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REQUIREMENTS FOR PHARMACEUTICALS FOR HUMAN USE**

ICH HARMONISED GUIDELINE

**BIOANALYTICAL METHOD VALIDATION
M10**

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81 **1. INTRODUCTION**

82 **1.1 Objective**

83 This guideline is intended to provide recommendations for the validation of bioanalytical assays
84 for chemical and biological drug quantification and their application in the analysis of study
85 samples. Adherence to the principles presented in this guideline will improve the quality and
86 consistency of the bioanalytical data in support of the development and market approval of both
87 chemical and biological drugs.

88 The objective of the validation of a bioanalytical assay is to demonstrate that it is suitable for
89 its intended purpose. Changes from the recommendations in this guideline may be acceptable
90 if appropriate scientific justification is provided. Applicants are encouraged to consult the
91 regulatory authority(ies) regarding significant changes in method validation approaches when
92 an alternate approach is proposed or taken.

93 **1.2 Background**

94 Concentration measurements of chemical and biological drug(s) and their metabolite(s) in
95 biological matrices are an important aspect of drug development. The results of pivotal
96 nonclinical toxicokinetic (TK)/pharmacokinetic (PK) studies and of clinical trials, including
97 comparative bioavailability/bioequivalence (BA/BE) studies, are used to make regulatory
98 decisions regarding the safety and efficacy of drug products. It is therefore critical that the
99 bioanalytical methods used are well characterised, appropriately validated and documented in
100 order to ensure reliable data to support regulatory decisions.

101 **1.3 Scope**

102 This guideline describes the method validation that is expected for bioanalytical assays that are
103 submitted to support regulatory submissions. The guideline is applicable to the validation of
104 bioanalytical methods used to measure concentrations of chemical and biological drug(s) and
105 their metabolite(s) in biological samples (e.g., blood, plasma, serum, other body fluids or
106 tissues) obtained in pivotal nonclinical TK/PK studies that are used to make regulatory
107 decisions and all phases of clinical trials in regulatory submissions. Full method validation is
108 expected for the primary matrix(ces) intended to support regulatory submissions. Additional
109 matrices should be partially validated as necessary. The analytes that should be measured in
110 nonclinical and clinical studies and the types of studies necessary to support a regulatory
111 submission are described in other ICH and regional regulatory documents.

112 For studies that are not submitted for regulatory approval or not considered for regulatory
113 decisions regarding safety, efficacy or labelling (e.g., exploratory investigations), applicants
114 may decide on the level of qualification that supports their own internal decision making.

115 The information in this guideline applies to the quantitative analysis by ligand binding assays
116 (LBAs) and chromatographic methods such as liquid chromatography (LC) or gas
117 chromatography (GC), which are typically used in combination with mass spectrometry (MS)
118 detection and occasionally with other detectors.

119 For studies that are subject to Good Laboratory Practice (GLP) or Good Clinical Practice (GCP)
120 the bioanalysis of study samples should also conform to their requirements.

121 The bioanalysis of biomarkers and bioanalytical methods used for the assessment of
122 immunogenicity are not within the scope of this guideline.

123 **2. GENERAL PRINCIPLES**

124 **2.1 Method Development**

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

128 Before the development of a bioanalytical method, the applicant should understand the analyte
129 of interest (e.g., the physicochemical properties of the drug, *in vitro* and *in vivo* metabolism and
130 protein binding) and consider aspects of any prior analytical methods that may be applicable.

131 Method development involves optimising the procedures and conditions involved with
132 extracting and detecting the analyte. Method development can include the optimisation of the
133 following bioanalytical parameters to ensure that the method is suitable for validation:

- 134 • Reference standards
- 135 • Critical reagents
- 136 • Calibration curve
- 137 • Quality control samples (QCs)
- 138 • Selectivity and specificity
- 139 • Sensitivity
- 140 • Accuracy

- 141 • Precision
- 142 • Recovery
- 143 • Stability of the analyte in the matrix
- 144 • Minimum Required Dilution (MRD)

145 Bioanalytical method development does not require extensive record keeping or notation.
146 However, the applicant should record the changes to procedures as well as any issues and their
147 resolutions to provide a rationale for any changes made to validated methods immediately prior
148 to or in the course of analysing study samples for pivotal studies.

149 Once the method has been developed, bioanalytical method validation proves that the optimised
150 method is suited to the analysis of the study samples.

151 **2.2 Method Validation**

152 **2.2.1 Full Validation**

153 Bioanalytical method validation is essential to ensure the acceptability of assay performance
154 and the reliability of analytical results. A bioanalytical method is defined as a set of procedures
155 used for measuring analyte concentrations in biological samples. A full validation of a
156 bioanalytical method should be performed when establishing a bioanalytical method for the
157 quantification of an analyte in clinical and in pivotal nonclinical studies. Full validation should
158 also be performed when implementing an analytical method that is reported in the literature and
159 when a commercial kit is repurposed for bioanalytical use in drug development. Usually one
160 analyte has to be determined, but on occasion it may be appropriate to measure more than one
161 analyte. This may involve two different drugs, a parent drug with its metabolites or the
162 enantiomers or isomers of a drug. In these cases, the principles of validation and analysis apply
163 to all analytes of interest.

164 For chromatographic methods a full validation should include the following elements:
165 selectivity, specificity (if necessary), matrix effect, calibration curve (response function), range
166 (lower limit of quantification (LLOQ) to upper limit of quantification (ULOQ)), accuracy,
167 precision, carry-over, dilution integrity, stability and reinjection reproducibility.

168 For LBAs the following elements should be evaluated: specificity, selectivity, calibration curve
169 (response function), range (LLOQ to ULOQ), accuracy, precision, carry-over (if necessary),
170 dilution linearity, parallelism (if necessary, conducted during sample analysis) and stability.

171 The matrix used for analytical method validation should be the same as the matrix of the study
172 samples, including anticoagulants and additives. In some cases, it may be difficult to obtain an
173 identical matrix to that of the study samples (e.g., rare matrices such as tissue, cerebrospinal
174 fluid, bile). In such cases surrogate matrices may be acceptable for analytical method validation.
175 The surrogate matrix should be selected and justified scientifically for use in the analytical
176 method.

177 A specific, detailed, written description of the bioanalytical method should be established *a*
178 *priori*. This description may be in the form of a protocol, study plan, report, or Standard
179 Operating Procedure (SOP).

180 **2.2.2 Partial Validation**

181 Modifications to a fully validated analytical method may be evaluated by partial validation.
182 Partial validation can range from as little as one accuracy and precision determination to a
183 nearly full validation (Refer to Section 6.1). The items in a partial validation are determined
184 according to the extent and nature of the changes made to the method.

185 **2.2.3 Cross Validation**

186 Where data are obtained from different methods within or across studies, or when data are
187 obtained within a study from different laboratories applying the same method, comparison of
188 those data is needed and a cross validation of the applied analytical methods should be carried
189 out (Refer to Section 6.2).

190 **3. CHROMATOGRAPHY**

191 **3.1 Reference Standards**

192 During method validation and the analysis of study samples, a blank biological matrix is spiked
193 with the analyte(s) of interest using solutions of reference standard(s) to prepare calibration
194 standards, QCs and stability QCs. Calibration standards and QCs should be prepared from
195 separate stock solutions. However, calibration standards and QCs may be prepared from the
196 same stock solution provided the accuracy and stability of the stock solution have been verified.
197 A suitable internal standard (IS) should be added to all calibration standards, QCs and study
198 samples during sample processing. The absence of an IS should be technically justified.

199 It is important that the reference standard is well characterised and the quality (purity, strength,
200 identity) of the reference standard and the suitability of the IS is ensured, as the quality will
201 affect the outcome of the analysis and, therefore, the study data. The reference standard used
202 during validation and study sample analysis should be obtained from an authentic and traceable

203 source. The reference standard should be identical to the analyte. If this is not possible, an
204 established form (e.g., salt or hydrate) of known quality may be used.

205 Suitable reference standards include compendial standards, commercially available standards
206 or sufficiently characterised standards prepared in-house or by an external non-commercial
207 organisation. A certificate of analysis (CoA) or an equivalent alternative is required to ensure
208 quality and to provide information on the purity, storage conditions, retest/expiration date and
209 batch number of the reference standard.

210 A CoA is not required for the IS as long as the suitability for use is demonstrated, e.g., a lack of
211 analytical interference is shown for the substance itself or any impurities thereof.

212 When MS detection is used, the use of the stable isotope-labelled analyte as the IS is
213 recommended whenever possible. However, it is essential that the labelled standard is of high
214 isotope purity and that no isotope exchange reaction occurs. The presence of unlabelled analyte
215 should be checked and if unlabelled analyte is detected, the potential influence should be
216 evaluated during method validation.

217 Stock and working solutions can only be prepared from reference standards that are within the
218 stability period as documented in the CoA (either expiration date or the retest date in early
219 development phase).

220 **3.2 Validation**

221 **3.2.1 Selectivity**

222 Selectivity is the ability of an analytical method to differentiate and measure the analyte in the
223 presence of potential interfering substances in the blank biological matrix.

224 Selectivity is evaluated using blank samples (matrix samples processed without addition of an
225 analyte or IS) obtained from at least 6 individual sources/lots (non-haemolysed and non-
226 lipaemic). Use of fewer sources may be acceptable in the case of rare matrices. Selectivity for
227 the IS should also be evaluated.

228 The evaluation of selectivity should demonstrate that no significant response attributable to
229 interfering components is observed at the retention time(s) of the analyte or the IS in the blank
230 samples. Responses detected and attributable to interfering components should not be more
231 than 20% of the analyte response at the LLOQ and not more than 5% of the IS response in the
232 LLOQ sample for each matrix.

233 For the investigation of selectivity in lipaemic matrices at least one source of matrix should be
234 used. To be scientifically meaningful, the matrix used for these tests should be representative
235 as much as possible of the expected study samples. A naturally lipaemic matrix with abnormally
236 high levels of triglycerides should be obtained from donors. Although it is recommended to use
237 lipaemic matrix from donors, if this is difficult to obtain, it is acceptable to spike matrix with
238 triglycerides even though it may not be representative of study samples. However, if the drug
239 impacts lipid metabolism or if the intended patient population is hyperlipidaemic, the use of
240 spiked samples is discouraged. This evaluation is not necessary for preclinical studies unless
241 the drug impacts lipid metabolism or is administered in a particular animal strain that is
242 hyperlipidaemic.

243 For the investigation of selectivity in haemolysed matrices at least one source of matrix should
244 be used. Haemolysed matrices are obtained by spiking matrix with haemolysed whole blood (at
245 least 2% V/V) to generate a visibly detectable haemolysed sample.

246 3.2.2 Specificity

247 Specificity is the ability of a bioanalytical method to detect and differentiate the analyte from
248 other substances, including its related substances (e.g., substances that are structurally similar
249 to the analyte, metabolites, isomer, impurities, degradation products formed during sample
250 preparation, or concomitant medications that are expected to be used in the treatment of patients
251 with the intended indication).

252 If the presence of related substances is anticipated in the biological matrix of interest, the impact
253 of such substances should be evaluated during method validation, or alternatively, in the pre-
254 dose study samples. In the case of LC-MS based methods, to assess the impact of such
255 substances, the evaluation may include comparing the molecular weight of a potential
256 interfering related substance with the analyte and chromatographic separation of the related
257 substance from the analyte.

258 Responses detected and attributable to interfering components should not be more than 20% of
259 the analyte response at the LLOQ and not more than 5% of the IS response in the LLOQ sample.

260 The possibility of back-conversion of a metabolite into the parent analyte during the successive
261 steps of the analysis (including extraction procedures or in the MS source) should also be
262 evaluated when relevant (i.e., potentially unstable metabolites such as ester analytes to
263 ester/acidic metabolites, unstable N-oxides or glucuronide metabolites, lactone-ring structures).
264 It is acknowledged that this evaluation will not be possible in the early stages of drug
265 development of a new chemical entity when the metabolism is not yet evaluated. However, it

266 is expected that this issue should be investigated and partial validation performed if needed.
267 The extent of back-conversion, if any, should be established and the impact on the study results
268 discussed in the Bioanalytical Report.

269 **3.2.3 Matrix Effect**

270 A matrix effect is defined as an alteration of the analyte response due to interfering and often
271 unidentified component(s) in the sample matrix. During method validation it is necessary to
272 evaluate the matrix effect between different independent sources/lots.

