



1 13 March 2019  
2 EMA/CHMP/ICH/172948/2019  
3 Committee for Human Medicinal Products

4 **ICH guideline M10 on bioanalytical method validation**  
5 **Step 2b**

Transmission to CHMP	28 February 2019
Adoption by CHMP	28 February 2019
Release for public consultation	14 March 2019
Deadline for comments	1 September 2019

6  
7

Comments should be provided using this [template](#). The completed comments form should be sent to [ich@ema.europa.eu](mailto:ich@ema.europa.eu)

8



10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50

**INTERNATIONAL COUNCIL FOR HARMONISATION OF TECHNICAL  
REQUIREMENTS FOR PHARMACEUTICALS FOR HUMAN USE**

**ICH HARMONISED GUIDELINE**

**BIOANALYTICAL METHOD VALIDATION  
M10**

Draft version

*Currently under public consultation*

*At Step 2 of the ICH Process, a consensus draft text or guideline, agreed by the appropriate ICH Expert Working Group, is transmitted by the ICH Assembly to the regulatory authorities of the ICH regions for internal and external consultation, according to national or regional procedures.*

51  
52  
53

**M10**  
**Document History**

Code	History	Date
M10	Endorsement by the Members of the ICH Assembly under <i>Step 2</i> and release for public consultation (	

54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
66  
67  
68  
69  
70  
71  
72  
73  
74  
75  
76  
77  
78  
79  
80  
81  
82  
83  
84  
85  
86  
87  
88  
89  
90  
91  
92

**Legal notice:** This document is protected by copyright and may, with the exception of the ICH logo, be used, reproduced, incorporated into other works, adapted, modified, translated or distributed under a public license provided that ICH's copyright in the document is acknowledged at all times. In case of any adaption, modification or translation of the document, reasonable steps must be taken to clearly label, demarcate or otherwise identify that changes were made to or based on the original document. Any impression that the adaption, modification or translation of the original document is endorsed or sponsored by the ICH must be avoided.

The document is provided "as is" without warranty of any kind. In no event shall the ICH or the authors of the original document be liable for any claim, damages or other liability arising from the use of the document.

The above-mentioned permissions do not apply to content supplied by third parties. Therefore, for documents where the copyright vests in a third party, permission for reproduction must be obtained from this copyright holder.

94

## ICH HARMONISED GUIDELINE

95

# BIOANALYTICAL METHOD VALIDATION

96

## M10

97

ICH Consensus Guideline

### 98 TABLE OF CONTENTS

99	<b>1. INTRODUCTION.....</b>	<b>4</b>
100	1.1 Objective .....	4
101	1.2 Background .....	4
102	1.3 Scope .....	4
103	<b>2. GENERAL PRINCIPLES.....</b>	<b>5</b>
104	2.1 Method Development.....	5
105	2.2 Method Validation .....	6
106	2.2.1 Full Validation .....	6
107	2.2.2 Partial Validation .....	7
108	2.2.3 Cross Validation .....	7
109	<b>3. CHROMATOGRAPHY.....</b>	<b>7</b>
110	3.1 Reference Standards .....	7
111	3.2 Validation.....	8
112	3.2.1 Selectivity .....	8
113	3.2.2 Specificity .....	9
114	3.2.3 Matrix Effect .....	10
115	3.2.4 Calibration Curve and Range.....	10
116	3.2.5 Accuracy and Precision.....	11
117	3.2.5.1 Preparation of Quality Control Samples .....	11
118	3.2.5.2 Evaluation of Accuracy and Precision.....	12
119	3.2.6 Carry-over .....	12
120	3.2.7 Dilution Integrity .....	13
121	3.2.8 Stability .....	13

122	3.2.9 Reinjection Reproducibility .....	16
123	3.3 Study Sample Analysis .....	16
124	3.3.1 Analytical Run.....	16
125	3.3.2 Acceptance Criteria for an Analytical Run .....	17
126	3.3.3 Calibration Range.....	18
127	3.3.4 Reanalysis of Study Samples .....	19
128	3.3.5 Reinjection of Study Samples .....	20
129	3.3.6 Integration of Chromatograms .....	20
130	<b>4. LIGAND BINDING ASSAYS.....</b>	<b>21</b>
131	4.1 Key Reagents .....	21
132	4.1.1 Reference Standard .....	21
133	4.1.2 Critical Reagents .....	21
134	4.2 Validation.....	22
135	4.2.1 Specificity .....	22
136	4.2.2 Selectivity .....	23
137	4.2.3 Calibration Curve and Range.....	23
138	4.2.4 Accuracy and Precision.....	24
139	4.2.4.1 Preparation of Quality Control Samples .....	24
140	4.2.4.2 Evaluation of Accuracy and Precision.....	24
141	4.2.5 Carry-over .....	25
142	4.2.6 Dilution Linearity and Hook Effect.....	25
143	4.2.7 Stability .....	26
144	4.3 Study Sample Analysis .....	27
145	4.3.1 Analytical Run.....	27
146	4.3.2 Acceptance Criteria for an Analytical Run .....	27
147	4.3.3 Calibration Range.....	28
148	4.3.4 Reanalysis of Study Samples .....	29
149		
150	<b>5. INCURRED SAMPLE REANALYSIS .....</b>	<b>30</b>
151	<b>6. PARTIAL AND CROSS VALIDATION.....</b>	<b>31</b>
152	6.1 Partial Validation.....	31
153	6.2 Cross Validation.....	33

154	<b>7. ADDITIONAL CONSIDERATIONS.....</b>	<b>33</b>
155	7.1 Analytes that are also Endogenous Compounds .....	33
156	7.1.1 Quality Control Samples.....	35
157	7.1.2 Calibration Standards .....	36
158	7.1.3 Selectivity, Recovery and Matrix Effects .....	36
159	7.1.4 Parallelism .....	37
160	7.1.5 Accuracy and Precision.....	37
161	7.1.6 Stability .....	37
162	7.2 Parallelism .....	37
163	7.3 Recovery .....	38
164	7.4 Minimum Required Dilution .....	38
165	7.5 Commercial and Diagnostic Kits .....	38
166	7.6 New or Alternative Technologies .....	39
167	7.6.1 Dried Matrix Methods.....	40
168	<b>8. DOCUMENTATION.....</b>	<b>40</b>
169	8.1 Summary Information .....	41
170	8.2 Documentation for Validation and Bioanalytical Reports .....	41
171	<b>9. GLOSSARY .....</b>	<b>49</b>
172		
173		

## 174 **1. INTRODUCTION**

### 175 ***1.1 Objective***

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

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

### 186 ***1.2 Background***

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

### 194 ***1.3 Scope***

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

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

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

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

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

## 216 **2. GENERAL PRINCIPLES**

### 217 ***2.1 Method Development***

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

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

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

- 227 • Reference standards
- 228 • Critical reagents
- 229 • Calibration curve
- 230 • Quality control samples (QCs)
- 231 • Selectivity and specificity
- 232 • Sensitivity
- 233 • Accuracy



- 234 • Precision
- 235 • Recovery
- 236 • Stability of the analyte in the matrix
- 237 • Minimum Required Dilution (MRD)

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

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

## 243 ***2.2 Method Validation***

### 244 ***2.2.1 Full Validation***

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

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

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

262 The matrix used for analytical method validation should be the same as the matrix of the study  
263 samples, including anticoagulants and additives. In some cases, it may be difficult to obtain an  
264 identical matrix to that of the study samples (e.g., rare matrices such as tissue, cerebrospinal fluid,

265 bile). In such cases surrogate matrices may be acceptable for analytical method validation. The  
266 surrogate matrix should be selected and justified scientifically for use in the analytical method.

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

### 270 **2.2.2 Partial Validation**

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

### 275 **2.2.3 Cross Validation**

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

## 280 **3. CHROMATOGRAPHY**

### 281 **3.1 Reference Standards**

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

289 It is important that the reference standard is well characterised and the quality (purity, strength,  
290 identity) of the reference standard and the suitability of the IS is ensured, as the quality will affect  
291 the outcome of the analysis and, therefore, the study data. The reference standard used during  
292 validation and study sample analysis should be obtained from an authentic and traceable source.  
293 The reference standard should be identical to the analyte. If this is not possible, an established  
294 form (e.g., salt or hydrate) of known quality may be used.

