub> and in the elimination  
774 phase for all study subjects.
- 775 • Two-thirds (67%) of the repeated sample results should be within 20% for small  
776 molecules and 30% for large molecules. The percentage difference of the results is  
777 determined with the following equation:

778 
$$\frac{(\text{Repeat} - \text{Original})}{\text{Mean}} * 100$$

779

780 Written procedures should be in place to guide an investigation in the event of ISR failure for the  
781 purpose of resolving the lack of reproducibility. All aspects of ISR evaluations should be  
782 documented to reconstruct the study conduct as well as any investigations thereof. ISR results  
783 should be included in the final report of the respective study.

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### 784 **VI. ADDITIONAL ISSUES**

785

#### 786 **A. Endogenous Compounds**

787

788 For analytes that are also endogenous compounds, the accuracy of the measurement of the  
789 analytes poses a challenge when the assay cannot distinguish between the therapeutic and the  
790 endogenous counterpart. In such situations, the following approaches are recommended to  
791 validate and monitor assay performance. Other approaches, if justified by scientific principles,  
792 may also be considered.

793

794 • The biological matrix used to prepare calibration standards should be the same as the  
795 study samples and free of the endogenous analyte. To address the suitability of an  
796 analyte-free biological matrix, the matrix should be demonstrated to have (1) no  
797 measurable endogenous analyte and (2) no matrix effect or interference when compared  
798 to the biological matrix. The use of alternate matrices (e.g., buffers, dialyzed serum) for  
799 the preparation of calibration standards is generally not recommended unless an analyte-  
800 free biological matrix is not readily available or cannot be prepared. In such cases, use of  
801 an alternate analyte-free matrix should be justified, and the calibration standard in the  
802 alternate matrix should be demonstrated to have no matrix effect when compared to the  
803 actual biological matrix of the study samples.

804 • The QCs should be prepared by spiking known quantities of analyte(s) in the same  
805 biological matrix as the study samples. The endogenous concentrations of the analyte in  
806 the biological matrix should be evaluated prior to QC preparation (e.g., by replicate  
807 analysis). The concentrations for the QCs should account for the endogenous  
808 concentrations in the biological matrix (i.e., additive) and be representative of the  
809 expected study concentrations.

810

#### 811 **B. Biomarkers**

812

813 The recommendations in this guidance pertain only to the validation of assays to measure in vivo  
814 biomarker concentrations in biological matrices such as blood or urine. Considerable effort also  
815 goes into defining the biological function of biomarkers, and confusion may arise regarding  
816 terminology. Information about defining the biological role of a biomarker is available on the  
817 FDA Drug Development Tools website.

818

819 Biomarkers are increasingly used to assess the effects of new drugs and therapeutic biological  
820 products in patient populations. Because of the important roles biomarkers can play in  
821 evaluating the safety and/or effectiveness of a new medical product, it is critical to ensure the  
822 integrity of the data generated by assays used to measure them. Biomarkers can be used for a  
823 wide variety of purposes during drug development; therefore, a fit-for-purpose approach should  
824 be used when evaluating the extent of method validation that is appropriate. When biomarker  
825 data will be used to support a regulatory action, such as the pivotal determination of safety  
826 and/or effectiveness or to support labeled dosing instructions, the assay should be fully validated.

827

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828 For assays intended to support early drug development (e.g., candidate selection, go-no-go  
829 decisions, proof-of-concept), the sponsor should incorporate the extent of method validation they  
830 deem appropriate.

831  
832 Method validation for biomarker assays should address the same questions as method validation  
833 for PK assays. The accuracy, precision, selectivity, range, reproducibility, and stability of a  
834 biomarker assay are important characteristics that define the method. The approach used for PK  
835 assays should be the starting point for validation of biomarker assays, although FDA realizes that  
836 some characteristics may not apply or that different considerations may need to be addressed.