273 The matrix effect should be evaluated by analysing at least 3 replicates of low and high QCs,
274 each prepared using matrix from at least 6 different sources/lots. The accuracy should be within
275 $\pm 15\%$ of the nominal concentration and the precision (per cent coefficient of variation (%CV))
276 should not be greater than 15% in all individual matrix sources/lots. Use of fewer sources/lots
277 may be acceptable in the case of rare matrices.

278 The matrix effect should also be evaluated in relevant patient populations or special populations
279 (e.g., hepatically impaired or renally impaired) when available. An additional evaluation of the
280 matrix effect is recommended using haemolysed or lipaemic matrix samples during method
281 validation on a case by case basis, especially when these conditions are expected to occur within
282 the study.

283 **3.2.4 Calibration Curve and Range**

284 The calibration curve demonstrates the relationship between the nominal analyte concentration
285 and the response of the analytical platform to the analyte. Calibration standards, prepared by
286 spiking matrix with a known quantity of analyte, span the calibration range and comprise the
287 calibration curve. Calibration standards should be prepared in the same biological matrix as the
288 study samples. The calibration range is defined by the LLOQ, which is the lowest calibration
289 standard, and the ULOQ, which is the highest calibration standard. There should be one
290 calibration curve for each analyte studied during method validation and for each analytical run.

291 A calibration curve should be generated with a blank sample, a zero sample (blank sample
292 spiked with IS), and at least 6 concentration levels of calibration standards, including the LLOQ
293 and the ULOQ.

294 A simple regression model that adequately describes the concentration-response relationship
295 should be used. The selection of the regression model should be directed by written procedures.
296 The regression model, weighting scheme and transformation should be determined during the
297 method validation. Blank and zero samples should not be included in the determination of the

298 regression equation for the calibration curve. Each calibration standard may be analysed in
299 replicate, in which case data from all acceptable replicates should be used in the regression
300 analysis.

301 The calibration curve parameters should be reported (slope and intercept in the case of a linear
302 model). The back-calculated concentrations of the calibration standards should be presented
303 together with the calculated mean accuracy values. All acceptable curves obtained during
304 validation, based on a minimum of 3 independent runs over several days, should be reported.
305 The accuracy of the back-calculated concentrations of each calibration standard should be
306 within $\pm 20\%$ of the nominal concentration at the LLOQ and within $\pm 15\%$ at all the other levels.
307 At least 75% of the calibration standards with a minimum of 6 calibration standard levels should
308 meet the above criteria.

309 In the case that replicates are used, the criteria (within $\pm 15\%$ or $\pm 20\%$ for LLOQ) should also
310 be fulfilled for at least 50% of the calibration standards tested per concentration level. In the
311 case that a calibration standard does not comply with these criteria, this calibration standard
312 sample should be rejected, and the calibration curve without this calibration standard should be
313 re-evaluated, including regression analysis. For accuracy and precision runs, if all replicates of
314 the LLOQ or the ULOQ calibration standard in a run are rejected then the run should be rejected
315 the possible source of the failure should be determined and the method revised if necessary. If
316 the next validation run also fails, then the method should be revised before restarting validation.

317 The calibration curve should be prepared using freshly spiked calibration standards in at least
318 one assessment. Subsequently, frozen calibration standards can be used within their defined
319 period of stability.

320 ***3.2.5 Accuracy and Precision***

321 ***3.2.5.1 Preparation of Quality Control Samples***

322 The QCs are intended to mimic study samples and should be prepared by spiking matrix with
323 a known quantity of analyte, storing them under the conditions anticipated for study samples
324 and analysing them to assess the validity of the analytical method.

325 Calibration standards and the QCs should be prepared from separate stock solutions in order to
326 avoid biased estimations which are not related to the analytical performance of the method.
327 However, calibration standards and the QCs may be prepared from the same stock solution,
328 provided the accuracy and stability of the stock solution have been verified. A single source of
329 blank matrix may be used, which should be free of interference or matrix effects, as described
330 in Section 3.2.3.

331 During method validation the QCs should be prepared at a minimum of 4 concentration levels
332 within the calibration curve range: the LLOQ, within three times of the LLOQ (low QC), around
333 30 - 50% of the calibration curve range (medium QC) and at least 75% of the ULOQ (high QC).

334 **3.2.5.2 Evaluation of Accuracy and Precision**

335 Accuracy and precision should be determined by analysing the QCs within each run (within-
336 run) and in different runs (between-run). Accuracy and precision should be evaluated using the
337 same runs and data.

338 Within-run accuracy and precision should be evaluated by analysing at least 5 replicates at each
339 QC concentration level in each analytical run. Between-run accuracy and precision should be
340 evaluated by analysing each QC concentration level in at least 3 analytical runs over at least
341 two days. To enable the evaluation of any trends over time within one run, it is recommended
342 to demonstrate accuracy and precision of the QCs over at least one of the runs in a size
343 equivalent to a prospective analytical run of study samples. Reported method validation data
344 and the determination of accuracy and precision should include all results obtained, including
345 individual QCs outside of the acceptance criteria, except those cases where errors are obvious
346 and documented. Within-run accuracy and precision data should be reported for each run. If the
347 within-run accuracy or precision criteria are not met in all runs, an overall estimate of within-
348 run accuracy and precision for each QC level should be calculated. Between-run (intermediate)
349 precision and accuracy should be calculated by combining the data from all runs.

350 The calibration curves for these assessments should be prepared using freshly spiked calibration
351 standards in at least one run. If freshly spiked calibration standards are not used in the other
352 runs, stability of the frozen calibration standards should be demonstrated.

353 The overall accuracy at each concentration level should be within $\pm 15\%$ of the nominal
354 concentration, except at the LLOQ, where it should be within $\pm 20\%$. The precision (%CV) of
355 the concentrations determined at each level should not exceed 15%, except at the LLOQ, where
356 it should not exceed 20%.

357 **3.2.6 Carry-over**

358 Carry-over is an alteration of a measured concentration due to residual analyte from a preceding
359 sample that remains in the analytical instrument.

360 Carry-over should be assessed and minimised during method development. During validation
361 carry-over should be assessed by analysing blank samples after the calibration standard at the
362 ULOQ. Carry-over in the blank samples following the highest calibration standard should not

363 be greater than 20% of the analyte response at the LLOQ and 5% of the response for the IS. If
 364 it appears that carry-over is unavoidable, study samples should not be randomised. Specific
 365 measures should be considered, tested during the validation and applied during the analysis of
 366 the study samples, so that carry-over does not affect accuracy and precision. This could include
 367 the injection of blank sample(s) after samples with an expected high concentration, before the
 368 next study sample.

369 **3.2.7 Dilution Integrity**

370 Dilution integrity is the assessment of the sample dilution procedure, when required, to confirm
 371 that it does not impact the accuracy and precision of the measured concentration of the analyte.
 372 The same matrix from the same species used for preparation of the QCs should be used for
 373 dilution.

374 Dilution QCs should be prepared with analyte concentrations in matrix that are greater than the
 375 ULOQ and then diluted with blank matrix. At least 5 replicates per dilution factor should be
 376 tested in one run to determine if concentrations are accurately and precisely measured within
 377 the calibration range. The dilution ratio(s) applied during study sample analysis should be
 378 within the range of the dilution ratios evaluated during validation. The mean accuracy of the
 379 dilution QCs should be within $\pm 15\%$ of the nominal concentration and the precision (%CV)
 380 should not exceed 15%.

381 In the cases of rare matrices use of a surrogate matrix for dilution may be acceptable, as long
 382 as it has been demonstrated that this does not affect precision and accuracy.

383 **3.2.8 Stability**

384 Stability evaluations should be carried out to ensure that every step taken during sample
 385 preparation, processing and analysis as well as the storage conditions used do not affect the
 386 concentration of the analyte.

387 The storage and analytical conditions applied to the stability tests, such as the sample storage
 388 times and temperatures, sample matrix, anticoagulant and container materials, should reflect
 389 those used for the study samples. Reference to data published in the literature is not considered
 390 sufficient. Validation of storage periods should be performed on stability QCs that have been
 391 stored for a time that is equal to or longer than the study sample storage periods.

392 Stability of the analyte in the studied matrix is evaluated using low and high concentration
 393 stability QCs. Aliquots of the low and high stability QCs are analysed at time zero and after the

394 applied storage conditions that are to be evaluated. A minimum of three stability QCs should
395 be prepared and analysed per concentration level/storage condition/timepoint.

396 The stability QCs are analysed against a calibration curve, obtained from freshly spiked
397 calibration standards in a run with its corresponding freshly prepared QCs or QCs for which
398 stability has been proven. The mean concentration at each QC level should be within $\pm 15\%$ of
399 the nominal concentration. If the concentrations of the study samples are consistently higher
400 than the ULOQ of the calibration range, the concentration of the high stability QC should be
401 adjusted to reflect these higher concentrations. It is recognised that this may not be possible in
402 nonclinical studies due to solubility limitations.

403 If multiple analytes are present in the study samples (e.g., studies with a fixed combination, or
404 due to a specific drug regimen) the stability test of an analyte in matrix should be conducted
405 with the matrix containing all of the analytes.

406 The following stability tests should be evaluated:

407 1) Stability of stock and working solutions

408 The stability of the stock and working solutions of the analyte and IS should be determined
409 under the storage conditions used during the analysis of study samples by using the lowest
410 and the highest concentrations of these solutions. They are assessed using the response of
411 the detector. Stability of the stock and working solutions should be tested with an
412 appropriate dilution, taking into consideration the linearity and measuring range of the
413 detector. If the stability varies with concentration, then the stability of all concentrations of
414 the stock and working solutions needs to be assessed. If no isotopic exchange occurs for the
415 stable isotope-labelled IS under the same storage conditions as the analyte for which the
416 stability is demonstrated, then no additional stability determinations for the IS are necessary.
417 If the reference standard expires, or it is past the retest date, the stability of the stock
418 solutions made previously with this lot of reference standard are defined by the expiration
419 or retest date established for the stock solution. The routine practice of making stock and
420 working solutions from reference standards solely for extending the expiry date for the use
421 of the reference standard is not acceptable.

422 2) Freeze-thaw matrix stability

423 To assess the impact of repeatedly removing samples from frozen storage, the stability of
424 the analyte should be assessed after multiple cycles of freezing and thawing. Low and high
425 stability QCs should be thawed and analysed according to the same procedures as the study
426 samples. Stability QCs should be kept frozen for at least 12 hours between the thawing

427 cycles. Stability QCs for freeze-thaw stability should be assessed using freshly prepared
428 calibration standards and QCs or QCs for which stability has been proven. The number of
429 freeze-thaw cycles validated should equal or exceed that of the freeze-thaw cycles
430 undergone by the study samples, but a minimum of three cycles should be conducted.

431 3) Bench top (short-term) matrix stability

432 Bench top matrix stability experiments should be designed and conducted to cover the
433 laboratory handling conditions for the study samples.

434 Low and high stability QCs should be thawed in the same manner as the study samples and
435 kept on the bench top at the same temperature and for at least the same duration as the study
436 samples.

437 The total time on the bench top should be concurrent; it is not acceptable to use additive
438 exposure to bench top conditions (i.e., adding up time from each freeze-thaw evaluation is
439 not acceptable).

440 4) Processed sample stability

441 The stability of processed samples, including the time until completion of analysis (in the
442 autosampler/instrument), should be determined. For example:

- 443 • Stability of the processed sample at the storage conditions to be used during the analysis
444 of study samples (dry extract or in the injection phase)
- 445 • On-instrument/ autosampler stability of the processed sample at injector or autosampler
446 temperature.

447 5) Long-term matrix stability

448 The long-term stability of the analyte in matrix stored in the freezer should be established.
449 Low and high stability QCs should be stored in the freezer under the same storage
450 conditions and at least for the same duration as the study samples.

451 For chemical drugs, it is considered acceptable to extrapolate the stability at one temperature
452 (e.g., -20°C) to lower temperatures (e.g., -70°C).

453 For biological drugs, it is acceptable to apply a bracketing approach, e.g., in the case that
454 the stability has been demonstrated at -70°C and at -20°C, then it is not necessary to
455 investigate the stability at temperatures in between those two points at which study samples
456 will be stored.