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

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

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

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

## 310 ***3.2 Validation***

### 311 ***3.2.1 Selectivity***

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

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

318 The evaluation of selectivity should demonstrate that no significant response attributable to  
319 interfering components is observed at the retention time(s) of the analyte or the IS in the blank  
320 samples. Responses detected and attributable to interfering components should not be more than  
321 20% of the analyte response at the LLOQ and not more than 5% of the IS response in the LLOQ  
322 sample for each matrix.

323 For the investigation of selectivity in lipaemic matrices at least one source of matrix should be  
324 used. To be scientifically meaningful, the matrix used for these tests should be representative as  
325 much as possible of the expected study samples. A naturally lipaemic matrix with abnormally high  
326 levels of triglycerides should be obtained from donors. Although it is recommended to use lipaemic

327 matrix from donors, if this is difficult to obtain, it is acceptable to spike matrix with triglycerides  
328 even though it may not be representative of study samples. However, if the drug impacts lipid  
329 metabolism or if the intended patient population is hyperlipidaemic, the use of spiked samples is  
330 discouraged. This evaluation is not necessary for preclinical studies unless the drug impacts lipid  
331 metabolism or is administered in a particular animal strain that is hyperlipidaemic.

332 For the investigation of selectivity in haemolysed matrices at least one source of matrix should be  
333 used. Haemolysed matrices are obtained by spiking matrix with haemolysed whole blood (at least  
334 2% V/V) to generate a visibly detectable haemolysed sample.

### 335 **3.2.2 Specificity**

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

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

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

348 The possibility of back-conversion of a metabolite into the parent analyte during the successive  
349 steps of the analysis (including extraction procedures or in the MS source) should also be evaluated  
350 when relevant (i.e., potentially unstable metabolites such as ester analytes to ester/acidic  
351 metabolites, unstable N-oxides or glucuronide metabolites, lactone-ring structures). It is  
352 acknowledged that this evaluation will not be possible in the early stages of drug development of a  
353 new chemical entity when the metabolism is not yet evaluated. However, it is expected that this  
354 issue should be investigated and partial validation performed if needed. The extent of back-  
355 conversion, if any, should be established and the impact on the study results discussed in the  
356 Bioanalytical Report.

### 357 **3.2.3 Matrix Effect**

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

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

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

### 371 **3.2.4 Calibration Curve and Range**

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

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

382 A simple regression model that adequately describes the concentration-response relationship  
383 should be used. The selection of the regression model should be directed by written procedures.  
384 The regression model, weighting scheme and transformation should be determined during the  
385 method validation. Blank and zero samples should not be included in the determination of the  
386 regression equation for the calibration curve. Each calibration standard may be analysed in  
387 replicate, in which case data from all acceptable replicates should be used in the regression  
388 analysis.

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

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

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

### 408 ***3.2.5 Accuracy and Precision***

#### 409 ***3.2.5.1 Preparation of Quality Control Samples***

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

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

419 During method validation the QCs should be prepared at a minimum of 4 concentration levels  
420 within the calibration curve range: the LLOQ, within three times of the LLOQ (low QC), around 30 -  
421 50% of the calibration curve range (medium QC) and at least 75% of the ULOQ (high QC).

### 422 **3.2.5.2 Evaluation of Accuracy and Precision**

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

426 Within-run accuracy and precision should be evaluated by analysing at least 5 replicates at each  
427 QC concentration level in each analytical run. Between-run accuracy and precision should be  
428 evaluated by analysing each QC concentration level in at least 3 analytical runs over at least two  
429 days. To enable the evaluation of any trends over time within one run, it is recommended to  
430 demonstrate accuracy and precision of the QCs over at least one of the runs in a size equivalent to  
431 a prospective analytical run of study samples. Reported method validation data and the  
432 determination of accuracy and precision should include all results obtained, including individual QCs  
433 outside of the acceptance criteria, except those cases where errors are obvious and documented.  
434 Within-run accuracy and precision data should be reported for each run. If the within-run accuracy  
435 or precision criteria are not met in all runs, an overall estimate of within-run accuracy and precision  
436 for each QC level should be calculated. Between-run (intermediate) precision and accuracy should  
437 be calculated by combining the data from all runs.

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

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

### 445 **3.2.6 Carry-over**

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

448 Carry-over should be assessed and minimised during method development. During validation  
449 carry-over should be assessed by analysing blank samples after the calibration standard at the  
450 ULOQ. Carry-over in the blank samples following the highest calibration standard should not be  
451 greater than 20% of the analyte response at the LLOQ and 5% of the response for the IS. If it  
452 appears that carry-over is unavoidable, study samples should not be randomised. Specific  
453 measures should be considered, tested during the validation and applied during the analysis of the  
454 study samples, so that carry-over does not affect accuracy and precision. This could include the

455 injection of blank sample(s) after samples with an expected high concentration, before the next  
456 study sample.

### 457 ***3.2.7 Dilution Integrity***

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

462 Dilution QCs should be prepared with analyte concentrations in matrix that are greater than the  
463 ULOQ and then diluted with blank matrix. At least 5 replicates per dilution factor should be tested  
464 in one run to determine if concentrations are accurately and precisely measured within the  
465 calibration range. The dilution ratio(s) applied during study sample analysis should be within the  
466 range of the dilution ratios evaluated during validation. The mean accuracy of the dilution QCs  
467 should be within  $\pm 15\%$  of the nominal concentration and the precision (%CV) should not exceed  
468 15%.

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

### 471 ***3.2.8 Stability***

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

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

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

484 The stability QCs are analysed against a calibration curve, obtained from freshly spiked calibration  
485 standards in a run with its corresponding freshly prepared QCs or QCs for which stability has been  
486 proven. The mean concentration at each QC level should be within  $\pm 15\%$  of the nominal



487 concentration. If the concentrations of the study samples are consistently higher than the ULOQ of  
488 the calibration range, the concentration of the high stability QC should be adjusted to reflect these  
489 higher concentrations. It is recognised that this may not be possible in nonclinical studies due to  
490 solubility limitations.

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

494 The following stability tests should be evaluated:

495 1) Stability of stock and working solutions

496 The stability of the stock and working solutions of the analyte and IS should be determined  
497 under the storage conditions used during the analysis of study samples by using the lowest and  
498 the highest concentrations of these solutions. They are assessed using the response of the  
499 detector. Stability of the stock and working solutions should be tested with an appropriate  
500 dilution, taking into consideration the linearity and measuring range of the detector. If the  
501 stability varies with concentration, then the stability of all concentrations of the stock and  
502 working solutions needs to be assessed. If no isotopic exchange occurs for the stable isotope-  
503 labelled IS under the same storage conditions as the analyte for which the stability is  
504 demonstrated, then no additional stability determinations for the IS are necessary. If the  
505 reference standard expires, or it is past the retest date, the stability of the stock solutions  
506 made previously with this lot of reference standard are defined by the expiration or retest date  
507 established for the stock solution. The routine practice of making stock and working solutions  
508 from reference standards solely for extending the expiry date for the use of the reference  
509 standard is not acceptable.

510 2) Freeze-thaw matrix stability

511 To assess the impact of repeatedly removing samples from frozen storage, the stability of the  
512 analyte should be assessed after multiple cycles of freezing and thawing. Low and high stability  
513 QCs should be thawed and analysed according to the same procedures as the study samples.  
514 Stability QCs should be kept frozen for at least 12 hours between the thawing cycles. Stability  
515 QCs for freeze-thaw stability should be assessed using freshly prepared calibration standards  
516 and QCs or QCs for which stability has been proven. The number of freeze-thaw cycles  
517 validated should equal or exceed that of the freeze-thaw cycles undergone by the study  
518 samples, but a minimum of three cycles should be conducted.