### 837 838 **C. Diagnostic Kits**

839  
840 Diagnostic kits are sometimes co-developed with new drug or therapeutic biologic products. The  
841 recommendations in this section of the guidance do not apply to commercial diagnostic kits that  
842 are intended for point-of-care patient diagnosis, but rather to analytical methods that are used  
843 during the development of new drugs and therapeutic biologics. The reader should refer to the  
844 appropriate CDRH guidance documents regarding FDA expectations for commercial diagnostic  
845 kits. Furthermore, these recommendations do not apply to Clinical Laboratory Improvements  
846 Amendments (CLIA)-regulated entities or to assays designed to quantify or identify genes or  
847 genetic polymorphisms.

848  
849 If a sponsor uses a commercially available diagnostic kit to measure a biomarker, drug, or  
850 therapeutic biologic concentration during the development of a novel drug or therapeutic  
851 biologic product, FDA makes the following recommendations.

852  
853 Ligand binding assay (LBA) kits with various detection platforms are sometimes used to  
854 determine analyte concentrations in PK or PD studies when the reported results must exhibit  
855 sufficient precision and accuracy. Because such kits are generally developed for use as clinical  
856 diagnostic tools, their suitability for use in PK or PD studies should be demonstrated.

857  
858 Diagnostic kit validation data provided by the manufacturer may not ensure reliability of the kit  
859 method for drug development purposes. The performance of diagnostic kits should be assessed  
860 in the facility conducting the sample analysis. Validation considerations for kit assays include,  
861 but are not limited to, the following examples:

- 862
- 863 • Site-specific validation should be performed. Specificity, accuracy, precision, and  
864 stability should be demonstrated under actual conditions of use. Modifications from kit  
865 processing instructions should be validated completely.
  - 866 • Kits that use sparse calibration standards (e.g., one- or two-point calibration curves)  
867 should include in-house validation experiments to establish the calibration curve with a  
868 sufficient number of standards across the calibration range.
  - 869 • Actual QC concentrations should be known. Concentrations of QCs expressed as ranges  
870 are not sufficient for quantitative applications. In such cases, QCs with known  
871 concentrations should be prepared and used, independent of the kit-supplied QCs.

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- 872 • Standards and QCs should be prepared in the same matrix as the subject samples. Kits  
873 with standards and QCs prepared in a matrix different from the subject samples should be  
874 justified, and appropriate cross-validation experiments should be performed. Refer to the  
875 endogenous compounds section of this guidance for additional discussion (see VI.A).
- 876 • If the analyte source (reference standard) in the kit differs from that of the subject  
877 samples (e.g., protein isoform variation), testing should evaluate differences in  
878 immunological activity with the kit reagents.
- 879 • If multiple kit lots are used within a study, lot-to-lot variability and comparability should  
880 be addressed for critical reagents.
- 881 • Individual batches using multiple assay plates (e.g., 96-well ELISA plates) should  
882 include sufficient replicate QCs on each plate to monitor accuracy. Acceptance criteria  
883 should be established for the individual plates and overall analytical run.

884

### **D. New Technologies**

885

886  
887 FDA encourages the development and use of new bioanalytical technologies. Generally, the use  
888 and submission of data based on new technologies should be supported with data generated by  
889 established technology, until the new approaches become accepted practice.

890

891 Although the Dried Blood Spot (DBS) methodology has been successful in individual cases, the  
892 method has not yet been widely accepted. Benefits of DBS include reduced blood sample  
893 volumes collected for drug analysis and ease of collection, storage, and transportation. A  
894 comprehensive validation will be essential prior to using DBS in regulated studies. This  
895 validation should address, at a minimum, the effects of the following issues: storage and  
896 handling temperature, homogeneity of sample spotting, hematocrit, stability, carryover, and  
897 reproducibility including ISR. Correlative studies with traditional sampling should be conducted  
898 during drug development. Sponsors are encouraged to seek feedback from the appropriate FDA  
899 review division early in drug development.