457 In addition, the following test should be performed if applicable:

458 6) Whole blood stability

459 Sufficient attention should be paid to the stability of the analyte in the sampled matrix
460 (blood) directly after collection from subjects and prior to preparation for storage to ensure
461 that the concentrations obtained by the analytical method reflect the concentrations of the
462 analyte in the subject's blood at the time of sample collection.

463 If the matrix used is plasma or serum, the stability of the analyte in blood should be
464 evaluated during method development (e.g., using an exploratory method in blood) or
465 during method validation. The results should be provided in the Validation Report.

466 **3.2.9 Reinjection Reproducibility**

467 Reproducibility of the method is assessed by replicate measurements of the QCs and is usually
468 included in the assessment of precision and accuracy. However, if samples could be reinjected
469 (e.g., in the case of instrument interruptions or other reasons such as equipment failure),
470 reinjection reproducibility should be evaluated and included in the Validation Report or
471 provided in the Bioanalytical Report of the study where it was conducted.

472 **3.3 Study Sample Analysis**

473 The analysis of study samples can be carried out after validation has been completed, however,
474 it is understood that some parameters may be completed at a later stage (e.g., long-term
475 stability). By the time the data are submitted to a regulatory authority, the bioanalytical method
476 validation should have been completed. The study samples, QCs and calibration standards
477 should be processed in accordance with the validated analytical method. If system suitability is
478 assessed, a predefined specific study plan, protocol or SOP should be used. System suitability,
479 including apparatus conditioning and instrument performance, should be determined using
480 samples that are independent of the calibration standards and QCs for the run. Subject samples
481 should not be used for system suitability. The IS responses of the study samples should be
482 monitored to determine whether there is systemic IS variability. Refer to Table 1 for
483 expectations regarding documentation.

484 **3.3.1 Analytical Run**

485 An analytical run consists of a blank sample (processed matrix sample without analyte and
486 without IS), a zero sample (processed matrix with IS), calibration standards at a minimum of 6
487 concentration levels, at least 3 levels of QCs (low, medium and high) in duplicate (or at least
488 5% of the number of study samples, whichever is higher) and the study samples to be analysed.

489 The QCs should be divided over the run in such a way that the accuracy and precision of the
490 whole run is ensured. Study samples should always be bracketed by QCs.

491 The calibration standards and QCs should be spiked independently using separately prepared
492 stock solutions, unless the accuracy and stability of the stock solutions have been verified. All
493 samples (calibration standards, QCs and study samples) should be processed and extracted as
494 one single batch of samples in the order in which they are intended to be analysed. A single
495 batch is comprised of study samples and QCs which are handled during a fixed period of time
496 and by the same group of analysts with the same reagents under homogeneous conditions.
497 Analysing samples that were processed as several separate batches in a single analytical run is
498 discouraged. If such an approach cannot be avoided, for instance due to bench top stability
499 limitations, each batch of samples should include low, medium and high QCs.

500 Acceptance criteria should be pre-established in an SOP or in the study plan and should be
501 defined for the whole analytical run and the separate batches in the run, if applicable. For
502 comparative BA/BE studies it is advisable to analyse all samples of one subject together in one
503 analytical run to reduce variability.

504 The impact of any carry-over that occurs during study sample analysis should be assessed and
505 reported (Refer to Section 3.2.6). If carry-over is detected its impact on the measured
506 concentrations should be mitigated (e.g., non-randomisation of study samples, injection of
507 blank samples after samples with an expected high concentration) or the validity of the reported
508 concentrations should be justified in the Bioanalytical Report.

509 *3.3.2 Acceptance Criteria for an Analytical Run*

510 Criteria for the acceptance or rejection of an analytical run should be defined in the protocol, in
511 the study plan or in an SOP. In the case that a run contains multiple batches, acceptance criteria
512 should be applied to the whole run and to the individual batches. It is possible for the run to
513 meet acceptance criteria, even if a batch within that run is rejected for failing to meet the batch
514 acceptance criteria.

515 The back-calculated concentrations of the calibration standards should be within $\pm 15\%$ of the
516 nominal value, except for the LLOQ for which it should be within $\pm 20\%$. At least 75% of the
517 calibration standard concentrations, with a minimum of six concentration levels, should fulfil
518 these criteria. If more than 6 calibration standard levels are used and one of the calibration
519 standards does not meet the criteria, this calibration standard should be rejected and the
520 calibration curve without this calibration standard should be re-evaluated and a new regression
521 analysis performed.

522 If the rejected calibration standard is the LLOQ, the new lower limit for this analytical run is
523 the next lowest acceptable calibration standard of the calibration curve. This new lower limit
524 calibration standard will retain its original acceptance criteria (i.e., $\pm 15\%$). If the highest
525 calibration standard is rejected, the ULOQ for this analytical run is the next acceptable highest
526 calibration standard of the calibration curve. The revised calibration range should cover at least
527 3 QC concentration levels (low, medium and high). Study samples outside of the revised range
528 should be reanalysed. If replicate calibration standards are used and only one of the LLOQ or
529 ULOQ standards fails, the calibration range is unchanged.

530 At least 2/3 of the total QCs and at least 50% at each concentration level should be within $\pm 15\%$
531 of the nominal values. If these criteria are not fulfilled the analytical run should be rejected. A
532 new analytical batch needs to be prepared for all study samples within the failed analytical run
533 for subsequent analysis. In the cases where the failure is due to an assignable technical cause,
534 samples may be reinjected.

535 Analytical runs containing samples that are diluted and reanalysed should include dilution QCs
536 to verify the accuracy and precision of the dilution method during study sample analysis. The
537 concentration of the dilution QCs should exceed that of the study samples being diluted (or of
538 the ULOQ) and they should be diluted using the same dilution factor. The within-run acceptance
539 criteria of the dilution QC(s) will only affect the acceptance of the diluted study samples and
540 not the outcome of the analytical run.

541 When several analytes are assayed simultaneously, there should be one calibration curve for
542 each analyte studied. If an analytical run is acceptable for one analyte but has to be rejected for
543 another analyte, the data for the accepted analyte should be used. The determination of the
544 rejected analyte requires a reextracted analytical batch and analysis.

545 The back-calculated concentrations of the calibration standards and QCs of passed and
546 accepted runs should be reported. The overall (between-run) accuracy and precision of the QCs
547 of all accepted runs should be calculated at each concentration level and reported in the
548 analytical report (Refer to Section 8 Documentation and Table 1). If the overall mean accuracy
549 or precision fails the 15% criterion, an investigation to determine the cause of the deviation
550 should be conducted. In the case of comparative BA/BE studies it may result in the rejection of
551 the data.

552 **3.3.3 Calibration Range**

553 If a narrow range of analyte concentrations of the study samples is known or anticipated before
554 the start of study sample analysis, it is recommended to either narrow the calibration curve

555 range, adapt the concentrations of the QCs, or add new QCs at different concentration levels as
556 appropriate, to adequately reflect the concentrations of the study samples.

557 At the intended therapeutic dose(s), if an unanticipated clustering of study samples at one end
558 of the calibration curve is encountered after the start of sample analysis, the analysis should be
559 stopped and either the standard calibration range narrowed (i.e., partial validation), existing QC
560 concentrations revised, or QCs at additional concentrations added to the original curve within
561 the observed range before continuing with study sample analysis. It is not necessary to reanalyse
562 samples analysed before optimising the calibration curve range or QC concentrations.

563 The same applies if a large number of the analyte concentrations of the study samples are above
564 the ULOQ. The calibration curve range should be changed, if possible, and QC(s) added or
565 their concentrations modified. If it is not possible to change the calibration curve range or the
566 number of samples with a concentration above the ULOQ is not large, samples should be diluted
567 according to the validated dilution method.

568 At least 2 QC levels should fall within the range of concentrations measured in study samples.
569 If the calibration curve range is changed, the bioanalytical method should be revalidated (partial
570 validation) to verify the response function and to ensure accuracy and precision.

571 *3.3.4 Reanalysis of Study Samples*

572 Possible reasons for reanalysis of study samples, the number of replicates and the decision
573 criteria to select the value to be reported should be predefined in the protocol, study plan or
574 SOP, before the actual start of the analysis of the study samples.

575 The number of samples (and percentage of total number of samples) that have been reanalysed
576 should be reported and discussed in the Bioanalytical Report.

577 Some examples of reasons for study sample reanalysis are:

- 578 • Rejection of an analytical run because the run failed the acceptance criteria with regard to
579 accuracy of the calibration standards and/or the precision and accuracy of the QCs
- 580 • IS response significantly different from the response for the calibration standards and QCs
581 (as pre-defined in an SOP)
- 582 • The concentration obtained is above the ULOQ

- 583 • The concentration observed is below the revised LLOQ in runs where the lowest calibration
584 standard has been rejected from a calibration curve, resulting in a higher LLOQ compared
585 with other runs
- 586 • Improper sample injection or malfunction of equipment
- 587 • The diluted study sample is below the LLOQ
- 588 • Identification of quantifiable analyte levels in pre-dose samples, control or placebo samples
- 589 • Poor chromatography (as pre-defined in an SOP)

590 For comparative BA/BE studies, reanalysis of study samples for a PK reason (e.g., a sample
591 concentration does not fit with the expected profile) is not acceptable, as it may bias the study
592 result.

593 Any reanalysed samples should be identified in the Bioanalytical Report and the initial value,
594 the reason for reanalysis, the values obtained in the reanalyses, the final accepted value and a
595 justification for the acceptance should be provided. Further, a summary table of the total number
596 of samples that have been reanalysed for each reason should be provided. In cases where the
597 first analysis yields a non-reportable result, a single reanalysis is considered sufficient (e.g.,
598 concentration above the ULOQ or equipment malfunction). In cases where the value needs to
599 be confirmed (e.g., pre-dose sample with measurable concentrations) replicate determinations
600 are required if sample volume allows.

601 The safety of trial subjects should take precedence over any other aspect of the trial.
602 Consequently, there may be other circumstances when it is necessary to reanalyse specific study
603 samples for the purpose of an investigation.

604 **3.3.5 Reinjection of Study Samples**

605 Reinjection of processed samples can be made in the case of equipment failure if reinjection
606 reproducibility has been demonstrated during validation or provided in the Bioanalytical Report
607 where it was conducted. Reinjection of a full analytical run or of individual calibration
608 standards or QCs simply because the calibration standards or QCs failed, without any identified
609 analytical cause, is not acceptable.

610 **3.3.6 Integration of Chromatograms**

611 Chromatogram integration and reintegration should be described in a study plan, protocol or
612 SOP. Any deviation from the procedures described *a priori* should be discussed in the
613 Bioanalytical Report. The list of chromatograms that required reintegration, including any

614 manual integrations, and the reasons for reintegration should be included in the Bioanalytical
615 Report. Original and reintegrated chromatograms and initial and repeat integration results
616 should be kept for future reference and submitted in the Bioanalytical Report for comparative
617 BA/BE studies.

618 **4. LIGAND BINDING ASSAYS**

619 **4.1 Key Reagents**

620 *4.1.1 Reference Standard*

621 The reference standard should be well characterised and documented (e.g., CoA and origin). A
622 biological drug has a highly complex structure and its reactivity with binding reagents for
623 bioanalysis may be influenced by a change in the manufacturing process of the drug substance.
624 It is recommended that the manufacturing batch of the reference standard used for the
625 preparation of calibration standards and QCs is derived from the same batch of drug substance
626 as that used for dosing in the nonclinical and clinical studies whenever possible. If the reference
627 standard batch used for bioanalysis is changed, bioanalytical evaluation should be carried out
628 prior to use to ensure that the performance characteristics of the method are within the
629 acceptance criteria.

630 *4.1.2 Critical Reagents*

631 Critical reagents, including binding reagents (e.g., binding proteins, aptamers, antibodies or
632 conjugated antibodies) and those containing enzymatic moieties, have direct impact on the
633 results of the assay and, therefore, their quality should be assured. Critical reagents bind the
634 analyte and, upon interaction, lead to an instrument signal corresponding to the analyte
635 concentration. The critical reagents should be identified and defined in the assay method.

636 Reliable procurement of critical reagents, whether manufactured in-house or purchased
637 commercially, should be considered early in method development. The data sheet for the critical
638 reagent should include at a minimum identity, source, batch/lot number, purity (if applicable),
639 concentration (if applicable) and stability/storage conditions (Refer to Table 1). Additional
640 characteristics may be warranted.