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

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

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

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

528 4) Processed sample stability

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

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

535 5) Long-term matrix stability

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

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

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

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

546 6) Whole blood stability

547 Sufficient attention should be paid to the stability of the analyte in the sampled matrix (blood)  
548 directly after collection from subjects and prior to preparation for storage to ensure that the

549 concentrations obtained by the analytical method reflect the concentrations of the analyte in  
550 the subject's blood at the time of sample collection.

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

### 554 **3.2.9 Reinjection Reproducibility**

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

## 560 **3.3 Study Sample Analysis**

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

### 571 **3.3.1 Analytical Run**

572 An analytical run consists of a blank sample (processed matrix sample without analyte and without  
573 IS), a zero sample (processed matrix with IS), calibration standards at a minimum of 6  
574 concentration levels, at least 3 levels of QCs (low, medium and high) in duplicate (or at least 5% of  
575 the number of study samples, whichever is higher) and the study samples to be analysed. The QCs  
576 should be divided over the run in such a way that the accuracy and precision of the whole run is  
577 ensured. Study samples should always be bracketed by QCs.

578 The calibration standards and QCs should be spiked independently using separately prepared stock  
579 solutions, unless the accuracy and stability of the stock solutions have been verified. All samples  
580 (calibration standards, QCs and study samples) should be processed and extracted as one single  
581 batch of samples in the order in which they are intended to be analysed. A single batch is

582 comprised of study samples and QCs which are handled during a fixed period of time and by the  
583 same group of analysts with the same reagents under homogeneous conditions. Analysing samples  
584 that were processed as several separate batches in a single analytical run is discouraged. If such  
585 an approach cannot be avoided, for instance due to bench top stability limitations, each batch of  
586 samples should include low, medium and high QCs.

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

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

### 596 ***3.3.2 Acceptance Criteria for an Analytical Run***

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

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

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

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

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

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

631 The back-calculated concentrations of the calibration standards and QCs of passed and accepted  
632 runs should be reported. The overall (between-run) accuracy and precision of the QCs of all  
633 accepted runs should be calculated at each concentration level and reported in the analytical report  
634 (Refer to Section 8 Documentation and Table 1). If the overall mean accuracy or precision fails the  
635 15% criterion, an investigation to determine the cause of the deviation should be conducted. In the  
636 case of comparative BA/BE studies it may result in the rejection of the data.

### 637 ***3.3.3 Calibration Range***

638 If a narrow range of analyte concentrations of the study samples is known or anticipated before the  
639 start of study sample analysis, it is recommended to either narrow the calibration curve range,  
640 adapt the concentrations of the QCs, or add new QCs at different concentration levels as  
641 appropriate, to adequately reflect the concentrations of the study samples.

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

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

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

### 656 ***3.3.4 Reanalysis of Study Samples***

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

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

662 Some examples of reasons for study sample reanalysis are:

- 663 • Rejection of an analytical run because the run failed the acceptance criteria with regard  
664 to accuracy of the calibration standards and/or the precision and accuracy of the QCs
- 665 • IS response significantly different from the response for the calibration standards and  
666 QCs (as pre-defined in an SOP)
- 667 • The concentration obtained is above the ULOQ
- 668 • The concentration observed is below the revised LLOQ in runs where the lowest  
669 calibration standard has been rejected from a calibration curve, resulting in a higher  
670 LLOQ compared with other runs
- 671 • Improper sample injection or malfunction of equipment
- 672 • The diluted study sample is below the LLOQ
- 673 • Identification of quantifiable analyte levels in pre-dose samples, control or placebo  
674 samples
- 675 • Poor chromatography (as pre-defined in an SOP)

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

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

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

### 690 ***3.3.5 Reinjection of Study Samples***

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

### 696 ***3.3.6 Integration of Chromatograms***

697 Chromatogram integration and reintegration should be described in a study plan, protocol or SOP.  
698 Any deviation from the procedures described *a priori* should be discussed in the Bioanalytical  
699 Report. The list of chromatograms that required reintegration, including any manual integrations,  
700 and the reasons for reintegration should be included in the Bioanalytical Report. Original and  
701 reintegrated chromatograms and initial and repeat integration results should be kept for future  
702 reference and submitted in the Bioanalytical Report for comparative BA/BE studies.

## 703 **4. LIGAND BINDING ASSAYS**

### 704 ***4.1 Key Reagents***

#### 705 ***4.1.1 Reference Standard***

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

#### 714 ***4.1.2 Critical Reagents***

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

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

725 A critical reagent lifecycle management procedure is necessary to ensure consistency between the  
726 original and new batches of critical reagents. Reagent performance should be evaluated using the  
727 bioanalytical assay. Minor changes to critical reagents would not be expected to influence the assay  
728 performance, whereas major changes may significantly impact the performance. If the change is  
729 minor (e.g., the source of one reagent is changed), a single comparative accuracy and precision  
730 assessment is sufficient for characterisation. If the change is major, then additional validation  
731 experiments are necessary. Ideally, assessment of changes will compare the assay with the new  
732 reagents to the assay with the old reagents directly. Major changes include, but are not limited to,  
733 change in production method of antibodies, additional blood collection from animals for polyclonal  
734 antibodies and new clones or new supplier for monoclonal antibody production.



735 Retest dates and validation parameters should be documented in order to support the extension or  
736 replacement of the critical reagent. Stability testing of the reagents should be based upon the  
737 performance in the bioanalytical assay and be based upon general guidance for reagent storage  
738 conditions and can be extended beyond the expiry date from the supplier. The performance  
739 parameters should be documented in order to support the extension or replacement of the critical  
740 reagent.

## 741 **4.2 Validation**

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

### 750 **4.2.1 Specificity**

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

753 The accuracy of the target analyte at the LLOQ and at the ULOQ should be investigated in the  
754 presence of related molecules at the maximal concentration(s) anticipated in study samples.  
755 The response of blank samples spiked with related molecules should be below the LLOQ. The  
756 accuracy of the target analyte in presence of related molecules should be within  $\pm 25\%$  of the  
757 nominal values.

758 In the event of non-specificity, the impact on the method should be evaluated by spiking  
759 increasing concentrations of interfering molecules in blank matrix and measuring the  
760 accuracy of the target analyte at the LLOQ and ULOQ. It is essential to determine the  
761 minimum concentration of the related molecule where interference occurs. Appropriate  
762 mitigation during sample analysis should be employed, e.g., it may be necessary to adjust the  
763 LLOQ/ULOQ accordingly or consider a new method.

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

### 767 **4.2.2 Selectivity**

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

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

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

779 Selectivity should be evaluated in lipaemic samples and haemolysed samples (Refer to Section  
780 3.2.1). For lipaemic and haemolysed samples, tests can be evaluated once using a single source of  
781 matrix. Selectivity should be assessed in samples from relevant patient populations. In the case of  
782 relevant patient populations there should be at least five individual patients.

### 783 **4.2.3 Calibration Curve and Range**

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

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

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

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

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

#### 809 ***4.2.4 Accuracy and Precision***

##### 810 ***4.2.4.1 Preparation of Quality Control Samples***

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

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

##### 820 ***4.2.4.2 Evaluation of Accuracy and Precision***

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

824 Accuracy and precision should be determined by analysing at least 3 replicates per run at each QC  
825 concentration level (LLOQ, low, medium, high, ULOQ) in at least 6 runs over 2 or more days.  
826 Reported method validation data and the determination of accuracy and precision should include all  
827 results obtained, except those cases where errors are obvious and documented. Within-run  
828 accuracy and precision data should be reported for each run. If the within-run accuracy or precision  
829 criteria are not met in all runs, an overall estimate of within-run accuracy and precision for each

830 QC level should be calculated. Between-run (intermediate) precision and accuracy should be  
831 calculated by combining the data from all runs.

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

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

#### 838 ***4.2.5 Carry-over***

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

#### 842 ***4.2.6 Dilution Linearity and Hook Effect***

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

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

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

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

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

#### 862 **4.2.7 Stability**

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

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

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

875 The stability QCs are analysed against a calibration curve, obtained from freshly spiked calibration  
876 standards in a run with its corresponding freshly prepared QCs or QCs for which stability has been  
877 proven. While the use of freshly prepared calibration standards and QCs is the preferred approach,  
878 it is recognised that in some cases, for macromolecules, it may be necessary to freeze them  
879 overnight. In such cases, valid justification should be provided and freeze-thaw stability  
880 demonstrated. The mean concentration at each level should be within  $\pm 20\%$  of the nominal  
881 concentration.