900

901

## **VII. DOCUMENTATION**

902

903  
904 General and specific SOPs and good record keeping are essential to a properly validated  
905 analytical method. The validity of an analytical method should be established and verified by  
906 laboratory studies, and the documentation of successful completion of such studies should be  
907 provided in the assay validation report. The data generated for bioanalytical method  
908 establishment and the QCs should be documented and available for data audit and inspection.  
909 Documentation for submission to FDA should include the following:

910

- 911 • Method development and validation data and reports.
- 912 • Bioanalytical reports of the application of any methods to study sample analysis.
- 913 • Overall summary information including limitations to use.

914 All relevant documentation necessary for reconstructing the study as it was conducted and

915 reported should be maintained in a secure environment. Relevant documentation includes, but is



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916 not limited to, source data; protocols and reports; records supporting procedural, operational, and  
917 environmental concerns; and correspondence records between the involved parties.

918  
919 Regardless of the documentation format (i.e., paper or electronic), records should be  
920 contemporaneous with the event, and subsequent alterations should not obscure the original data.  
921 The basis for changing or reprocessing data should be documented with sufficient detail, and the  
922 original record should be maintained. Electronic audit trails should be available for all  
923 chromatography acquisition and data processing software and other means of electronic data  
924 capture. Information related to each bioanalytical run should be maintained at the laboratory and  
925 should include the analysts performing the run, start and stop times (duration), raw data,  
926 integration codes, and/or other reporting codes.

927

### **A. System Suitability/Equilibration**

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929  
930 System suitability is routinely assessed before an analytical run. Data generated from system  
931 suitability checks should be maintained in a specific file on-site and should be available for  
932 inspection. System suitability samples should be different from the study samples, standards, and  
933 QCs to be analyzed in the run. Therefore, study samples, standards, or QCs should not be used  
934 as their own system suitability samples within the analytical run.

935

### **B. Summary Information**

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937  
938 Summary information should include:

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- 940 • A summary of assay methods used for each study protocol. Each summary should  
941 provide the protocol number, protocol title, assay type, assay method identification code,  
942 bioanalytical report code, and effective date of the method.
- 943 • For each analyte, a summary table of all the relevant method validation reports should be  
944 provided including partial validation, and cross-validation reports. The table should  
945 include assay method identification code, type of assay, the reason for the new method or  
946 additional validation (e.g., to lower the limit of quantification), and the dates of final  
947 reports. Changes made to the method should be clearly identified.
- 948 • A summary table cross-referencing multiple identification codes should be provided  
949 when an assay has different codes for the assay method, validation reports, and  
950 bioanalytical reports.

951

### **C. Documentation for Method Validation**

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954 Documentation for method validation should include:

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- 956 • An operational description of the analytical method used in the study.
- 957 • A detailed description of the assay procedure (analyte, IS, sample pre-treatment, method  
958 of extraction, and analysis).

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- 959 • A description of the preparation of the calibration standards and QCs including blank  
960 matrix, anticoagulant if applicable, dates of preparation, and storage conditions.
- 961 • Evidence of purity and identity of drug, metabolites, and IS used at the time of the  
962 validation experiments. The chromatography of the analyte should be interference-free.  
963 The batch/lot numbers and storage conditions of the reference standards used to prepare  
964 the calibration standards and QCs of each assay should be provided.
- 965 • A description of potential interferences for the drug or metabolites in LBAs.
- 966 • A description of experiments conducted to determine accuracy, precision, recovery,  
967 selectivity, stability, limits of quantification, calibration curve (equations and weighting  
968 functions used), and a summary of the results including intra- and inter-assay precision  
969 and accuracy. QCs results that fail to meet the acceptance criteria should not be excluded  
970 from calculations of accuracy and precision unless there is an assignable cause.
- 971 • A description of cross-validation or partial validation experiments and supporting study  
972 data, if applicable.
- 973 • Legible annotated chromatograms or mass spectrograms, if applicable.
- 974 • Description and supporting data of significant investigations of unexpected results if  
975 applicable.
- 976 • Tabulated data including, but not limited to, the following:
  - 977 – All validation experiments with analysis dates, whether the experiments passed or  
978 failed and the reason for the failure.
  - 979 – Results of calibration standards from all validation experiments, including calibration  
980 range, response function, back-calculated concentrations, accuracy and precision.
  - 981 – QC results from all validation experiments (within- and between-run precision and  
982 accuracy).
  - 983 – Data from all stability experiments, i.e., storage temperatures, duration of storage,  
984 dates of analysis, and dates of preparation of QCs and calibration standards used in  
985 the stability experiments.
  - 986 – Data on selectivity, LLOQ, carry-over, extraction recovery, matrix effect if  
987 applicable, dilution integrity, anticoagulant effect if applicable.