641 A critical reagent lifecycle management procedure is necessary to ensure consistency between
642 the original and new batches of critical reagents. Reagent performance should be evaluated
643 using the bioanalytical assay. Minor changes to critical reagents would not be expected to
644 influence the assay performance, whereas major changes may significantly impact the
645 performance. If the change is minor (e.g., the source of one reagent is changed), a single

646 comparative accuracy and precision assessment is sufficient for characterisation. If the change
647 is major, then additional validation experiments are necessary. Ideally, assessment of changes
648 will compare the assay with the new reagents to the assay with the old reagents directly. Major
649 changes include, but are not limited to, change in production method of antibodies, additional
650 blood collection from animals for polyclonal antibodies and new clones or new supplier for
651 monoclonal antibody production.

652 Retest dates and validation parameters should be documented in order to support the extension
653 or replacement of the critical reagent. Stability testing of the reagents should be based upon the
654 performance in the bioanalytical assay and be based upon general guidance for reagent storage
655 conditions and can be extended beyond the expiry date from the supplier. The performance
656 parameters should be documented in order to support the extension or replacement of the critical
657 reagent.

658 **4.2 Validation**

659 When using LBA, study samples can be analysed using an assay format of 1 or more well(s)
660 per sample. The assay format should be specified in the protocol, study plan or SOP. If method
661 development and assay validation are performed using 1 or more well(s) per sample, then study
662 sample analysis should also be performed using 1 or more well(s) per sample, respectively. If
663 multiple wells per sample are used, the reportable sample concentration value should be
664 determined either by calculating the mean of the responses from the replicate wells or by
665 averaging the concentrations calculated from each response. Data evaluation should be
666 performed on reportable concentration values.

667 **4.2.1 Specificity**

668 Specificity is evaluated by spiking blank matrix samples with related molecules at the maximal
669 concentration(s) of the structurally related molecule anticipated in study samples.

670 The accuracy of the target analyte at the LLOQ and at the ULOQ should be investigated in the
671 presence of related molecules at the maximal concentration(s) anticipated in study samples. The
672 response of blank samples spiked with related molecules should be below the LLOQ. The
673 accuracy of the target analyte in presence of related molecules should be within $\pm 25\%$ of the
674 nominal values.

675 In the event of non-specificity, the impact on the method should be evaluated by spiking
676 increasing concentrations of interfering molecules in blank matrix and measuring the accuracy
677 of the target analyte at the LLOQ and ULOQ. It is essential to determine the minimum

678 concentration of the related molecule where interference occurs. Appropriate mitigation during
679 sample analysis should be employed, e.g., it may be necessary to adjust the LLOQ/ULOQ
680 accordingly or consider a new method.

681 During method development and early assay validation, these “related molecules” are
682 frequently not available. Additional evaluation of specificity may be conducted after the
683 original validation is completed.

684 **4.2.2 Selectivity**

685 Selectivity is the ability of the method to detect and differentiate the analyte of interest in the
686 presence of other “unrelated compounds” (non-specific interference) in the sample matrix. The
687 matrix can contain non-specific matrix component such as degrading enzymes, heterophilic
688 antibodies or rheumatoid factor which may interfere with the analyte of interest.

689 Selectivity should be evaluated at the low end of an assay where problems occur in most cases,
690 but it is recommended that selectivity is also evaluated at higher analyte concentrations.
691 Therefore, selectivity is evaluated using blank samples obtained from at least 10 individual
692 sources and by spiking the individual blank matrices at the LLOQ and at the high QC level.
693 The response of the blank samples should be below the LLOQ in at least 80% of the individual
694 sources.

695 The accuracy should be within $\pm 25\%$ at the LLOQ and within $\pm 20\%$ at the high QC level of the
696 nominal concentration in at least 80% of the individual sources evaluated.

697 Selectivity should be evaluated in lipaemic samples and haemolysed samples (Refer to Section
698 3.2.1). For lipaemic and haemolysed samples, tests can be evaluated once using a single source
699 of matrix. Selectivity should be assessed in samples from relevant patient populations. In the
700 case of relevant patient populations there should be at least five individual patients.

701 **4.2.3 Calibration Curve and Range**

702 The calibration curve demonstrates the relationship between the nominal analyte concentration
703 and the response of the analytical platform to the analyte. Calibration standards, prepared by
704 spiking matrix with a known quantity of analyte, span the calibration range and comprise the
705 calibration curve. Calibration standards should be prepared in the same biological matrix as the
706 study samples. The calibration range is defined by the LLOQ, which is the lowest calibration
707 standard, and the ULOQ, which is the highest calibration standard. There should be one
708 calibration curve for each analyte studied during method validation and for each analytical run.

709 A calibration curve should be generated with at least 6 concentration levels of calibration
710 standards, including LLOQ and ULOQ standards, plus a blank sample. The blank sample
711 should not be included in the calculation of calibration curve parameters. Anchor point samples
712 at concentrations below the LLOQ and above the ULOQ of the calibration curve may also be
713 used to improve curve fitting. The relationship between response and concentration for a
714 calibration curve is most often fitted by a 4- or 5-parameter logistic model if there are data
715 points near the lower and upper asymptotes, although other models may be used with suitable
716 justification.

717 A minimum of 6 independent runs should be evaluated over several days considering the factors
718 that may contribute to between-run variability.

719 The accuracy and precision of back-calculated concentrations of each calibration standard
720 should be within $\pm 25\%$ of the nominal concentration at the LLOQ and ULOQ, and within $\pm 20\%$
721 at all other levels. At least 75% of the calibration standards excluding anchor points, and a
722 minimum of 6 concentration levels of calibration standards, including the LLOQ and ULOQ,
723 should meet the above criteria. The anchor points do not require acceptance criteria since they
724 are beyond the quantifiable range of the curve.

725 The calibration curve should preferably be prepared using freshly spiked calibration standards.
726 If freshly spiked calibration standards are not used, the frozen calibration standards can be used
727 within their defined period of stability.

728 ***4.2.4 Accuracy and Precision***

729 ***4.2.4.1 Preparation of Quality Control Samples***

730 The QCs are intended to mimic study samples and should be prepared by spiking matrix with
731 a known quantity of analyte, stored under the conditions anticipated for study samples and
732 analysed to assess the validity of the analytical method.

733 The dilution series for the preparation of the QCs should be completely independent from the
734 dilution series for the preparation of calibration standard samples. They may be prepared from
735 a single stock provided that its accuracy has been verified or is known. The QCs should be
736 prepared at a minimum of 5 concentration levels within the calibration curve range: The analyte
737 should be spiked at the LLOQ, within three times of the LLOQ (low QC), around the geometric
738 mean of the calibration curve range (medium QC), and at least at 75% of the ULOQ (high QC)
739 and at the ULOQ.

740 **4.2.4.2 Evaluation of Accuracy and Precision**

741 Accuracy and precision should be determined by analysing the QCs within each run (within-
742 run) and in different runs (between-run). Accuracy and precision should be evaluated using the
743 same runs and data.

744 Accuracy and precision should be determined by analysing at least 3 replicates per run at each
745 QC concentration level (LLOQ, low, medium, high, ULOQ) in at least 6 runs over 2 or more
746 days. Reported method validation data and the determination of accuracy and precision should
747 include all results obtained, except those cases where errors are obvious and documented.
748 Within-run accuracy and precision data should be reported for each run. If the within-run
749 accuracy or precision criteria are not met in all runs, an overall estimate of within-run accuracy
750 and precision for each QC level should be calculated. Between-run (intermediate) precision and
751 accuracy should be calculated by combining the data from all runs.

752 The overall within-run and between-run accuracy at each concentration level should be within
753 $\pm 20\%$ of the nominal values, except for the LLOQ and ULOQ, which should be within $\pm 25\%$
754 of the nominal value. Within-run and between-run precision of the QC concentrations
755 determined at each level should not exceed 20%, except at the LLOQ and ULOQ, where it
756 should not exceed 25%.

757 Furthermore, the total error (i.e., sum of absolute value of the errors in accuracy (%) and
758 precision (%)) should be evaluated. The total error should not exceed 30% (40% at LLOQ and
759 ULOQ).

760 **4.2.5 Carry-over**

761 Carry-over is generally not an issue for LBA analyses. However, if the assay platform is prone
762 to carry-over, the potential of carry-over should be investigated by placing blank samples after
763 the calibration standard at the ULOQ. The response of blank samples should be below the
764 LLOQ.

765 **4.2.6 Dilution Linearity and Hook Effect**

766 Due to the narrow assay range in many LBAs, study samples may require dilution in order to
767 achieve analyte concentrations within the range of the assay. Dilution linearity is assessed to
768 confirm: (i) that measured concentrations are not affected by dilution within the calibration
769 range and (ii) that sample concentrations above the ULOQ of a calibration curve are not
770 impacted by hook effect (i.e., a signal suppression caused by high concentrations of the analyte),
771 whereby yielding an erroneous result.

772 The same matrix as that of the study sample should be used for preparation of the QCs for
773 dilution.

774 Dilution linearity should be demonstrated by generating a dilution QC, i.e., spiking the matrix
775 with an analyte concentration above the ULOQ, analysed undiluted (for hook effect) and
776 diluting this sample (to at least 3 different dilution factors) with blank matrix to a concentration
777 within the calibration range. For each dilution factor tested, at least 3 runs should be performed
778 using the number of replicates that will be used in sample analysis. The absence or presence of
779 response reduction (hook effect) is checked in the dilution QCs and, if observed, measures
780 should be taken to eliminate response reduction during the analysis of study samples.

781 The calculated concentration for each dilution should be within $\pm 20\%$ of the nominal
782 concentration after correction for dilution and the precision of the final concentrations across
783 all the dilutions should not exceed 20%.

784 The dilution factor(s) applied during study sample analysis should be within the range of
785 dilution factors evaluated during validation.

786 **4.2.7 Stability**

787 Stability evaluations should be carried out to ensure that every step taken during sample
788 preparation, processing and analysis as well as the storage conditions used do not affect the
789 concentration of the analyte.

790 The storage and analytical conditions applied to the stability tests, such as the sample storage
791 times and temperatures, sample matrix, anticoagulant, and container materials should reflect
792 those used for the study samples. Reference to data published in the literature is not considered
793 sufficient. Validation of storage periods should be performed on stability QCs that have been
794 stored for a time that is equal to or longer than the study sample storage periods.

795 Stability of the analyte in the studied matrix is evaluated using low and high concentration
796 stability QCs. Aliquots of the low and high stability QCs are analysed at time zero and after the
797 applied storage conditions that are to be evaluated. A minimum of three stability QCs should
798 be prepared and analysed per concentration level/storage condition/timepoint.

799 The stability QCs are analysed against a calibration curve, obtained from freshly spiked
800 calibration standards in a run with its corresponding freshly prepared QCs or QCs for which
801 stability has been proven. While the use of freshly prepared calibration standards and QCs is
802 the preferred approach, it is recognised that in some cases, for macromolecules, it may be
803 necessary to freeze them overnight. In such cases, valid justification should be provided and

804 freeze-thaw stability demonstrated. The mean concentration at each level should be within
805 $\pm 20\%$ of the nominal concentration.

806 Since sample dilution may be required for many LBA assays due to a narrow calibration range,
807 the concentrations of the study samples may be consistently higher than the ULOQ of the
808 calibration curve. If this is the case, the concentration of the stability QCs should be adjusted,
809 considering the applied sample dilution, to represent the actual sample concentration range.

810 As mentioned in Section 3.2.8, the investigation of stability should cover bench top (short-term)
811 stability at room temperature or sample preparation temperature and freeze-thaw stability. In
812 addition, long-term stability should be studied.

813 For chemical drugs, it is considered acceptable to extrapolate the stability at one temperature
814 (e.g., -20°C) to lower temperatures (e.g., -70°C).

815 For biological drugs, it is acceptable to apply a bracketing approach, e.g., in the case that the
816 stability has been demonstrated at -70°C and at -20°C , then it is not necessary to investigate the
817 stability at temperatures in between those two points at which study samples will be stored.

818 **4.3 Study Sample Analysis**

819 The analysis of study samples can be carried out after validation has been completed however
820 it is understood that some parameters may be completed at a later stage (e.g., long-term
821 stability). By the time the data are submitted to a regulatory authority, the bioanalytical method
822 validation should have been completed. The study samples, QCs and calibration standards
823 should be processed in accordance with the validated analytical method. Refer to Table 1 for
824 expectations regarding documentation.