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

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

889 For chemical drugs, it is considered acceptable to extrapolate the stability at one temperature  
890 (e.g.,  $-20^{\circ}\text{C}$ ) to lower temperatures (e.g.,  $-70^{\circ}\text{C}$ ).

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

### 894 **4.3 Study Sample Analysis**

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

#### 901 ***4.3.1 Analytical Run***

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

908 Most often microtitre plates are used for LBAs. An analytical run may comprise of one or more  
909 plate(s). Typically, each plate contains an individual set of calibration standards and QCs. If each  
910 plate contains its own calibration standards and QCs then each plate should be assessed on its  
911 own. However, for some platforms the sample capacity may be limited. In this case, sets of  
912 calibration standards may be placed on the first and the last plate, but QCs should be placed on  
913 every single plate. QCs should be placed at least at the beginning (before) and at the end (after) of  
914 the study samples of each plate. The QCs on each plate and each calibration curve should fulfil the  
915 acceptance criteria (Refer to Section 4.3.2). For the calculation of concentrations, the calibration  
916 standards should be combined to conduct one regression analysis. If the combined calibration  
917 curve does not pass the acceptance criteria the whole run fails.

#### 918 ***4.3.2 Acceptance Criteria for an Analytical Run***

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

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

931 If the rejected calibration standard is the LLOQ, the new lower limit for this analytical run is the  
932 next lowest acceptable calibration standard of the calibration curve. If the highest calibration  
933 standard is rejected, the new upper limit for this analytical run is the next acceptable highest  
934 calibration standard of the calibration curve. The new lower and upper limit calibration standard will  
935 retain their original acceptance criteria (i.e.,  $\pm 20\%$ ). The revised calibration range should cover all  
936 QCs (low, medium and high). The study samples outside of the revised assay range should be  
937 reanalysed.

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

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

### 948 ***4.3.3 Calibration Range***

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

#### 956 **4.3.4 Reanalysis of Study Samples**

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

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

962 Some examples of reasons for study sample reanalysis are:

- 963 • Rejection of an analytical run because the run failed the acceptance criteria with regard  
964 to accuracy of the calibration standards and/or the precision and accuracy of the QCs,
- 965 • The concentration obtained is above the ULOQ
- 966 • The concentration obtained is below the LLOQ in runs where the lowest calibration  
967 standard has been rejected from a calibration curve, resulting in a higher LLOQ compared  
968 with other runs
- 969 • Malfunction of equipment
- 970 • The diluted sample is below the LLOQ
- 971 • Identification of quantifiable analyte levels in pre-dose samples, control or placebo  
972 samples.
- 973 • When samples are analysed in more than one well and non-reportable values are  
974 obtained due to one replicate failing the pre-defined acceptance criteria (e.g., excessive  
975 variability between wells, one replicate being above the ULOQ or below the LLOQ).

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

979 The reanalysed samples should be identified in the Bioanalytical Report and the initial value, the  
980 reason for reanalysis, the values obtained in the reanalyses, the final accepted value and a  
981 justification for the acceptance should be provided. Further, a summary table of the total number  
982 of samples that have been reanalysed due to each reason should be provided. In cases where the  
983 first analysis yields a non-reportable result, a single reanalysis is considered sufficient (e.g.,  
984 concentration above the ULOQ or excessive variability between wells). The analysis of the samples  
985 should be based on the same number of wells per study sample as in the initial analysis. In cases



986 where the value needs to be confirmed, (e.g., pre-dose sample with measurable concentrations)  
987 multiple determinations are required where sample volume allows.

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

## 991 **5. INCURRED SAMPLE REANALYSIS (ISR)**

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

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

1000 ISR should be performed at least in the following situations:

- 1001 • For preclinical studies, ISR should, in general, be performed for the main nonclinical TK  
1002 studies once per species. However, ISR in a PK study instead of a TK study might also be  
1003 acceptable, as long as the respective study has been conducted as a pivotal study, used  
1004 to make regulatory decisions.
- 1005 • All pivotal comparative BA/BE studies
- 1006 • First clinical trial in subjects
- 1007 • Pivotal early patient trial(s), once per patient population
- 1008 • First or pivotal trial in patients with impaired hepatic and/or renal function

1009 ISR is conducted by repeating the analysis of a subset of samples from a given study in separate  
1010 (i.e., different to the original) runs on different days using the same bioanalytical method.

1011 The extent of ISR depends upon the analyte and the study samples and should be based upon an  
1012 in-depth understanding of the analytical method and analyte. However, as a minimum, if the total  
1013 number of study samples is less than 1000, then 10% of the samples should be reanalysed; if the  
1014 total number of samples is greater than 1000, then 10% of the first 1000 samples (100) plus 5%  
1015 of the number of samples that exceed 1000 samples should be assessed. Objective criteria for

1016 choosing the subset of study samples for ISR should be predefined in the protocol, study plan or an  
1017 SOP. While the subjects should be picked as randomly as possible from the dosed study population,  
1018 adequate coverage of the PK profile in its entirety is important. Therefore, it is recommended that  
1019 the samples for ISR be chosen around the maximum concentration (C<sub>max</sub>) and some in the  
1020 elimination phase. Additionally, the samples chosen should be representative of the whole study.

1021 Samples should not be pooled, as pooling may limit anomalous findings. ISR samples and QCs  
1022 should be prepared in the same manner as in the original analysis. ISR should be performed within  
1023 the stability window of the analyte, but not on the same day as the original analysis.

1024 The percent difference between the initial concentration and the concentration measured during the  
1025 repeat analysis should be calculated in relation to their mean value using the following equation:

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

1026 For chromatographic methods, the percent difference should be ≤ 20% for at least 2/3 of the  
1027 repeats. For LBAs, the percent difference should be ≤ 30% for at least 2/3 of the repeats.

1028 If the overall ISR results fail the acceptance criteria, an investigation should be conducted and the  
1029 causes remediated. There should be an SOP that directs how investigations are triggered and  
1030 conducted. If an investigation does not identify the cause of the failure, the potential impact of an  
1031 ISR failure on study validity should also be provided in the Bioanalytical Report. If ISR meets the  
1032 acceptance criteria yet shows large or systemic differences between results for multiple samples,  
1033 this may indicate analytical issues and it is advisable to investigate this further.

1034 Examples of trends that are of concern include:

- 1035 • All samples from one subject fail
- 1036 • All of samples from one run fail

1037 All aspects of ISR evaluations should be documented to allow reconstruction of the study and any  
1038 investigations. Individual samples that are quite different from the original value (e.g., > 50%,  
1039 “flyers”) should not trigger reanalysis of the original sample and do not need to be investigated.  
1040 ISR sample data should not replace the original study sample data.

## 1041 **6. PARTIAL AND CROSS VALIDATION**

### 1042 ***6.1 Partial Validation***

1043 Partial validations evaluate modifications to already fully validated bioanalytical methods. Partial  
1044 validation can range from as little as one within-run accuracy and precision determination, to a

1045 nearly full validation. If stability is established at one facility it does not necessarily need to be  
 1046 repeated at another facility.