988  
989 All measurements with the individual calculated concentrations should be presented in the  
990 validation report.

### 991 **D. Documentation for Bioanalytical Report**

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993  
994 Documentation of the application of validated bioanalytical methods to routine drug analysis  
995 should include:

- 996 • Evidence of purity at the time of use and identity of drug standards, metabolite standards,  
997 and internal standards used during routine analyses, and expiration or retest dates.
- 998 • Step-by-step description of procedures for preparation of QCs and calibrators.

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- Sample identification, collection dates, storage prior to shipment, information on shipment batch, and storage prior to analysis. Information should include dates, times, and sample condition.
- 1002
- 1003
- Any deviations from the validated method, significant equipment and material changes, SOPs, protocols, and justifications for deviations.
- 1004
- Equations and regression methods for calculation of concentration results.
- 1005
- 1006
- 1007
- 1008
- 1009
- 1010
- Complete serial chromatograms from 5-20% of subjects, with standards and QCs from those analytical runs. For pivotal bioequivalence studies used to support approval, chromatograms from 20% of serially selected subjects should be included. In other studies, chromatograms from 5% of randomly selected subjects in each study should be included. Subjects whose chromatograms are to be submitted should be defined prior to the analysis of any clinical samples.
- 1011
- Reasons for missing samples.
- 1012
- 1013
- 1014
- 1015
- Repeat analyses should be documented with the reason(s) for the repeat analysis, the initial and repeat analysis results, the reported result, assay run identification, and the manager authorizing reanalysis. Repeat analysis of a clinical or nonclinical sample should be performed only under a predefined SOP.
- 1016
- 1017
- 1018
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- 1020
- Data from reintegrated chromatograms should be documented with the reason for reintegration, initial and repeat integration results, the method used for reintegration, the reported result, assay run identification, and the manager authorizing reintegration. Reintegration of a clinical or nonclinical sample should be performed only under a predefined SOP.
- 1021
- 1022
- 1023
- The following tables should be included:
- 1024
- 1025
- Summary of intra- and inter-assay values of QCs and calibration curve standards used for accepting the analytical run. QC graphs and trend analyses are encouraged.
- 1026
- 1027
- 1028
- 1029
- A table listing all of the accepted and rejected analytical runs of clinical or nonclinical samples. The table should include assay run identification, assay method, and the subjects that were analyzed in each run. Tables with the individual back-calculated results for all study samples should be submitted.
- 1030
- 1031
- Examples of tabular listings of analytical data for reports can be found in the Appendix (IX. Appendix)
- 1032