825 **4.3.1 Analytical Run**

826 An analytical run consists of a blank sample, calibration standards at a minimum of 6
827 concentration levels, at least 3 levels of QCs (low, medium and high) applied as two sets (or at
828 least 5% of the number of study samples, whichever is higher) and the study samples to be
829 analysed. The blank sample should not be included in the calculation of calibration curve
830 parameters. The QCs should be placed in the run in such a way that the accuracy and precision
831 of the whole run is ensured taking into account that study samples should always be bracketed
832 by QCs.

833 Most often microtitre plates are used for LBAs. An analytical run may comprise of one or more
834 plate(s). Typically, each plate contains an individual set of calibration standards and QCs. If

835 each plate contains its own calibration standards and QCs then each plate should be assessed
836 on its own. However, for some platforms the sample capacity may be limited. In this case, sets
837 of calibration standards may be placed on the first and the last plate, but QCs should be placed
838 on every single plate. QCs should be placed at least at the beginning (before) and at the end
839 (after) of the study samples of each plate. The QCs on each plate and each calibration curve
840 should fulfil the acceptance criteria (Refer to Section 4.3.2). For the calculation of
841 concentrations, the calibration standards should be combined to conduct one regression analysis.
842 If the combined calibration curve does not pass the acceptance criteria the whole run fails.

843 ***4.3.2 Acceptance Criteria for an Analytical Run***

844 Criteria for the acceptance or rejection of an analytical run should be defined in the protocol, in
845 the study plan or in an SOP. In the case that a run contains multiple batches, acceptance criteria
846 should be applied to the whole run and to the individual batches. It is possible for the run to
847 meet acceptance criteria, even if a batch within that run is rejected for failing to meet the batch
848 acceptance criteria.

849 The back-calculated concentrations of the calibration standards should be within $\pm 20\%$ of the
850 nominal value at each concentration level, except for the LLOQ and the ULOQ, for which it
851 should be within $\pm 25\%$. At least 75% of the calibration standards, with a minimum of 6
852 concentration levels, should fulfil this criterion. This requirement does not apply to anchor
853 calibration standards. If more than 6 calibration standards are used and one of the calibration
854 standards does not meet these criteria, this calibration standard should be rejected and the
855 calibration curve without this calibration standard should be re-evaluated and a new regression
856 analysis performed.

857 If the rejected calibration standard is the LLOQ, the new lower limit for this analytical run is
858 the next lowest acceptable calibration standard of the calibration curve. If the highest calibration
859 standard is rejected, the new upper limit for this analytical run is the next acceptable highest
860 calibration standard of the calibration curve. The new lower and upper limit calibration standard
861 will retain their original acceptance criteria (i.e., $\pm 20\%$). The revised calibration range should
862 cover all QCs (low, medium and high). The study samples outside of the revised assay range
863 should be reanalysed.

864 Each run should contain at least 3 levels of QCs (low, medium and high). During study sample
865 analysis, the calibration standards and QCs should mimic the analysis of the study sample with
866 regard to the number of wells used per study sample. At least 2/3 of the QCs and 50% at each
867 concentration level should be within $\pm 20\%$ of the nominal value at each concentration level.
868 Exceptions to these criteria should be justified and predefined in the SOP or protocol.

869 The overall mean accuracy and precision of the QCs of all accepted runs should be calculated
870 at each concentration level and reported in the analytical report. In the case that the overall
871 mean accuracy and/or precision exceeds 20%, additional investigations should be conducted to
872 determine the cause(s) of this deviation. In the case of comparative BA/BE studies it may result
873 in the rejection of the data.

874 **4.3.3 Calibration Range**

875 At least 2 QC sample levels should fall within the range of concentrations measured in study
876 samples. At the intended therapeutic dose(s), if an unanticipated clustering of study samples at
877 one end of the calibration curve is encountered after the start of sample analysis, the analysis
878 should be stopped and either the standard calibration range narrowed (i.e., partial validation),
879 existing QC concentrations revised, or QCs at additional concentrations added to the original
880 curve within the observed range before continuing with study sample analysis. It is not
881 necessary to reanalyse samples analysed before optimising the calibration curve range or QC
882 concentrations.

883 **4.3.4 Reanalysis of Study Samples**

884 Possible reasons for reanalysis of study samples, the number of reanalyses and the decision
885 criteria to select the value to be reported should be predefined in the protocol, study plan or
886 SOP, before the actual start of the analysis of the study samples.

887 The number of samples (and percentage of total number of samples) that have been reanalysed
888 should be reported and discussed in the Bioanalytical Report.

889 Some examples of reasons for study sample reanalysis are:

- 890 • Rejection of an analytical run because the run failed the acceptance criteria with regard to
891 accuracy of the calibration standards and/or the precision and accuracy of the QCs,
- 892 • The concentration obtained is above the ULOQ
- 893 • The concentration obtained is below the LLOQ in runs where the lowest calibration
894 standard has been rejected from a calibration curve, resulting in a higher LLOQ compared
895 with other runs
- 896 • Malfunction of equipment
- 897 • The diluted sample is below the LLOQ
- 898 • Identification of quantifiable analyte levels in pre-dose samples, control or placebo samples.

- 899 • When samples are analysed in more than one well and non-reportable values are obtained
900 due to one replicate failing the pre-defined acceptance criteria (e.g., excessive variability
901 between wells, one replicate being above the ULOQ or below the LLOQ).

902 For comparative BA/BE studies, reanalysis of study samples for a PK reason (e.g., a sample
903 concentration does not fit with the expected profile) is not acceptable, as it may bias the study
904 result.

905 The reanalysed samples should be identified in the Bioanalytical Report and the initial value,
906 the reason for reanalysis, the values obtained in the reanalyses, the final accepted value and a
907 justification for the acceptance should be provided. Further, a summary table of the total number
908 of samples that have been reanalysed due to each reason should be provided. In cases where the
909 first analysis yields a non-reportable result, a single reanalysis is considered sufficient (e.g.,
910 concentration above the ULOQ or excessive variability between wells). The analysis of the
911 samples should be based on the same number of wells per study sample as in the initial analysis.
912 In cases where the value needs to be confirmed, (e.g., pre-dose sample with measurable
913 concentrations) multiple determinations are required where sample volume allows.

914 The safety of trial subjects should take precedence over any other aspect of the trial.
915 Consequently, there may be other circumstances when it is necessary to reanalyse specific study
916 samples for the purpose of an investigation.

917 **5. INCURRED SAMPLE REANALYSIS (ISR)**

918 The performance of study samples may differ from that of the calibration standards and QCs
919 used during method validation, which are prepared by spiking blank matrix. Differences in
920 protein binding, back-conversion of known and unknown metabolites, sample inhomogeneity,
921 concomitant medications or biological components unique to the study samples may affect the
922 accuracy and precision of analysis of the analyte in study samples.

923 Therefore, ISR is a necessary component of bioanalytical method validation. It is intended to
924 verify the reliability of the reported sample analyte concentrations and to critically support the
925 precision and accuracy measurements established with spiked QCs.

926 ISR should be performed at least in the following situations:

- 927 • For preclinical studies, ISR should, in general, be performed for the main nonclinical TK
928 studies once per species. However, ISR in a PK study instead of a TK study might also be
929 acceptable, as long as the respective study has been conducted as a pivotal study, used to
930 make regulatory decisions.

- 931 • All pivotal comparative BA/BE studies
- 932 • First clinical trial in subjects
- 933 • Pivotal early patient trial(s), once per patient population
- 934 • First or pivotal trial in patients with impaired hepatic and/or renal function

935 ISR is conducted by repeating the analysis of a subset of samples from a given study in separate
 936 (i.e., different to the original) runs on different days using the same bioanalytical method.

937 The extent of ISR depends upon the analyte and the study samples and should be based upon
 938 an in-depth understanding of the analytical method and analyte. However, as a minimum, if the
 939 total number of study samples is less than 1000, then 10% of the samples should be reanalysed;
 940 if the total number of samples is greater than 1000, then 10% of the first 1000 samples (100)
 941 plus 5% of the number of samples that exceed 1000 samples should be assessed. Objective
 942 criteria for choosing the subset of study samples for ISR should be predefined in the protocol,
 943 study plan or an SOP. While the subjects should be picked as randomly as possible from the
 944 dosed study population, adequate coverage of the PK profile in its entirety is important.
 945 Therefore, it is recommended that the samples for ISR be chosen around the maximum
 946 concentration (C_{max}) and some in the elimination phase. Additionally, the samples chosen
 947 should be representative of the whole study.

948 Samples should not be pooled, as pooling may limit anomalous findings. ISR samples and QCs
 949 should be prepared in the same manner as in the original analysis. ISR should be performed
 950 within the stability window of the analyte, but not on the same day as the original analysis.

951 The percent difference between the initial concentration and the concentration measured during
 952 the repeat analysis should be calculated in relation to their mean value using the following
 953 equation:

$$954 \quad \% \text{ difference} = \frac{\text{repeat value} - \text{initial value}}{\text{mean value}} \times 100$$

955 For chromatographic methods, the percent difference should be ≤ 20% for at least 2/3 of the
 956 repeats. For LBAs, the percent difference should be ≤ 30% for at least 2/3 of the repeats.

957 If the overall ISR results fail the acceptance criteria, an investigation should be conducted and
 958 the causes remediated. There should be an SOP that directs how investigations are triggered
 959 and conducted. If an investigation does not identify the cause of the failure, the potential impact
 960 of an ISR failure on study validity should also be provided in the Bioanalytical Report. If ISR

961 meets the acceptance criteria yet shows large or systemic differences between results for
962 multiple samples, this may indicate analytical issues and it is advisable to investigate this further.

963 Examples of trends that are of concern include:

- 964 • All samples from one subject fail
- 965 • All of samples from one run fail

966 All aspects of ISR evaluations should be documented to allow reconstruction of the study and
967 any investigations. Individual samples that are quite different from the original value (e.g., >
968 50%, “flyers”) should not trigger reanalysis of the original sample and do not need to be
969 investigated. ISR sample data should not replace the original study sample data.

970 **6. PARTIAL AND CROSS VALIDATION**

971 **6.1 Partial Validation**

972 Partial validations evaluate modifications to already fully validated bioanalytical methods.
973 Partial validation can range from as little as one within-run accuracy and precision
974 determination, to a nearly full validation. If stability is established at one facility it does not
975 necessarily need to be repeated at another facility.

976 For chromatographic methods, typical bioanalytical method modifications or changes that fall
977 into this category include, but are not limited to, the following situations:

- 978 • Analytical site change using same method (i.e., bioanalytical method transfers between
979 laboratories)
- 980 • A change in analytical methodology (e.g., change in detection systems, platform)
- 981 • A change in sample processing procedures
- 982 • A change in sample volume (e.g., the smaller volume of paediatric samples)
- 983 • Changes to the calibration concentration range
- 984 • A change in anticoagulant (but not changes in the counter-ion) in biological fluids (e.g.,
985 heparin to ethylenediaminetetraacetic acid (EDTA))
- 986 • Change from one matrix within a species to another (e.g., switching from human plasma to
987 serum or cerebrospinal fluid) or changes to the species within the matrix (e.g., switching
988 from rat plasma to mouse plasma)

- 989 • A change in storage conditions

990 For LBAs, typical bioanalytical method modifications or changes that fall into this category
991 include, but are not limited to, the following situations:

- 992 • Changes in LBA critical reagents (e.g., lot-to-lot changes)
- 993 • Changes in MRD
- 994 • A change in storage conditions
- 995 • Changes to the calibration concentration range
- 996 • A change in analytical methodology (e.g., change in detection systems, platform)
- 997 • Analytical site change using same method (i.e., bioanalytical method transfers between
998 laboratories)
- 999 • A change in sample preparation

1000 Partial validations are acceptable if the parameters tested meet the full validation criteria. If
1001 these criteria are not satisfied, additional investigation and validation is warranted.

1002 **6.2 Cross Validation**

1003 Cross validation is required to compare data under the following situations:

- 1004 • Data are obtained from different fully validated methods within a study
- 1005 • Data are obtained from different fully validated methods across studies that are going to be
1006 combined or compared to support special dosing regimens, or regulatory decisions
1007 regarding safety, efficacy and labelling.
- 1008 • Data are obtained within a study from different laboratories with the same bioanalytical
1009 method.