1047 For chromatographic methods, typical bioanalytical method modifications or changes that fall into  
 1048 this category include, but are not limited to, the following situations:

- 1049 • Analytical site change using same method (i.e., bioanalytical method transfers between  
 1050 laboratories)
- 1051 • A change in analytical methodology (e.g., change in detection systems, platform)
- 1052 • A change in sample processing procedures
- 1053 • A change in sample volume (e.g., the smaller volume of paediatric samples)
- 1054 • Changes to the calibration concentration range
- 1055 • A change in anticoagulant (but not changes in the counter-ion) in biological fluids (e.g., heparin  
 1056 to ethylenediaminetetraacetic acid (EDTA))
- 1057 • Change from one matrix within a species to another (e.g., switching from human plasma to  
 1058 serum or cerebrospinal fluid) or changes to the species within the matrix (e.g., switching from  
 1059 rat plasma to mouse plasma)
- 1060 • A change in storage conditions

1061 For LBAs, typical bioanalytical method modifications or changes that fall into this category include,  
 1062 but are not limited to, the following situations:

- 1063 • Changes in LBA critical reagents (e.g., lot-to-lot changes)
- 1064 • Changes in MRD
- 1065 • A change in storage conditions
- 1066 • Changes to the calibration concentration range
- 1067 • A change in analytical methodology (e.g., change in detection systems, platform)
- 1068 • Analytical site change using same method (i.e., bioanalytical method transfers between  
 1069 laboratories)
- 1070 • A change in sample preparation

1071 Partial validations are acceptable if the parameters tested meet the full validation criteria. If these  
 1072 criteria are not satisfied, additional investigation and validation is warranted.

## 1073 **6.2 Cross Validation**

1074 Cross validation is required to compare data under the following situations:

- 1075 • Data are obtained from different fully validated methods within a study
- 1076 • Data are obtained from different fully validated methods across studies that are going to be  
1077 combined or compared to support special dosing regimens, or regulatory decisions regarding  
1078 safety, efficacy and labelling.
- 1079 • Data are obtained within a study from different laboratories with the same bioanalytical  
1080 method.

1081 Cross validation is not generally required to compare data obtained across studies from different  
1082 laboratories using the same validated method at each site.

1083 Cross validation should be performed in advance of study samples being analysed, if possible.

1084 Cross validation should be assessed by measuring the same set of QCs (low, medium and high) in  
1085 triplicate and study samples that span the study sample concentration range (if available  $n \geq 30$ )  
1086 with both assays or in both laboratories.

1087 Bias can be assessed by Bland-Altman plots or Deming regression. Other methods appropriate for  
1088 assessing agreement between two assays (e.g., concordance correlation coefficient) may be used  
1089 too. Alternatively, the concentration vs. time curves for incurred samples could be plotted for  
1090 samples analysed by each method to assess bias. If disproportionate bias is observed between  
1091 methods, the impact on the clinical data interpretation should be assessed.

1092 The use of multiple bioanalytical methods in the conduct of one comparative BA/BE study is  
1093 strongly discouraged.

## 1094 **7. ADDITIONAL CONSIDERATIONS**

### 1095 **7.1 Analytes that are also Endogenous Compounds**

1096 For analytes that are also endogenous compounds, the accuracy of the measurement of the  
1097 analytes poses a challenge when the assay cannot distinguish between the therapeutic agent and  
1098 the endogenous counterpart.

1099 The endogenous levels may vary because of age, gender, diurnal variations, illness or as a side  
1100 effect of drug treatment. If available, biological matrix with an adequate signal-to-noise ratio (i.e.,  
1101 endogenous level sufficiently low for the desired LLOQ, e.g., <20% of the LLOQ) should be used as  
1102 blank matrix to prepare calibration standards and QCs since the biological matrix used to prepare

1103 calibration standards and QCs should be the same as the study samples (i.e., authentic biological  
1104 matrix) and should be free of matrix effect and endogenous analyte at the level that causes  
1105 interference.

1106 In those cases where matrices without interference are not available, there are four possible  
1107 approaches to calculate the concentration of the endogenous analyte in calibration standards, QCs  
1108 and, consequently, study samples: 1) the standard addition approach, 2) the background  
1109 subtraction approach, 3) the surrogate matrix (neat, artificial or stripped matrices) approach and  
1110 4) the surrogate analyte approach.

1111 1) Standard Addition Approach:

1112 Every study sample is divided into aliquots of equal volume. All aliquots, but one, are  
1113 separately spiked with known and varying amounts of the analyte standards to  
1114 construct a calibration curve for every study sample. The study sample concentration  
1115 is then determined as the negative x-intercept of the standard calibration curve  
1116 prepared in that particular study sample.

## 1117 2) Background Subtraction Approach:

1118 The endogenous background concentrations of analytes in a pooled/representative  
1119 matrix are subtracted from the concentrations of the added standards, subsequently  
1120 the subtracted concentrations are used to construct the calibration curve.

## 1121 3) Surrogate Matrix Approach:

1122 The matrix of the study samples is substituted by a surrogate matrix. Surrogate  
1123 matrices can vary widely in complexity from simple buffers or artificial matrices that  
1124 try to mimic the authentic one, to stripped matrices.

## 1125 4) Surrogate Analyte Approach:

1126 Stable-isotope labelled analytes are used as surrogate standards to construct the  
1127 calibration curves for the quantification of endogenous analytes. In this method it is  
1128 assumed that the physicochemical properties of the authentic and surrogates  
1129 analytes are the same with the exception of molecular weight. However, isotope  
1130 standards may differ in retention time and MS sensitivity, therefore, before  
1131 application of this approach, the ratio of the labelled to unlabelled analyte MS  
1132 responses (i.e., the response factor) should be close to unity and constant over the  
1133 entire calibration range. If the response factor does not comply with these  
1134 requirements, it should be incorporated into the regression equation of the  
1135 calibration curve.

1136 Validation of an analytical method for an analyte that is also an endogenous compound will require  
1137 the following considerations.

1138 **7.1.1 Quality Control Samples**

1139 The endogenous concentrations of the analyte in the biological matrix should be evaluated prior to  
1140 QC preparation (e.g., by replicate analysis). The blank matrices with the minimum level of the  
1141 endogenous analyte should be used. The concentrations of the QCs should account for the  
1142 endogenous concentrations in the biological matrix (i.e., additive) and be representative of the  
1143 expected study concentrations.

1144 The QCs used for validation should be aliquots of the authentic biological matrix unspiked and  
1145 spiked with known amounts of the authentic analyte. In spiked samples, the added amount should  
1146 be enough to provide concentrations that are statistically different from the endogenous  
1147 concentration.

### 1148 **7.1.2 Calibration Standards**

1149 In the Surrogate Matrix and Surrogate Analyte Approaches, these surrogates should be used only  
1150 for the preparation of the calibration standards.

1151 In the Standard Addition and Background Subtraction Approaches the same biological matrix and  
1152 analyte as the study samples is used to prepare the calibration standards. However, when the  
1153 background concentrations are lowered by dilution of the blank matrices before spiking with the  
1154 standards (e.g., if a lower LLOQ is required in the Background Subtraction Approach) the  
1155 composition of the matrices in the study samples and the calibration standards is different, which  
1156 may cause different recoveries and matrix effects.

### 1157 **7.1.3 Selectivity, Recovery and Matrix Effects**

1158 The assessment of selectivity is complicated by the absence of interference-free matrix. For  
1159 chromatography, peak purity should be investigated as part of method validation by analysing  
1160 matrices obtained from several donors using a discriminative detection system (e.g., tandem mass  
1161 spectrometry (MS/MS)). Other approaches, if justified by scientific principles, may also be  
1162 considered.

1163 For the Standard Addition and Background Subtraction Approaches, as the same biological matrix  
1164 and analyte are used for study samples and calibration standards, the same recovery and matrix  
1165 effect occurs in the study samples and the calibration standards. For the Surrogate Matrix and  
1166 Surrogate Analyte Approaches, the matrix effect and the extraction recovery may differ between  
1167 calibration standards and study samples.

1168       • If the Surrogate Matrix Approach is used, demonstration of similar matrix effect and  
1169 extraction recovery in both the surrogate and original matrix is required. This should  
1170 be investigated in an experiment using QCs spiked with analyte in the matrix against  
1171 the surrogate calibration curve and should be within  $\pm 15\%$  for chromatographic  
1172 assays and within  $\pm 20\%$  for LBA assays.

1173       • If the Surrogate Analyte Approach is used, demonstration of similarity in matrix effect  
1174 and recovery between surrogate and authentic endogenous analytes is required. This  
1175 should be investigated in an experiment within  $\pm 15\%$  for chromatographic assays and  
1176 within  $\pm 20\%$  for LBA assays.