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### VIII. GLOSSARY

- 1033  
1034  
1035 **Accuracy:** The degree of closeness of the determined value to the nominal or known true value  
1036 under prescribed conditions. This is sometimes termed *trueness*.
- 1037 **Analyte:** A specific chemical moiety being measured; it can be an intact drug, a biomolecule or  
1038 its derivative, a metabolite, and/or a degradation product in a biologic matrix.
- 1039 **Analytical run:** A complete set of analytical and study samples with appropriate number of  
1040 standards and QCs for their validation. Several runs may be completed in one day, or one run  
1041 may take several days to complete.
- 1042 **Biological matrix:** A discrete material of biological origin that can be sampled and processed in  
1043 a reproducible manner. Examples are blood, serum, plasma, urine, feces, cerebrospinal fluid,  
1044 saliva, sputum, and various discrete tissues.
- 1045 **Batch:** A batch is a number of unknown samples from one or more patients in a study and QCs  
1046 that are processed at one time.
- 1047 **Blank:** A sample of a biological matrix to which no analytes have been added, that is used to  
1048 assess the specificity of the bioanalytical method.
- 1049 **Calibration standard:** A biological matrix to which a known amount of analyte has been  
1050 added. Calibration standards are used to construct calibration curves from which the  
1051 concentrations of analytes in quality control samples and in unknown study samples are  
1052 determined.
- 1053 **Full validation:** Establishment of all validation parameters that apply to sample analysis for the  
1054 bioanalytical method for each analyte.
- 1055 **Incurred Sample Reanalysis (ISR):** A repeated measurement of analyte concentration from  
1056 study samples to demonstrate reproducibility.
- 1057 **Internal standard (IS):** Test compound(s) (e.g., structurally similar analog, stable labeled  
1058 compound) added to both calibration standards and samples at known and constant concentration  
1059 to facilitate quantification of the target analyte(s).
- 1060 **Limit of detection (LOD):** The lowest concentration of an analyte that the bioanalytical  
1061 procedure can reliably differentiate from background noise.
- 1062 **Lower limit of quantification (LLOQ):** The lowest amount of an analyte in a sample that can  
1063 be quantitatively determined with acceptable precision and accuracy.
- 1064 **Matrix effect:** The direct or indirect alteration or interference in response due to the presence of  
1065 unintended analytes (for analysis) or other interfering substances in the sample.
- 1066 **Method:** A comprehensive description of all procedures used in sample analysis.
- 1067 **Precision:** The closeness of agreement (i.e., *degree of scatter*) among a series of measurements  
1068 obtained from multiple sampling of the same homogenous sample under the prescribed  
1069 conditions.
- 1070 **Processed Sample:** The final extract (prior to instrumental analysis) of a sample that has been  
1071 subjected to various manipulations (e.g., extraction, dilution, concentration).

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1072 **Quality Control Sample (QCs):** A sample with a known quantity of analyte that is used to  
1073 monitor the performance of a bioanalytical method and to assess the integrity and validity of the  
1074 results of the unknown samples analyzed in an individual run.

1075 **Quantification range:** The range of concentrations, including ULOQ and LLOQ, that can be  
1076 reliably and reproducibly quantified with accuracy and precision through the use of a  
1077 concentration-response relationship.

1078 **Recovery:** The extraction efficiency of an analytical process, reported as a percentage of the  
1079 known amount of an analyte carried through the sample extraction and processing steps of the  
1080 method.

1081 **Reproducibility:** The precision between two laboratories. It also represents precision of the  
1082 method under the same operating conditions over a short period of time.

1083 **Sample:** A generic term encompassing controls, blanks, unknowns, and processed samples.

1084 **Selectivity/Specificity:** The ability of the bioanalytical method to measure and differentiate the  
1085 analytes in the presence of components that may be expected to be present. These could include  
1086 metabolites, impurities, degradants, or matrix components.

1087 **Sensitivity:** is defined as the lowest analyte concentration that can be measured with acceptable  
1088 accuracy and precision (i.e., LLOQ).

1089 **Stability:** The chemical stability of an analyte in a given matrix under specific conditions for  
1090 given time intervals.

1091 **Standard curve:** The relationship between the experimental response values and the analytical  
1092 concentrations (also called a *calibration curve*).

1093 **System suitability:** Determination of instrument performance (e.g., sensitivity and  
1094 chromatographic retention) by analysis of a set of reference standards conducted prior to the  
1095 analytical run.

1096 **Unknown:** A biological sample that is the subject of the analysis.

1097 **Upper limit of quantification (ULOQ):** The highest amount of an analyte in a sample that can  
1098 be quantitatively determined with precision and accuracy.