1010 Cross validation is not generally required to compare data obtained across studies from different
1011 laboratories using the same validated method at each site.

1012 Cross validation should be performed in advance of study samples being analysed, if possible.

1013 Cross validation should be assessed by measuring the same set of QCs (low, medium and high)
1014 in triplicate and study samples that span the study sample concentration range (if available
1015 $n \geq 30$) with both assays or in both laboratories.

1016 Bias can be assessed by Bland-Altman plots or Deming regression. Other methods appropriate
1017 for assessing agreement between two assays (e.g., concordance correlation coefficient) may be
1018 used too. Alternatively, the concentration vs. time curves for incurred samples could be plotted
1019 for samples analysed by each method to assess bias. If disproportionate bias is observed
1020 between methods, the impact on the clinical data interpretation should be assessed.

1021 The use of multiple bioanalytical methods in the conduct of one comparative BA/BE study is
1022 strongly discouraged.

1023 **7. ADDITIONAL CONSIDERATIONS**

1024 **7.1 Analytes that are also Endogenous Compounds**

1025 For analytes that are also endogenous compounds, the accuracy of the measurement of the
1026 analytes poses a challenge when the assay cannot distinguish between the therapeutic agent and
1027 the endogenous counterpart.

1028 The endogenous levels may vary because of age, gender, diurnal variations, illness or as a side
1029 effect of drug treatment. If available, biological matrix with an adequate signal-to-noise ratio
1030 (i.e., endogenous level sufficiently low for the desired LLOQ, e.g., <20% of the LLOQ) should
1031 be used as blank matrix to prepare calibration standards and QCs since the biological matrix
1032 used to prepare calibration standards and QCs should be the same as the study samples (i.e.,
1033 authentic biological matrix) and should be free of matrix effect and endogenous analyte at the
1034 level that causes interference.

1035 In those cases where matrices without interference are not available, there are four possible
1036 approaches to calculate the concentration of the endogenous analyte in calibration standards,
1037 QCs and, consequently, study samples: 1) the standard addition approach, 2) the background
1038 subtraction approach, 3) the surrogate matrix (neat, artificial or stripped matrices) approach and
1039 4) the surrogate analyte approach.

1040 1) Standard Addition Approach:

1041 Every study sample is divided into aliquots of equal volume. All aliquots, but one,
1042 are separately spiked with known and varying amounts of the analyte standards to
1043 construct a calibration curve for every study sample. The study sample concentration
1044 is then determined as the negative x-intercept of the standard calibration curve
1045 prepared in that particular study sample.

1046 2) Background Subtraction Approach:

1047 The endogenous background concentrations of analytes in a pooled/representative
1048 matrix are subtracted from the concentrations of the added standards, subsequently
1049 the subtracted concentrations are used to construct the calibration curve.

1050 3) Surrogate Matrix Approach:

1051 The matrix of the study samples is substituted by a surrogate matrix. Surrogate
1052 matrices can vary widely in complexity from simple buffers or artificial matrices that
1053 try to mimic the authentic one, to stripped matrices.

1054 4) Surrogate Analyte Approach:

1055 Stable-isotope labelled analytes are used as surrogate standards to construct the
1056 calibration curves for the quantification of endogenous analytes. In this method it is
1057 assumed that the physicochemical properties of the authentic and surrogates analytes
1058 are the same with the exception of molecular weight. However, isotope standards may
1059 differ in retention time and MS sensitivity, therefore, before application of this
1060 approach, the ratio of the labelled to unlabelled analyte MS responses (i.e., the
1061 response factor) should be close to unity and constant over the entire calibration range.
1062 If the response factor does not comply with these requirements, it should be
1063 incorporated into the regression equation of the calibration curve.

1064 Validation of an analytical method for an analyte that is also an endogenous compound will
1065 require the following considerations.

1066 **7.1.1 Quality Control Samples**

1067 The endogenous concentrations of the analyte in the biological matrix should be evaluated prior
1068 to QC preparation (e.g., by replicate analysis). The blank matrices with the minimum level of
1069 the endogenous analyte should be used. The concentrations of the QCs should account for the
1070 endogenous concentrations in the biological matrix (i.e., additive) and be representative of the
1071 expected study concentrations.

1072 The QCs used for validation should be aliquots of the authentic biological matrix unspiked and
1073 spiked with known amounts of the authentic analyte. In spiked samples, the added amount
1074 should be enough to provide concentrations that are statistically different from the endogenous
1075 concentration.

1076 **7.1.2 Calibration Standards**

1077 In the Surrogate Matrix and Surrogate Analyte Approaches, these surrogates should be used
1078 only for the preparation of the calibration standards.

1079 In the Standard Addition and Background Subtraction Approaches the same biological matrix
1080 and analyte as the study samples is used to prepare the calibration standards. However, when
1081 the background concentrations are lowered by dilution of the blank matrices before spiking
1082 with the standards (e.g., if a lower LLOQ is required in the Background Subtraction Approach)
1083 the composition of the matrices in the study samples and the calibration standards is different,
1084 which may cause different recoveries and matrix effects.

1085 **7.1.3 Selectivity, Recovery and Matrix Effects**

1086 The assessment of selectivity is complicated by the absence of interference-free matrix. For
1087 chromatography, peak purity should be investigated as part of method validation by analysing
1088 matrices obtained from several donors using a discriminative detection system (e.g., tandem
1089 mass spectrometry (MS/MS)). Other approaches, if justified by scientific principles, may also
1090 be considered.

1091 For the Standard Addition and Background Subtraction Approaches, as the same biological
1092 matrix and analyte are used for study samples and calibration standards, the same recovery and
1093 matrix effect occurs in the study samples and the calibration standards. For the Surrogate Matrix
1094 and Surrogate Analyte Approaches, the matrix effect and the extraction recovery may differ
1095 between calibration standards and study samples.

1096

- If the Surrogate Matrix Approach is used, demonstration of similar matrix effect and
1097 extraction recovery in both the surrogate and original matrix is required. This should be
1098 investigated in an experiment using QCs spiked with analyte in the matrix against the
1099 surrogate calibration curve and should be within $\pm 15\%$ for chromatographic assays and
1100 within $\pm 20\%$ for LBA assays.

1101

- If the Surrogate Analyte Approach is used, demonstration of similarity in matrix effect
1102 and recovery between surrogate and authentic endogenous analytes is required. This
1103 should be investigated in an experiment within $\pm 15\%$ for chromatographic assays and
1104 within $\pm 20\%$ for LBA assays.

1105 Since the composition of the biological matrix might affect method performance, it is necessary
1106 to investigate matrices from different donors, except in the Standard Addition Approach, where
1107 each sample is analysed with its own calibration curve.

1108 **7.1.4 Parallelism**

1109 Parallelism should be evaluated in the Surrogate Matrix and Surrogate Analyte Approaches by
1110 means of the Standard Addition approach, spike recovery or dilutional linearity.

1111 **7.1.5 Accuracy and Precision**

1112 In case of using a surrogate matrix or analyte, the assessment of accuracy and precision should
1113 be performed by analysing the QCs against the surrogate calibration curve. In certain cases,
1114 dilution of the QCs with surrogate matrix may be necessary. These experiments should be
1115 repeated with authentic biological matrices from different donors to address variability due to
1116 the matrix. Analysis of the unspiked QCs will give the mean endogenous background
1117 concentration and only precision and no accuracy can be determined for this QCs.

1118 The concentration of the endogenous substance in the blank sample may be determined and
1119 subtracted from the total concentrations observed in the spiked samples. Accuracy is
1120 recommended to be calculated using the following formula:

$$1121 \quad \text{Accuracy (\%)} = 100 \times \frac{(\text{Measured concentration of spiked sample} - \text{endogenous concentration})}{\text{Nominal concentration}}$$

1122 **7.1.6 Stability**

1123 In order to mimic study samples as much as possible, stability experiments should be
1124 investigated with the authentic analyte in the authentic biological matrix and with unspiked and
1125 spiked samples. However, if a surrogate matrix is used for calibration standards, stability should
1126 also be demonstrated for the analyte in the surrogate matrix, as this could differ from stability
1127 in the authentic biological matrix.

1128 **7.2 Parallelism**

1129 Parallelism is defined as a parallel relationship between the calibration curve and serially
1130 diluted study samples to detect any influence of dilution on analyte measurement. Although
1131 lack of parallelism is a rare occurrence for PK assays, parallelism of LBA should be evaluated
1132 on a case-by-case basis, e.g., where interference caused by a matrix component (e.g., presence
1133 of endogenous binding protein) is suspected during study sample analysis. Parallelism
1134 investigation or the justification for its absence should be included in the Bioanalytical Report.
1135 As parallelism assessments are rarely possible during method development and method
1136 validation due to the unavailability of study samples and parallelism is strictly linked to the
1137 study samples (i.e., an assay may have perfectly suitable parallelism for a certain population of
1138 samples, yet lack it for another population), these experiments should be conducted during the

1139 analysis of the study samples. A high concentration study sample (preferably close to C_{max})
1140 should be diluted to at least three concentrations with blank matrix. The precision between
1141 samples in a dilution series should not exceed 30%. However, when applying the 30% criterion,
1142 data should be carefully monitored as results that pass this criterion may still reveal trends of
1143 non-parallelism. In the case that the sample does not dilute linearly (i.e., in a non-parallel
1144 manner), a procedure for reporting a result should be defined *a priori*.

1145 **7.3 Recovery**

1146 For methods that employ sample extraction, the recovery (extraction efficiency) should be
1147 evaluated. Recovery is reported as a percentage of the known amount of an analyte carried
1148 through the sample extraction and processing steps of the method. Recovery is determined by
1149 comparing the analyte response in a biological sample that is spiked with the analyte and
1150 processed, with the response in a biological blank sample that is processed and then spiked with
1151 the analyte. Recovery of the analyte does not need to be 100%, but the extent of recovery of an
1152 analyte and of the IS (if used) should be consistent. Recovery experiments are recommended to
1153 be performed by comparing the analytical results for extracted samples at multiple
1154 concentrations, typically three concentrations (low, medium and high).

1155 **7.4 Minimum Required Dilution**

1156 MRD is a dilution factor employed in samples that are diluted with buffer solution to reduce
1157 the background signal or matrix interference on the analysis using LBA. The MRD should be
1158 identical for all samples including calibration standards and the QCs and it should be
1159 determined during method development. If MRD is changed after establishment of the method,
1160 partial validation is necessary. MRD should be defined in the Validation Report of the analytical
1161 method.

1162 **7.5 Commercial and Diagnostic Kits**

1163 Commercial or diagnostic kits (referred to as kits) are sometimes co-developed with new drugs
1164 or therapeutic biological products for point-of-care patient diagnosis. The recommendations in
1165 this section of the guideline do not apply to the development of kits that are intended for point-
1166 of-care patient diagnosis (e.g., companion or complimentary diagnostic kits). Refer to the
1167 appropriate guideline documents regarding regulatory expectations for the development of
1168 these kits.

1169 If an applicant repurposes a kit (instead of developing a new assay) or utilises “research use
1170 only” kits to measure chemical or biological drug concentrations during the development of a

1171 novel drug, the applicant should assess the kit validation to ensure that it conforms to the drug
1172 development standards described in this guideline.

1173 Validation considerations for kit assays include, but are not limited to, the following:

1174 • If the reference standard in the kit differs from that of the study samples, testing should
1175 evaluate differences in assay performance of the kit reagents. The specificity, accuracy,
1176 precision and stability of the assay should be demonstrated under actual conditions of use
1177 in the facility conducting the sample analysis. Modifications from kit processing
1178 instructions should be completely validated.

1179 • Kits that use sparse calibration standards (e.g., one- or two-point calibration curves) should
1180 include in-house validation experiments to establish the calibration curve with a sufficient
1181 number of standards across the calibration range.

1182 • Actual QC concentrations should be known. Concentrations of QCs expressed as ranges are
1183 not sufficient for quantitative applications. In such cases QCs with known concentrations
1184 should be prepared and used, independent of the kit-supplied QCs.

1185 • Calibration standards and QCs should be prepared in the same matrix as the study samples.
1186 Kits with calibration standards and QCs prepared in a matrix different from the study
1187 samples should be justified and appropriate experiments should be performed.