1177 Since the composition of the biological matrix might affect method performance, it is necessary to  
1178 investigate matrices from different donors, except in the Standard Addition Approach, where each  
1179 sample is analysed with its own calibration curve.

#### 1180 **7.1.4 Parallelism**

1181 Parallelism should be evaluated in the Surrogate Matrix and Surrogate Analyte Approaches by  
1182 means of the Standard Addition approach, spike recovery or dilutional linearity.

#### 1183 **7.1.5 Accuracy and Precision**

1184 In case of using a surrogate matrix or analyte, the assessment of accuracy and precision should be  
1185 performed by analysing the QCs against the surrogate calibration curve. In certain cases, dilution  
1186 of the QCs with surrogate matrix may be necessary. These experiments should be repeated with  
1187 authentic biological matrices from different donors to address variability due to the matrix. Analysis  
1188 of the unspiked QCs will give the mean endogenous background concentration and only precision  
1189 and no accuracy can be determined for this QCs.

1190 The concentration of the endogenous substance in the blank sample may be determined and  
1191 subtracted from the total concentrations observed in the spiked samples. Accuracy is  
1192 recommended to be calculated using the following formula:

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

#### 1193 **7.1.6 Stability**

1194 In order to mimic study samples as much as possible, stability experiments should be investigated  
1195 with the authentic analyte in the authentic biological matrix and with unspiked and spiked samples.  
1196 However, if a surrogate matrix is used for calibration standards, stability should also be  
1197 demonstrated for the analyte in the surrogate matrix, as this could differ from stability in the  
1198 authentic biological matrix.

#### 1199 **7.2 Parallelism**

1200 Parallelism is defined as a parallel relationship between the calibration curve and serially diluted  
1201 study samples to detect any influence of dilution on analyte measurement. Although lack of  
1202 parallelism is a rare occurrence for PK assays, parallelism of LBA should be evaluated on a case-by-  
1203 case basis, e.g., where interference caused by a matrix component (e.g., presence of endogenous  
1204 binding protein) is suspected during study sample analysis. Parallelism investigation or the  
1205 justification for its absence should be included in the Bioanalytical Report. As parallelism  
1206 assessments are rarely possible during method development and method validation due to the  
1207 unavailability of study samples and parallelism is strictly linked to the study samples (i.e., an assay  
1208 may have perfectly suitable parallelism for a certain population of samples, yet lack it for another  
1209 population), these experiments should be conducted during the analysis of the study samples. A



1210 high concentration study sample (preferably close to C<sub>max</sub>) should be diluted to at least three  
1211 concentrations with blank matrix. The precision between samples in a dilution series should not  
1212 exceed 30%. However, when applying the 30% criterion, data should be carefully monitored as  
1213 results that pass this criterion may still reveal trends of non-parallelism. In the case that the  
1214 sample does not dilute linearly (i.e., in a non-parallel manner), a procedure for reporting a result  
1215 should be defined *a priori*.

### 1216 **7.3 Recovery**

1217 For methods that employ sample extraction, the recovery (extraction efficiency) should be  
1218 evaluated. Recovery is reported as a percentage of the known amount of an analyte carried  
1219 through the sample extraction and processing steps of the method. Recovery is determined by  
1220 comparing the analyte response in a biological sample that is spiked with the analyte and  
1221 processed, with the response in a biological blank sample that is processed and then spiked with  
1222 the analyte. Recovery of the analyte does not need to be 100%, but the extent of recovery of an  
1223 analyte and of the IS (if used) should be consistent. Recovery experiments are recommended to be  
1224 performed by comparing the analytical results for extracted samples at multiple concentrations,  
1225 typically three concentrations (low, medium and high).

### 1226 **7.4 Minimum Required Dilution**

1227 MRD is a dilution factor employed in samples that are diluted with buffer solution to reduce the  
1228 background signal or matrix interference on the analysis using LBA. The MRD should be identical  
1229 for all samples including calibration standards and the QCs and it should be determined during  
1230 method development. If MRD is changed after establishment of the method, partial validation is  
1231 necessary. MRD should be defined in the Validation Report of the analytical method.

### 1232 **7.5 Commercial and Diagnostic Kits**

1233 Commercial or diagnostic kits (referred to as kits) are sometimes co-developed with new drugs or  
1234 therapeutic biological products for point-of-care patient diagnosis. The recommendations in this  
1235 section of the guideline do not apply to the development of kits that are intended for point-of-care  
1236 patient diagnosis (e.g., companion or complimentary diagnostic kits). Refer to the appropriate  
1237 guideline documents regarding regulatory expectations for the development of these kits.

1238 If an applicant repurposes a kit (instead of developing a new assay) or utilises "research use only"  
1239 kits to measure chemical or biological drug concentrations during the development of a novel drug,  
1240 the applicant should assess the kit validation to ensure that it conforms to the drug development  
1241 standards described in this guideline.

1242 Validation considerations for kit assays include, but are not limited to, the following:

- 1243 • If the reference standard in the kit differs from that of the study samples, testing should  
1244 evaluate differences in assay performance of the kit reagents. The specificity, accuracy,  
1245 precision and stability of the assay should be demonstrated under actual conditions of  
1246 use in the facility conducting the sample analysis. Modifications from kit processing  
1247 instructions should be completely validated.
- 1248 • Kits that use sparse calibration standards (e.g., one- or two-point calibration curves)  
1249 should include in-house validation experiments to establish the calibration curve with a  
1250 sufficient number of standards across the calibration range.
- 1251 • Actual QC concentrations should be known. Concentrations of QCs expressed as ranges  
1252 are not sufficient for quantitative applications. In such cases QCs with known  
1253 concentrations should be prepared and used, independent of the kit-supplied QCs.
- 1254 • Calibration standards and QCs should be prepared in the same matrix as the study  
1255 samples. Kits with calibration standards and QCs prepared in a matrix different from the  
1256 study samples should be justified and appropriate experiments should be performed.
- 1257 • If multiple kit lots are used within a study, lot-to-lot variability and comparability should  
1258 be addressed for any critical reagents included in the kits.
- 1259 • If a kit using multiple assay plates is employed, sufficient replicate QCs should be used on  
1260 each plate to monitor the accuracy of the assay. Acceptance criteria should be  
1261 established for the individual plates and for the overall analytical run.

## 1262 ***7.6 New or Alternative Technologies***

1263 When a new or alternative technology is used as the sole bioanalytical technology from the onset of  
1264 drug development, cross validation with an existing technology is not required.

1265 The use of two different bioanalytical technologies for the development of a drug may generate  
1266 data for the same product that could be difficult to interpret. This outcome can occur when one  
1267 platform generates drug concentrations that differ from those obtained with another platform.  
1268 Therefore, when a new or alternative analytical platform is replacing a previous platform used in  
1269 the development of a drug it is important that the potential differences are well understood. The  
1270 data generated from the previous platform/technology should be cross validated to that of the new  
1271 or alternative platform/technology. Seeking feedback from the regulatory authorities is encouraged  
1272 early in drug development. The use of two methods or technologies within a comparative BA/BE  
1273 study is strongly discouraged.

1274 The use of new technology in regulated bioanalysis should be supported by acceptance criteria  
1275 established *a priori* based on method development and verified in validation.

### 1276 **7.6.1 Dried Matrix Methods**

1277 Dried matrix methods (DMM) is a sampling methodology that offers benefits such as collection of  
1278 reduced blood sample volumes as a microsampling technique for drug analysis and ease of  
1279 collection, storage and transportation. In addition to the typical methodological validation for LC-  
1280 MS or LBA, use of DMM necessitates further validation of this sampling approach before using DMM  
1281 in studies that support a regulatory application, such as:

- 1282 • Haematocrit (especially for spotting of whole blood into cards)
- 1283 • Sample homogeneity (especially for sub-punch of the sample on the card/device)
- 1284 • Reconstitution of the sample
- 1285 • DMM sample collection for ISR
  - 1286 ○ Care should be taken to ensure sufficient sample volumes or numbers of  
1287 replicates are retained for ISR
  - 1288 ○ Should be assessed by multiple punches of the sample or samples should be  
1289 taken in duplicate

1290 When DMM is used for clinical or nonclinical studies in addition to typical liquid approaches (e.g.,  
1291 liquid plasma samples) in the same studies, these two methods should be cross validated as  
1292 described (Refer to Section 6.2). For nonclinical TK studies, refer to Section 4.1 of ICH S3A Q&A.  
1293 Feedback from the appropriate regulatory authorities is encouraged in early drug development.