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**IX. APPENDIX**

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1102 Report Format examples for applications to CDER or CVM. Summary tables should be included  
1103 in Module 2 of the eCTD.  
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**TABLE 1-EXAMPLE OF AN OVERALL SUMMARY TABLE  
FOR A METHOD VALIDATION REPORT\***

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1108 **This table contains fictitious information, which serves illustrative purposes only.**

	Results	Hyperlink <sup>†</sup>	Comments
Methodology	LC/MS/MS	01-SOP-001	
Method Validation Report Number	MVR-001	MVR-001	
Biological matrix	Human plasma	MVR-001	
Anticoagulant (if applicable)	EDTA	MVR-001	
Calibration curve range	XXX-YYY ng/mL	<b>Summary tables</b> <a href="#">001MVR-01/CCTables</a>  <b>Report text</b> <a href="#">001MVR-01/CCText</a>	
Analyte of interest	Compound A	NA	
Internal standard	Compound A internal standard	NA	
Inter-run accuracy (for each QC concentration)	Low QC (AA ng/mL): X% Medium QC (e.g. BB ng/mL): Y% High QC (e.g. CC ng/mL): Z%	<b>Summary tables</b> <a href="#">001MVR-01/APTables</a>  <b>Report text</b> <a href="#">001MVR-01/APText</a>	
Inter-run precision (for each QC concentration)	Low QC (AA ng/mL): X% Medium QC (BB ng/mL): Y% High QC (CC ng/mL): Z%		
Dilution integrity (specify dilution factors and QC concentrations and matrix that were evaluated)	Dilution QC: CC ng/mL (dilution factor: X) Accuracy: Y% Precision: Z%	<b>Summary tables</b> <a href="#">001MVR-01/DILTables</a>  <b>Report text</b> <a href="#">001MVR-01/DILText</a>	
Selectivity	< 20% of the lower limit of quantification (LLOQ) -list drugs tested	<b>Summary tables</b> <a href="#">001MVR-01/SELTables</a>  <b>Report text</b> <a href="#">001MVR-01/SELText</a>	
Short term or bench	Demonstrated for X	<b>Summary tables</b>	

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Top temperature stability	hours at Y°C	<a href="#">001MVR-01/STSTables</a> <b>Report text</b> <a href="#">001MVR-01/STSText</a>	
Long-term stability	Demonstrated for X days at Y°C	<b>Summary tables</b> <a href="#">001MVR-01/LTSTables</a> <b>Report text</b> <a href="#">001MVR-01/LTSText</a>	
Freeze-thaw stability	Demonstrated for Y cycles at Z°C	<b>Summary tables</b> <a href="#">001MVR-01/FTSTables</a> <b>Report text</b> <a href="#">001MVR-01/FTSText</a>	
Stock solution stability	Demonstrated for X weeks at Y°C	<b>Summary tables</b> <a href="#">001MVR-01/SSSTables</a> <b>Report text</b> <a href="#">001MVR-01/SSSText</a>	
Processed Sample Stability	Demonstrated for Y hours at Z°C	<b>Summary tables</b> <a href="#">001MVR-01/PSSTables</a> <b>Report text</b> <a href="#">001MVR-01/PSSText</a>	
ISR	> 67% of samples acceptable	<b>Summary tables</b> <a href="#">001MVR-01/ISRTables</a> <b>Report text</b> <a href="#">001MVR-01/ISRText</a>	
Recovery: extraction efficiency	<b>Summary tables</b> <a href="#">001MVR-01/EXTTables</a> <b>Report text</b> <a href="#">001MVR-01/EXTText</a>		
Matrix effects	<b>Summary tables</b> <a href="#">001MVR-01/MATTables</a> <b>Report text</b> <a href="#">001MVR-01/MATText</a>		

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\*Failed method validation experiments should be listed, and data may be requested.

†For eCTD submissions, a hyperlink should be provided for the summary tables and report text.