1188 • If multiple kit lots are used within a study, lot-to-lot variability and comparability should be
1189 addressed for any critical reagents included in the kits.

1190 • If a kit using multiple assay plates is employed, sufficient replicate QCs should be used on
1191 each plate to monitor the accuracy of the assay. Acceptance criteria should be established
1192 for the individual plates and for the overall analytical run.

1193 **7.6 New or Alternative Technologies**

1194 When a new or alternative technology is used as the sole bioanalytical technology from the
1195 onset of drug development, cross validation with an existing technology is not required.

1196 The use of two different bioanalytical technologies for the development of a drug may generate
1197 data for the same product that could be difficult to interpret. This outcome can occur when one
1198 platform generates drug concentrations that differ from those obtained with another platform.
1199 Therefore, when a new or alternative analytical platform is replacing a previous platform used
1200 in the development of a drug it is important that the potential differences are well understood.

1201 The data generated from the previous platform/technology should be cross validated to that of
1202 the new or alternative platform/technology. Seeking feedback from the regulatory authorities is
1203 encouraged early in drug development. The use of two methods or technologies within a
1204 comparative BA/BE study is strongly discouraged.

1205 The use of new technology in regulated bioanalysis should be supported by acceptance criteria
1206 established *a priori* based on method development and verified in validation.

1207 **7.6.1 Dried Matrix Methods**

1208 Dried matrix methods (DMM) is a sampling methodology that offers benefits such as collection
1209 of reduced blood sample volumes as a microsampling technique for drug analysis and ease of
1210 collection, storage and transportation. In addition to the typical methodological validation for
1211 LC-MS or LBA, use of DMM necessitates further validation of this sampling approach before
1212 using DMM in studies that support a regulatory application, such as:

- 1213 • Haematocrit (especially for spotting of whole blood into cards)
- 1214 • Sample homogeneity (especially for sub-punch of the sample on the card/device)
- 1215 • Reconstitution of the sample
- 1216 • DMM sample collection for ISR
 - 1217 ○ Care should be taken to ensure sufficient sample volumes or numbers of
 - 1218 replicates are retained for ISR
 - 1219 ○ Should be assessed by multiple punches of the sample or samples should be
 - 1220 taken in duplicate

1221 When DMM is used for clinical or nonclinical studies in addition to typical liquid approaches
1222 (e.g., liquid plasma samples) in the same studies, these two methods should be cross validated
1223 as described (Refer to Section 6.2). For nonclinical TK studies, refer to Section 4.1 of ICH S3A
1224 Q&A. Feedback from the appropriate regulatory authorities is encouraged in early drug
1225 development.

1226 **8. DOCUMENTATION**

1227 General and specific SOPs and good record keeping are essential to a properly validated
1228 analytical method. The data generated for bioanalytical method validation should be
1229 documented and available for data audit and inspection. Table 1 describes the recommended
1230 documentation for submission to the regulatory authorities and documentation that should be

1231 available at the analytical site at times of inspection. This documentation may be stored at the
1232 analytical site or at another secure location. In this case the documentation should be readily
1233 available when requested.

1234 All relevant documentation necessary for reconstructing the study as it was conducted and
1235 reported should be maintained in a secure environment. Relevant documentation includes, but
1236 is not limited to, source data, protocols and reports, records supporting procedural, operational,
1237 and environmental concerns and correspondence records between all involved parties.

1238 Regardless of the documentation format (i.e., paper or electronic), records should be
1239 contemporaneous with the event and subsequent alterations should not obscure the original data.
1240 The basis for changing or reprocessing data should be documented with sufficient detail, and
1241 the original record should be maintained. Transcripts/copies of data derived from analyses in
1242 biohazardous areas should be maintained if applicable.

1243 **8.1 Summary Information**

1244 Summary information should include the following items in Section 2.6.4/2.7.1 of the Common
1245 Technical Document (CTD) or reports:

1246 • A summary of assay methods used for each study should be included. Each summary
1247 should provide the protocol number, the assay type, the assay method identification
1248 code, the Bioanalytical Report code, effective date of the method, and the associated
1249 Validation Report codes.

1250 • A summary table of all the relevant Validation Reports should be provided for each
1251 analyte, including Partial Validation and Cross Validation Reports. The table should
1252 include the assay method identification code, the type of assay, the reason for the
1253 new method or additional validation (e.g., to lower the limit of quantification).
1254 Changes made to the method should be clearly identified.

1255 • A summary table cross-referencing multiple identification codes should be provided
1256 when an assay has different codes for the assay method, the Validation Reports and
1257 the Bioanalytical Reports.

1258 • Discussion of method changes in the protocol (e.g., evolution of methods, reason(s)
1259 for revisions, unique aspects)

1260 • For comparative BA/BE studies a list of regulatory site inspections including dates
1261 and outcomes for each analytical site if available.

1262 **8.2 Documentation for Validation and Bioanalytical Reports**

1263 Table 1 describes the recommended documentation for the Validation and Bioanalytical Reports.

Table 1: Documentation and Reporting

Items	Documentation at the Analytical Site	Validation Report*	Bioanalytical Report*
Chromatographic System Suitability	<ul style="list-style-type: none"> Dates, times, and samples used for suitability testing 	<ul style="list-style-type: none"> Not applicable 	<ul style="list-style-type: none"> Not applicable
Synopsis Overview of Method Evolution	<ul style="list-style-type: none"> History/evolution of methods (e.g., to explain revisions, unique aspects with supportive data, if available) 	<ul style="list-style-type: none"> Not applicable 	<ul style="list-style-type: none"> Not applicable
Reference Standards	<ul style="list-style-type: none"> CoA or equivalent alternative to ensure quality (including purity), stability/expiration/retest date(s), batch number, and manufacturer or source Log records of receipt, use, and storage conditions. If expired, recertified CoA, or retest of quality and identity with retest dates 	<ul style="list-style-type: none"> A copy of the CoA or equivalent alternative including batch/lot number, source, quality (including purity), storage conditions, and expiration/retest date, or table with this information. If expired, quality and stability at the time of use and retest dates and retested values. 	<ul style="list-style-type: none"> A copy of the CoA or equivalent alternative including batch /lot number, source, quality (including purity), storage conditions, and expiration/retest date or a table with this information. If expired, quality and stability at the time of use and retest dates and retested values.
Internal Standard	<ul style="list-style-type: none"> IS quality or demonstration of suitability Log records of receipt, use, and storage conditions 	<ul style="list-style-type: none"> Name of reagent or standard Origin 	<ul style="list-style-type: none"> Name of reagent or standard Origin

Table 1 continued: Documentation and Reporting

Items	Documentation at the Analytical Site	Validation Report*	Bioanalytical Report*
Critical Reagents	<ul style="list-style-type: none"> Name of reagent Batch/ Lot number Source/Origin Concentration, if applicable Retest date (expiry date) Storage conditions 	<ul style="list-style-type: none"> Name of reagent Batch/ Lot number Source/ Origin Retest date (expiry date) Storage conditions 	<ul style="list-style-type: none"> Name of reagent Batch/ Lot number Source/ Origin Retest date (expiry date) Storage conditions
Stock Solutions	<ul style="list-style-type: none"> Log of preparation, and use of stock solutions Storage location and condition 	<ul style="list-style-type: none"> Notation that solutions were used within stability period Stock solution stability Storage conditions 	<ul style="list-style-type: none"> Notation that solutions were used within stability period Stock solution stability † Storage conditions†
Blank Matrix	<ul style="list-style-type: none"> Records of matrix descriptions, lot numbers, receipt dates, storage conditions, and source/supplier 	<ul style="list-style-type: none"> Description, lot number, receipt dates 	<ul style="list-style-type: none"> Description, lot number, receipt dates††
Calibration Standards and QCs	<ul style="list-style-type: none"> Records and date of preparation Record of storage temperature (e.g., log of in/out dates, analyst, temperatures, and freezer(s)) 	<ul style="list-style-type: none"> Description of preparation including matrix Batch number, preparation dates and stability period Storage conditions (temperatures, dates, duration, etc.) 	<ul style="list-style-type: none"> Description of preparation† Preparation dates and stability period Storage conditions†

Table 1 continued: Documentation and Reporting

Items	Documentation at the Analytical Site	Validation Report*	Bioanalytical Report*
SOPs	<p>SOPs for all aspects of analysis, such as:</p> <ul style="list-style-type: none"> • Method/procedure (validation/analytical) • Acceptance criteria (e.g., run, calibration curve, QCs) • Instrumentation • Reanalysis • ISR • Record of changes to SOP (change, date, reason, etc.) 	<ul style="list-style-type: none"> • A detailed description of the assay procedure 	<ul style="list-style-type: none"> • A list of SOPs/analytical protocols used for the assay procedure
Sample Tracking	<ul style="list-style-type: none"> • Study sample receipt, and condition on receipt • Records that indicate how samples were transported and received. Sample inventory and reasons for missing samples • Location of storage (e.g., freezer unit) • Tracking logs of QCs, calibration standards, and study samples • Freezer logs for QCs, calibration standards, and study samples entry and exit 	<ul style="list-style-type: none"> • Not applicable 	<ul style="list-style-type: none"> • Dates of receipt of shipments number of samples, and for comparative BA/BE studies the subject ID • Sample condition on receipt • Analytical site storage condition and location • Storage: total duration from sample collection to analysis • List of any deviations from planned storage conditions, and potential impact

Table 1 continued: Documentation and Reporting

Items	Documentation at the Analytical Site	Validation Report*	Bioanalytical Report*
Analysis	<ul style="list-style-type: none"> • Documentation and data for system suitability checks for chromatography • Instrument use log, including dates of analysis for each run • Sample extraction logs including documentation of processing of calibration standards, QCs, and study samples for each run, including dates of extraction • Identity of QCs and calibration standard lots, and study samples in each run • Documentation of instrument settings and maintenance • Laboratory information management system (LIMS) • Validation information, including documentation and data for: <ul style="list-style-type: none"> ○ Selectivity, (matrix effects), specificity, (interference) sensitivity, precision and accuracy, carry-over, dilution, recovery, matrix effect ○ Bench-top, freeze-thaw, long-term, extract, and stock solution stability ○ Cross/partial validations, if applicable 	<ul style="list-style-type: none"> • Table of all runs (including failed runs), and analysis dates • Instrument ID for each run in comparative BA/BE studies † • Table of calibration standard concentration and response functions results (calibration curve parameters) of all accepted runs with accuracy and precision. • Table of within- and between- run QC results (from accuracy and precision runs). Values outside should be clearly marked. • Include total error for LBA methods • Data on selectivity (matrix effect), specificity (interference), dilution linearity and sensitivity (LLOQ), carry-over, recovery. Bench-top, freeze-thaw, long-term, extract, and stock solution stability • Partial/cross-validation, if applicable • Append separate report for additional validation, if any 	<ul style="list-style-type: none"> • Table of all runs, status (accepted and failed), reason for failure, and analysis dates. • Instrument ID for each run in comparative BA/BE studies† • Table of calibration standard concentration and response function results (calibration curve parameters) of all accepted runs with accuracy and precision. • Table of QCs results of all accepted runs with accuracy and precision results of the QCs and between-run accuracy and precision results from accepted runs. • Table of reinjected runs with results from reinjected runs and reason(s) for reinjection • QCs graphs trend analysis encouraged • Study concentration results table. • For comparative BA/BE studies, IS response plots for each analytical run, including failed runs

Table 1 continued: Documentation and Reporting

Items	Documentation at the Analytical Site	Validation Report*	Bioanalytical Report*
<p>Chromatograms and Reintegration</p>	<ul style="list-style-type: none"> • Electronic audit trail: • 100% e-chromatograms of original and reintegration from accepted and fail runs • Reason for reintegration • Mode of reintegration 100% of run summary sheets of accepted and failed runs, including calibration curve, regression, weighting function, analyte and IS response and retention time, response ratio, integration type 	<ul style="list-style-type: none"> • Representative chromatograms (original and reintegration) • Reason for reintegration • For comparative BA/BE studies, 100% chromatograms of original and reintegration from accepted and fail runs. • Chromatograms may be submitted as a supplement • For comparative BA/BE studies, 100% of run summary sheets of accepted and failed runs, including calibration curve, regression, weighting function, analyte and IS responses and retention times and dilution factor if applicable. 	<ul style="list-style-type: none"> • For and comparative BA/BE studies, 100% of chromatograms. • Chromatograms may be submitted as a supplement • For comparative BA/BE studies, original and reintegrated chromatograms and initial and repeat integration results • For other studies, randomly selected chromatograms from 5% of studies submitted in application dossiers • Reason for reintegration • Identification and discussion of chromatograms with manual reintegration • SOP for reintegration, as applicable • For comparative BA/BE studies, 100% of run summary sheets of accepted and failed runs, including calibration curve, regression, weighting function, analyte and IS responses and retention times, and dilution factor if applicable.