## 1294 **8. DOCUMENTATION**

1295 General and specific SOPs and good record keeping are essential to a properly validated analytical  
1296 method. The data generated for bioanalytical method validation should be documented and  
1297 available for data audit and inspection. Table 1 describes the recommended documentation for  
1298 submission to the regulatory authorities and documentation that should be available at the  
1299 analytical site at times of inspection. This documentation may be stored at the analytical site or at  
1300 another secure location. In this case the documentation should be readily available when  
1301 requested.

1302 All relevant documentation necessary for reconstructing the study as it was conducted and  
1303 reported should be maintained in a secure environment. Relevant documentation includes, but is

1304 not limited to, source data, protocols and reports, records supporting procedural, operational, and  
1305 environmental concerns and correspondence records between all involved parties.

1306 Regardless of the documentation format (i.e., paper or electronic), records should be  
1307 contemporaneous with the event and subsequent alterations should not obscure the original data.  
1308 The basis for changing or reprocessing data should be documented with sufficient detail, and the  
1309 original record should be maintained. Transcripts/copies of data derived from analyses in  
1310 biohazardous areas should be maintained if applicable.

### 1311 ***8.1 Summary Information***

1312 Summary information should include the following items in Section 2.6.4/2.7.1 of the Common  
1313 Technical Document (CTD) or reports:

- 1314 • A summary of assay methods used for each study should be included. Each summary  
1315 should provide the protocol number, the assay type, the assay method identification  
1316 code, the Bioanalytical Report code, effective date of the method, and the associated  
1317 Validation Report codes.
- 1318 • A summary table of all the relevant Validation Reports should be provided for each  
1319 analyte, including Partial Validation and Cross Validation Reports. The table should  
1320 include the assay method identification code, the type of assay, the reason for the new  
1321 method or additional validation (e.g., to lower the limit of quantification). Changes  
1322 made to the method should be clearly identified.
- 1323 • A summary table cross-referencing multiple identification codes should be provided  
1324 when an assay has different codes for the assay method, the Validation Reports and  
1325 the Bioanalytical Reports.
- 1326 • Discussion of method changes in the protocol (e.g., evolution of methods, reason(s) for  
1327 revisions, unique aspects)
- 1328 • For comparative BA/BE studies a list of regulatory site inspections including dates and  
1329 outcomes for each analytical site if available.

### 1330 ***8.2 Documentation for Validation and Bioanalytical Reports***

1331 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*
<b>Chromatographic System Suitability</b>	<ul style="list-style-type: none"> <li>Dates, times, and samples used for suitability testing</li> </ul>	<ul style="list-style-type: none"> <li>Not applicable</li> </ul>	<ul style="list-style-type: none"> <li>Not applicable</li> </ul>
<b>Synopsis Overview of Method Evolution</b>	<ul style="list-style-type: none"> <li>History/evolution of methods (e.g., to explain revisions, unique aspects with supportive data, if available)</li> </ul>	<ul style="list-style-type: none"> <li>Not applicable</li> </ul>	<ul style="list-style-type: none"> <li>Not applicable</li> </ul>
<b>Reference Standards</b>	<ul style="list-style-type: none"> <li>CoA or equivalent alternative to ensure quality (including purity), stability/expiration/retest date(s), batch number, and manufacturer or source</li> <li>Log records of receipt, use, and storage conditions.</li> <li>If expired, recertified CoA, or retest of quality and identity with retest dates</li> </ul>	<ul style="list-style-type: none"> <li>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.</li> <li>If expired, quality and stability at the time of use and retest dates and retested values.</li> </ul>	<ul style="list-style-type: none"> <li>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.</li> <li>If expired, quality and stability at the time of use and retest dates and retested values.</li> </ul>
<b>Internal Standard</b>	<ul style="list-style-type: none"> <li>IS quality or demonstration of suitability</li> <li>Log records of receipt, use, and storage conditions</li> </ul>	<ul style="list-style-type: none"> <li>Name of reagent or standard</li> <li>Origin</li> </ul>	<ul style="list-style-type: none"> <li>Name of reagent or standard</li> <li>Origin</li> </ul>

Table 1 continued: Documentation and Reporting

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

Table 1 continued: Documentation and Reporting

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

Table 1 continued: Documentation and Reporting

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

Table 1 continued: Documentation and Reporting

Items	Documentation at the Analytical Site	Validation Report*	Bioanalytical Report*
-------	--------------------------------------	--------------------	-----------------------



<p><b>Chromatograms and Reintegration</b></p>	<ul style="list-style-type: none"> <li>• Electronic audit trail:</li> <li>• 100% e-chromatograms of original and reintegration from accepted and fail runs</li> <li>• Reason for reintegration</li> <li>• 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</li> </ul>	<ul style="list-style-type: none"> <li>• Representative chromatograms (original and reintegration)</li> <li>• Reason for reintegration</li> <li>• For comparative BA/BE studies, 100% chromatograms of original and reintegration from accepted and fail runs.</li> <li>• Chromatograms may be submitted as a supplement</li> <li>• 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.</li> </ul>	<ul style="list-style-type: none"> <li>• For and comparative BA/BE studies, 100% of chromatograms.</li> <li>• Chromatograms may be submitted as a supplement</li> <li>• For comparative BA/BE studies, original and reintegrated chromatograms and initial and repeat integration results</li> <li>• For other studies, randomly selected chromatograms from 5% of studies submitted in application dossiers</li> <li>• Reason for reintegration</li> <li>• Identification and discussion of chromatograms with manual reintegration</li> <li>• SOP for reintegration, as applicable</li> <li>• 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.</li> </ul>
---	--	--	---

Table 1 continued: Documentation and Reporting

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

1336 \*The applicant is expected to maintain data at the analytical site to support summary data submitted in Validation and Bioanalytical Reports. Validation  
 1337 and Bioanalytical Reports should be submitted in the application.

1338 † May append or link from Validation Report.

1339    <sup>††</sup>Submit            either            in            Validation            Report            or            in            Bioanalytical            Report

1340 **9. GLOSSARY**

1341 **Accuracy:**

1342 The degree of closeness of the measured value to the nominal or known true value under  
1343 prescribed conditions (or as measured by a particular method). In this document accuracy is  
1344 expressed as percent relative error of the nominal value.

1345 Accuracy (%) = ((Measured Value-Nominal Value)/Nominal Value) × 100

1346

1347 **Analysis:**

1348 A series of analytical procedures from sample processing/dilution to measurement on an analytical  
1349 instrument.

1350

1351 **Analyte:**

1352 A specific chemical moiety being measured, including an intact drug, a biomolecule or its derivative  
1353 or a metabolite in a biologic matrix.

1354

1355 **Analytical Procedure:**

1356 The analytical procedure refers to the way of performing the analysis. It should describe in detail  
1357 the steps necessary to perform each analysis.

1358

1359 **Analytical Run (also referred to as "Run"):**

1360 A complete set of analytical and study samples with appropriate number of calibration standards  
1361 and QCs for their validation. Several runs may be completed in one day or one run may take  
1362 several days to complete.

1363 **Anchor Calibration Standards/Anchor Points:**

1364 Spiked samples set at concentrations below the LLOQ or above the ULOQ of the calibration curve  
1365 and analysed to improve curve fitting in LBAs.

1366

1367 **Batch (for Bioanalysis):**

1368 A batch is comprised of QCs and study samples which are handled during a fixed period of time and  
1369 by the same group of analysts with the same reagents under homogenous conditions.

1370

1371 **Batch (for Reference Standards and Reagents):**

1372 A specific quantity of material produced in a process or series of processes so that it is  
1373 expected to be homogeneous within specified limits. Also referred to as "Lot".