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1112 **TABLE 2-EXAMPLE OF INFORMATION FOR REFERENCE STANDARDS**  
1113 **FOR METHOD VALIDATION CONDUCTED IN PLASMA MATRIX\***  
1114

1115 Include information linking the use of specific lots of reference standards for the analyte and  
1116 internal standard to specific method validation experiments\*  
1117

1118 **This table contains fictitious information, which serves illustrative purposes only.**

Reference standard	Retest/expiration date	Lot Numbers	Validation experiment	Dates of Analysis	Evidence of purity (Hyperlink)	Comments
Compound A	MM/DD/YY	RS01	Runs 1-3 (accuracy and precision) Run 3 (selectivity experiment)	MM/DD/YY	<a href="#">001MVR-01/RS01</a>	
Compound A internal standard	MM/DD/YY	RS02	Runs 1-3 (accuracy and precision) Run 3 (selectivity experiment)	MM/DD/YY	<a href="#">001MVR-01/RS02</a>	

1119 \* A similar table would be included in the bioanalytical study report linking the use of reference standards to  
1120 specific batches or analytical runs.  
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**TABLE 3-EXAMPLE OF METHOD VALIDATION SUMMARY  
AND STUDY INFORMATION FOR CLINICAL STUDY XXXXX**

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**This table contains fictitious information, which serves illustrative purposes only.**

	Results	Comments
Methodology	LC-MS/MS	
Biological matrix	Human plasma	
Anticoagulant (if applicable)	EDTA	
Calibration curve range	XXX-YYY ng/ml	
Analyte of interest	Compound A	
Internal standard	Compound A internal standard	
<b>Method validation summary</b>		
Method Validation Report Number	MVR-001	
Inter-run accuracy (for each QC concentration)	Low QC (AA ng/mL): X% Medium QC (e.g. BB ng/mL): Y% High QC (e.g. CC ng/mL): Z%	
Inter-run precision (for each QC concentration)	Low QC (AA ng/mL): X% Medium QC (BB ng/mL): Y% High QC (CC ng/mL): Z%	
Long-term stability	Demonstrated for X days at Y°C	
Freeze-thaw stability	Demonstrated for Y cycles at Z°C	
<b>Study Information</b>		
ISR (include the percentage of samples analyzed)	> 67% of samples acceptable	
Duration from time sample was first drawn to date of last sample analysis (including ISR)	XXX months	
Actual sample storage temperature*	Y°C at AAA Z°C at BBB	

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\* list the sample storage temperature at each site

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**TABLE 4-EXAMPLE OF SUMMARY ANALYTICAL RUNS  
FOR A BIOANALYTICAL STUDY REPORT \***

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Provide a table summarizing both the failed and accepted runs for each study.

**This table contains fictitious information, which serves illustrative purposes only.**

**Clinical Study XXYY-0032456**

Analytical run *	Batch number within analytical run	Dates of Analysis	Results (Accepted /Rejected)	Hyperlink <sup>†</sup>	Comments (e.g. information on runs that failed)
001-100-01	Not applicable	MM/DD/YY	Rejected	<b>Summary tables for calibration curve standards and QCs</b>  <a href="#">001BR-01/01CALTables</a> <a href="#">001BR-01/01QCTables</a>  <b>Report text</b> <a href="#">001BR-01/01CALText</a> <a href="#">001BR-01/01QCText</a>  <b>Raw Data</b> <a href="#">001BR-01/01CALData</a> <a href="#">001BR-01/01QCData</a>	<a href="#">001BR-01/01Failure</a> 67% of the QCs passed; however both QCs that exceeded $\pm 15\%$ were at the low QC concentration. The follow-up investigation concluded that the LC/MS/MS instrument required a recalibration:
001-100-02	Not applicable	MM/DD/YY	Accepted	<b>Summary tables for calibration curve standards and QCs</b>  <a href="#">001BR-01/02CALTables</a> <a href="#">001BR-01/02QCTables</a>  <b>Report text</b> <a href="#">001BR-01/02CALText</a> <a href="#">001BR-01/02QCText</a>  <b>Raw Data</b> <a href="#">001BR-01/02CALData</a> <a href="#">001BR-01/02QCData</a>	This is the reanalysis of the samples from run 001-100-01

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\*If multiple batches are analyzed within an analytical run, each batch should be separately evaluated to determine if the batch meets acceptance criteria.

<sup>†</sup>For eCTD submissions, a hyperlink should be provided for the summary tables, report text, and raw data.