Table 1 continued: Documentation and Reporting

Items	Documentation at the Analytical Site	Validation Report*	Bioanalytical Report*
Deviations from Procedures	<ul style="list-style-type: none"> • Contemporaneous documentation of deviations/ unexpected events • Investigation of unexpected events • Impact assessment 	<ul style="list-style-type: none"> • Description of Deviations • Impact on study results • Description and supporting data of significant investigations 	<ul style="list-style-type: none"> • Description of deviations • Impact on study results • Description and supporting data of significant investigations
Repeat Analysis	<ul style="list-style-type: none"> • SOP for conducting reanalysis/ repeat analysis (define reasons for reanalysis, etc.) • Retain 100% of repeat/reanalysed data • Contemporaneous records of reason for repeats 	<ul style="list-style-type: none"> • Not applicable 	<ul style="list-style-type: none"> • Table of sample IDs, reason for reassay, original and reassay values, reason for reported values, run IDs • Reanalysis SOP, if requested
ISR	<ul style="list-style-type: none"> • SOP for ISR • ISR data: Run IDs, run summary sheets, chromatograms or other electronic instrument data files • Document ISR failure investigations, if any 	<ul style="list-style-type: none"> • Not applicable 	<ul style="list-style-type: none"> • ISR data table (original and reanalysis values and run IDs, percent difference, percent passed) • ISR failure investigations, if any^{††} • SOP for ISR^{††} (if requested)
Communication	<ul style="list-style-type: none"> • Between involved parties (Applicant, contract research organizations (CROs), and consultants) related to study/assay 	<ul style="list-style-type: none"> • Not applicable 	<ul style="list-style-type: none"> • Not applicable
Audits and Inspections	<ul style="list-style-type: none"> • Audit and inspection report 	<ul style="list-style-type: none"> • Not applicable 	<ul style="list-style-type: none"> • Not applicable

1268 *The applicant is expected to maintain data at the analytical site to support summary data submitted in Validation and Bioanalytical Reports.
 1269 Validation and Bioanalytical Reports should be submitted in the application.

1270 † May append or link from Validation Report.

1271 †† Submit either in Validation Report or in Bioanalytical Report

1272 **9. GLOSSARY**

1273 **Accuracy:**

1274 The degree of closeness of the measured value to the nominal or known true value under
1275 prescribed conditions (or as measured by a particular method). In this document accuracy is
1276 expressed as percent relative error of the nominal value.

1277 Accuracy (%) = ((Measured Value-Nominal Value)/Nominal Value) × 100

1278

1279 **Analysis:**

1280 A series of analytical procedures from sample processing/dilution to measurement on an
1281 analytical instrument.

1282

1283 **Analyte:**

1284 A specific chemical moiety being measured, including an intact drug, a biomolecule or its
1285 derivative or a metabolite in a biologic matrix.

1286

1287 **Analytical Procedure:**

1288 The analytical procedure refers to the way of performing the analysis. It should describe in
1289 detail the steps necessary to perform each analysis.

1290

1291 **Analytical Run (also referred to as “Run”):**

1292 A complete set of analytical and study samples with appropriate number of calibration standards
1293 and QCs for their validation. Several runs may be completed in one day or one run may take
1294 several days to complete.

1295 **Anchor Calibration Standards/Anchor Points:**

1296 Spiked samples set at concentrations below the LLOQ or above the ULOQ of the calibration
1297 curve and analysed to improve curve fitting in LBAs.

1298

1299 **Batch (for Bioanalysis):**

1300 A batch is comprised of QCs and study samples which are handled during a fixed period of time
1301 and by the same group of analysts with the same reagents under homogenous conditions.

1302

1303 **Batch (for Reference Standards and Reagents):**

1304 A specific quantity of material produced in a process or series of processes so that it is
1305 expected to be homogeneous within specified limits. Also referred to as “Lot”.

1306

1307 **Biological Drugs:**

1308 Drugs manufactured by using biotechnology (e.g., therapeutic proteins). Also referred to as
1309 large molecule drugs.

1310

1311 **Biological Matrix:**

1312 A biological material including, but not limited to, blood, serum, plasma and urine.

1313

1314 **Binding Reagent:**

1315 A reagent that directly binds to the analyte in LBA-based bioanalytical methods.

1316

1317 **Blank Sample:**

1318 A sample of a biological matrix to which no analyte and no IS has been added.

1319 **Calibration Curve:**

1320 The relationship between the instrument response (e.g., peak area, height or signal) and the
1321 concentration (amount) of analyte in the sample within a given range. Also referred to as
1322 Standard Curve.

1323

1324 **Calibration Range:**

1325 The calibration range of an analytical procedure is the interval between the upper and lower
1326 concentration (amounts) of analyte in the sample (including these concentrations) for which it
1327 has been demonstrated that the analytical procedure meets the requirements for precision,
1328 accuracy and response function.

1329

1330 **Calibration Standard:**

1331 A matrix to which a known amount of analyte has been added or spiked. Calibration standards
1332 are used to construct calibration curves.

1333

1334 **Carry-over:**

1335 The appearance of an analyte signal in a sample from a preceding sample.

1336

1337 **Chemical Drugs:**

1338 Chemically synthesised drugs. Also referred to as small molecule drugs.

1339

1340 **Critical Reagent:**

1341 Critical reagents for LBAs include binding reagents (e.g., antibodies, binding proteins,
1342 peptides) and those containing enzymatic moieties that have a direct impact on the results of
1343 the assay.

1344 **Cross Validation:**

1345 Comparison of two bioanalytical methods or the same bioanalytical method in different
1346 laboratories in order to demonstrate that the reported data are comparable.

1347

1348 **Dilution Integrity:**

1349 Assessment of the sample dilution procedure to confirm that the procedure does not impact the
1350 measured concentration of the analyte.

1351

1352 **Dilution Linearity:**

1353 A parameter demonstrating that the method can appropriately analyse samples at a
1354 concentration exceeding the ULOQ of the calibration curve without influence of hook effect or
1355 prozone effect and that the measured concentrations are not affected by dilution within the
1356 calibration range in LBAs.

1357

1358 **Full Validation:**

1359 Establishment of all validation parameters that ensure the integrity of the method when applied
1360 to sample analysis.

1361

1362 **Hook Effect:**

1363 Suppression of response due to very high concentrations of a particular analyte. A hook effect
1364 may occur in LBAs that use a liquid-phase reaction step for incubating the binding reagents
1365 with the analyte. Also referred to as prozone.

1366

1367 **Incurred Sample:**

1368 A sample obtained from study subjects or animals.

1369 **Incurred Sample Reanalysis (ISR):**

1370 Reanalysis of a portion of the incurred samples in a separate analytical run on a different day
1371 to determine whether the original analytical results are reproducible.

1372

1373 **Interfering Substance:**

1374 A substance that is present in the matrix that may affect the analysis of an analyte.

1375

1376 **Internal Standard (IS):**

1377 A structurally similar analogue or stable isotope labelled compound added to calibration
1378 standards, QCs and study samples at a known and constant concentration to facilitate
1379 quantification of the target analyte.

1380

1381 **Ligand Binding Assay (LBA):**

1382 A method to analyse an analyte of interest using reagents that specifically bind to the analyte.
1383 The analyte is detected using reagents labelled with e.g. an enzyme, radioisotope, fluorophore
1384 or chromophore. Reactions are carried out in microtitre plates, test tubes, disks, etc.

1385

1386 **Lower Limit of Quantification (LLOQ):**

1387 The lowest amount of an analyte in a sample that can be quantitatively determined with
1388 predefined precision and accuracy.

1389

1390 **Matrix Effect:**

1391 The direct or indirect alteration or interference in response due to the presence of unintended
1392 analytes or other interfering substances in the sample.

1393 **Method:**

1394 A comprehensive description of all procedures used in sample analysis.

1395

1396 **Minimum Required Dilution (MRD):**

1397 The initial dilution factor by which biological samples are diluted with buffer solution for the
1398 analysis by LBAs. The MRD may not necessarily be the ultimate dilution but should be
1399 identical for all samples including calibration standards and QCs. However, samples may
1400 require further dilution.

1401

1402 **Nominal Concentration:**

1403 Theoretical or expected concentration.

1404

1405 **Parallelism:**

1406 Parallelism demonstrates that the serially diluted incurred sample response curve is parallel to
1407 the calibration curve. Parallelism is a performance characteristic that can detect potential matrix
1408 effects.

1409

1410 **Partial Validation:**

1411 Evaluation of modifications to already fully validated analytical methods.

1412

1413 **Precision:**

1414 The closeness of agreement (i.e., degree of scatter) among a series of measurements. Precision
1415 is expressed as the coefficient of variation (CV) or the relative standard deviation (RSD)
1416 expressed as a percentage.

1417 Precision (%) = (Standard Deviation / Mean) x 100

1418 **Processed Sample:**

1419 The final sample that has been subjected to various manipulations (e.g., extraction, dilution,
1420 concentration).

1421

1422 **Quality Control Sample (QC):**

1423 A sample spiked with a known quantity of analyte that is used to monitor the performance of a
1424 bioanalytical method and assess the integrity and validity of the results of the unknown samples
1425 analysed in an individual batch or run.

1426

1427 **Recovery:**

1428 The extraction efficiency of an analytical process, reported as a percentage of the known amount
1429 of an analyte carried through the sample extraction and processing steps of the method.

1430

1431 **Reproducibility:**

1432 The extent to which consistent results are obtained when an experiment is repeated.

1433

1434 **Response Function:**

1435 A function which adequately describes the relationship between instrument response (e.g., peak
1436 area or height ratio or signal) and the concentration (amount) of analyte in the sample. Response
1437 function is defined within a given range. See also Calibration Curve.

1438

1439 **Selectivity:**

1440 Ability of an analytical method to differentiate and measure the analyte in the presence of
1441 interfering substances in the biological matrix (non-specific interference).

1442 **Sensitivity:**

1443 The lowest analyte concentration that can be measured with acceptable accuracy and precision
1444 (i.e., LLOQ).

1445

1446 **Specificity:**

1447 Ability of an analytical method to detect and differentiate the analyte from other substances,
1448 including its related substances (e.g., substances that are structurally similar to the analyte,
1449 metabolites, isomers, impurities or concomitant medications).

1450

1451 **Standard Curve:**

1452 The relationship between the instrument response (e.g., peak area, height or signal) and the
1453 concentration (amount) of analyte in the sample within a given range. Also referred to as
1454 calibration Curve.

1455

1456 **Standard Operating Procedure (SOP):**

1457 Detailed written instructions to achieve uniformity of the performance of a specific function.

1458

1459 **Surrogate Matrix:**

1460 An alternative to a study matrix of limited availability (e.g., tissue, cerebrospinal fluid, bile) or
1461 where the study matrix contains an interfering endogenous counterpart.

1462

1463 **System Suitability:**

1464 Determination of instrument performance (e.g., sensitivity and chromatographic retention) by
1465 analysis of a set of reference standards conducted prior to the analytical run.

1466 **Total Error:**

1467 The sum of the absolute value of the errors in accuracy (%) and precision (%). Total error is
1468 reported as percent (%) error.

1469

1470 **Upper Limit of Quantification (ULOQ):**

1471 The upper limit of quantification of an individual analytical procedure is the highest amount of
1472 analyte in a sample that can be quantitatively determined with pre-defined precision and
1473 accuracy.

1474

1475 **Validation:**

1476 Demonstration that a bioanalytical method is suitable for its intended purpose.

1477

1478 **Working Solution:**

1479 A non-matrix solution prepared by diluting the stock solution in an appropriate solvent. It is
1480 mainly added to matrix to prepare calibration standards and QCs.

1481

1482 **Zero Sample:**

1483 A blank sample spiked with an IS.