1374

1375 **Biological Drugs:**

1376 Drugs manufactured by using biotechnology (e.g., therapeutic proteins). Also referred to as large  
1377 molecule drugs.

1378

1379 **Biological Matrix:**

1380 A biological material including, but not limited to, blood, serum, plasma and urine.

1381

1382 **Binding Reagent:**

1383 A reagent that directly binds to the analyte in LBA-based bioanalytical methods.

1384

1385 **Blank Sample:**

1386 A sample of a biological matrix to which no analyte and no IS has been added.

1387 **Calibration Curve:**

1388 The relationship between the instrument response (e.g., peak area, height or signal) and the  
1389 concentration (amount) of analyte in the sample within a given range. Also referred to as Standard  
1390 Curve.

1391

1392 **Calibration Range:**

1393 The calibration range of an analytical procedure is the interval between the upper and lower  
1394 concentration (amounts) of analyte in the sample (including these concentrations) for which it has  
1395 been demonstrated that the analytical procedure meets the requirements for precision, accuracy  
1396 and response function.

1397

1398 **Calibration Standard:**

1399 A matrix to which a known amount of analyte has been added or spiked. Calibration standards are  
1400 used to construct calibration curves.

1401

1402 **Carry-over:**

1403 The appearance of an analyte signal in a sample from a preceding sample.

1404

1405 **Chemical Drugs:**

1406 Chemically synthesised drugs. Also referred to as small molecule drugs.

1407

1408 **Critical Reagent:**

1409 Critical reagents for LBAs include binding reagents (e.g., antibodies, binding proteins, peptides)  
1410 and those containing enzymatic moieties that have a direct impact on the results of the assay.

1411 **Cross Validation:**

1412 Comparison of two bioanalytical methods or the same bioanalytical method in different laboratories  
1413 in order to demonstrate that the reported data are comparable.

1414

1415 **Dilution Integrity:**

1416 Assessment of the sample dilution procedure to confirm that the procedure does not impact the  
1417 measured concentration of the analyte.

1418

1419 **Dilution Linearity:**

1420 A parameter demonstrating that the method can appropriately analyse samples at a concentration  
1421 exceeding the ULOQ of the calibration curve without influence of hook effect or prozone effect and  
1422 that the measured concentrations are not affected by dilution within the calibration range in LBAs.

1423

1424 **Full Validation:**

1425 Establishment of all validation parameters that ensure the integrity of the method when applied to  
1426 sample analysis.

1427

1428 **Hook Effect:**

1429 Suppression of response due to very high concentrations of a particular analyte. A hook effect may  
1430 occur in LBAs that use a liquid-phase reaction step for incubating the binding reagents with the  
1431 analyte. Also referred to as prozone.

1432

1433 **Incurred Sample:**

1434 A sample obtained from study subjects or animals.

1435 **Incurred Sample Reanalysis (ISR):**

1436 Reanalysis of a portion of the incurred samples in a separate analytical run on a different day to  
1437 determine whether the original analytical results are reproducible.

1438

1439 **Interfering Substance:**

1440 A substance that is present in the matrix that may affect the analysis of an analyte.

1441

1442 **Internal Standard (IS):**

1443 A structurally similar analogue or stable isotope labelled compound added to calibration standards,  
1444 QCs and study samples at a known and constant concentration to facilitate quantification of the  
1445 target analyte.

1446

1447 **Ligand Binding Assay (LBA):**

1448 A method to analyse an analyte of interest using reagents that specifically bind to the analyte. The  
1449 analyte is detected using reagents labelled with e.g. an enzyme, radioisotope, fluorophore or  
1450 chromophore. Reactions are carried out in microtitre plates, test tubes, disks, etc.

1451

1452 **Lower Limit of Quantification (LLOQ):**

1453 The lowest amount of an analyte in a sample that can be quantitatively determined with predefined  
1454 precision and accuracy.

1455

1456 **Matrix Effect:**

1457 The direct or indirect alteration or interference in response due to the presence of unintended  
1458 analytes or other interfering substances in the sample.



1459 **Method:**

1460 A comprehensive description of all procedures used in sample analysis.

1461

1462 **Minimum Required Dilution (MRD):**

1463 The initial dilution factor by which biological samples are diluted with buffer solution for the  
1464 analysis by LBAs. The MRD may not necessarily be the ultimate dilution but should be identical for  
1465 all samples including calibration standards and QCs. However, samples may require further  
1466 dilution.

1467

1468 **Nominal Concentration:**

1469 Theoretical or expected concentration.

1470

1471 **Parallelism:**

1472 Parallelism demonstrates that the serially diluted incurred sample response curve is parallel to the  
1473 calibration curve. Parallelism is a performance characteristic that can detect potential matrix  
1474 effects.

1475

1476 **Partial Validation:**

1477 Evaluation of modifications to already fully validated analytical methods.

1478

1479 **Precision:**

1480 The closeness of agreement (i.e., degree of scatter) among a series of measurements. Precision is  
1481 expressed as the coefficient of variation (CV) or the relative standard deviation (RSD) expressed as  
1482 a percentage.

1483 Precision (%) = (Standard Deviation / Mean) x 100

1484 **Processed Sample:**

1485 The final sample that has been subjected to various manipulations (e.g., extraction, dilution,  
1486 concentration).

1487

1488 **Quality Control Sample (QC):**

1489 A sample spiked with a known quantity of analyte that is used to monitor the performance of a  
1490 bioanalytical method and assess the integrity and validity of the results of the unknown samples  
1491 analysed in an individual batch or run.

1492

1493 **Recovery:**

1494 The extraction efficiency of an analytical process, reported as a percentage of the known amount of  
1495 an analyte carried through the sample extraction and processing steps of the method.

1496

1497 **Reproducibility:**

1498 The extent to which consistent results are obtained when an experiment is repeated.

1499

1500 **Response Function:**

1501 A function which adequately describes the relationship between instrument response (e.g., peak  
1502 area or height ratio or signal) and the concentration (amount) of analyte in the sample. Response  
1503 function is defined within a given range. See also Calibration Curve.

1504

1505 **Selectivity:**

1506 Ability of an analytical method to differentiate and measure the analyte in the presence of  
1507 interfering substances in the biological matrix (non-specific interference).

1508 **Sensitivity:**

1509 The lowest analyte concentration that can be measured with acceptable accuracy and precision  
1510 (i.e., LLOQ).

1511

1512 **Specificity:**

1513 Ability of an analytical method to detect and differentiate the analyte from other substances,  
1514 including its related substances (e.g., substances that are structurally similar to the analyte,  
1515 metabolites, isomers, impurities or concomitant medications).

1516

1517 **Standard Curve:**

1518 The relationship between the instrument response (e.g., peak area, height or signal) and the  
1519 concentration (amount) of analyte in the sample within a given range. Also referred to as  
1520 calibration Curve.

1521

1522 **Standard Operating Procedure (SOP):**

1523 Detailed written instructions to achieve uniformity of the performance of a specific function.

1524

1525 **Surrogate Matrix:**

1526 An alternative to a study matrix of limited availability (e.g., tissue, cerebrospinal fluid, bile) or  
1527 where the study matrix contains an interfering endogenous counterpart.

1528

1529 **System Suitability:**

1530 Determination of instrument performance (e.g., sensitivity and chromatographic retention) by  
1531 analysis of a set of reference standards conducted prior to the analytical run.

1532 **Total Error:**

1533 The sum of the absolute value of the errors in accuracy (%) and precision (%). Total error is  
1534 reported as percent (%) error.

1535

1536 **Upper Limit of Quantification (ULOQ):**

1537 The upper limit of quantification of an individual analytical procedure is the highest amount of  
1538 analyte in a sample that can be quantitatively determined with pre-defined precision and accuracy.

1539

1540 **Validation:**

1541 Demonstration that a bioanalytical method is suitable for its intended purpose.

1542

1543 **Working Solution:**

1544 A non-matrix solution prepared by diluting the stock solution in an appropriate solvent. It is mainly  
1545 added to matrix to prepare calibration standards and QCs.

1546

1547 **Zero Sample:**

1548 A blank sample spiked with an IS.

1549