29 October 2019
EMA/485652/2019

Overview of comments received on ICH guideline M10 on bioanalytical method validation (EMA/CHMP/ICH/172948/2019)

Comments from:

Stakeholder no.	Name of organisation or individual
1	Medicines for Europe
2	F. Hoffmann- La Roche Ltd, Basel, Switzerland
3	EFPIA – Giovanna Rizzetto (giovanna.rizzetto@efpia.eu)
4	European Bioanalysis Forum vzw
5	ORBIS project - Piotr Rudzki (p.rudzki@ifarm.eu / pj.rudzki@wp.pl)
6	AstraZeneca Pharmaceuticals

Please note that comments will be sent to the relevant **ICH EWG** for consideration in the context of Step 3 of the ICH process.

Temporary visiting address Spark building • Orlyplein 24 • 1043 DP Amsterdam • The Netherlands
For deliveries refer to www.ema.europa.eu/how-to-find-us
Send us a question via www.ema.europa.eu/contacts **Telephone** +31(0)88 781 6000 An agency of the European Union

1. General comments

Stakeholder number	General comment (if any)
1	This guideline aims to provide harmonized and practical recommendations on regulatory requirements for bioanalytical method development and validation. In doing so, it is critical to integrate current regulatory guidance from multiple regulatory agencies as well as the current best practices of the industry. To reduce any misunderstanding and extend the utility of the guideline, further elaboration on certain procedures and their criteria might be necessary.
3	Overall comment from our industry is that the document is well written. Nevertheless, the EFPIA has suggestions to ensure that the final ICH M10 is not only a harmonised guideline for BMV, but also supports method development, validation/qualification and study sample analysis for all phases of pharmaceutical R&D in the most resource effective way (from ICH mission: ICH's mission is to achieve greater harmonisation worldwide to ensure that safe, effective, and high quality medicines are developed and registered in the most resource-efficient manner. *Ref: https://www.ich.org/about/mission.html).* The detailed comments in this document are the consolidated opinion from the EFPIA member companies (R*ef: https://www.efpia.eu*). They were gathered through internal surveys amongst the EFPIA members, and from discussions as part of the European Bioanalysis Forum (EBF) and AAPS workshops on ICH M10, held in Barcelona, Spain (May 2019) and Silver Spring, USA (June 2019) respectively, to which senior experts from EFPIA member companies actively participated. General remarks (refinements as proposed in our comments further in the document are of significant importance for industry): • "Scope" is generally perceived as too broad and ambiguous. If unchanged, all studies, all matrices and all analytes are at risk of becoming in scope. • Some parts of stability assessment are perceived as too broad. Example given is co-med stability assessment. • Consider harmonised decision-based acceptance criteria rather than technology-based ones (LCMS vs LBA). (*Ref: Bioanalysis (2018) 10(16), 1255–1259*). Also, this would prepare the Guideline for future technologies entering the regulatory BMV space. • "Table 1: Documentation" and "Paragraph 2.1: Method Development" carry the risk of becoming overinterpreted and are increasing the resource requirements for industry, whilst stifling scientific freedom required in the method development arena (and

Stakeholder number	General comment (if any)
	not aligned with the mission of ICH). ➢ For "Documentation" we suggest to limit the requirements in table 1 to BA/BE-studies, and allow reporting of other studies to be less detailed (i.e. less in reports but allow documentation to be available at the analytical site) ➢ For "Method Development," we suggest to limit to scope to changes to already validated methods in later stages of development. • 3Rs: EFPIA feels that a sustainable and science based guideline should consider animal welfare and not require unnecessary use of animals. (*Ref: https://www.nc3rs.org.uk/the-3rs*) ➢ Replace = allow surrogate matrix used when proven valid (e.g. sample dilutions, calibrators,..) ➢ Reduce = using smaller volumes/less replicates of sample or matrix in preclinical assays ➢ Refine = facilitate micro-sampling assays More details on above general comments are provided below. Suggested changes are given in red. Where available, the justification is provided in blue
4	This document contains the consolidated comments from the European Bioanalysis Forum vzw (EBF vzw, non-profit). Following companies (Pharma R&D and CRO) are member of the EBF vzw A&M Labor Abbott Healthcare Products Abbvie ABL ABS Accelera Alderley Analytical Almirall, S.A.

Stakeholder number	General comment (if any)
	Amgen
	Aptuit an Evotec Company
	ARCinova
	Argenx
	Ascendis Pharma
	AstraZeneca
	AZ Biopharma
	Atlanbio-Citoxlab
	Bayer Pharma AG
	Bioagilytix
	Boehringer-Ingelheim
	Celerion
	Charles River
	Comac Medical
	Concept Life Sciences
	Covance
	Dynakin
	Eurofins
	F. Hoffmann-La Roche
	Ferring Pharmaceuticals
	GlaxoSmithKline

Stakeholder number	General comment (if any)
	Genmab
	Grünenthal
	Intertek Pharmaceutical Services Manchester
	Idorsia Pharmaceuticals Ltd
	Janssen R&D / Biologics
	Kymos
	Leo Pharma a/s
	LGC
	Lundbeck a/s
	Merck Serono
	MSD
	Novartis
	Novimmune
	NovoNordisk
	Nuvisan GmbH
	Orion Corporation Orion Pharma
	Orphazyme
	pharm-analyt Labor GmbH
	Pharmaron-UK
	Pierre Fabre
	PRA Health Sciences

Stakeholder number	General comment (if any)
	PSIOXUS QPS Netherlands Recipharm Sanofi Servier SGS Swiss BioQuant Symphogen SYNLAB Analytics & Services Switzerland AG Synthon Syrinx Bioanalytics UCB Biopharma Unilabs Svar Life sciences Overall comment from the European Bioanalysis Forum (EBF) community is that the document is well written. Nevertheless, the EBF has suggestions to ensure that the final ICH M10 is not only a harmonised guideline for BMV, but also supports method development, validation/qualification and study sample analysis for all phases of pharmaceutical R&D in the most resource effective way (from ICH mission: ICH's mission is to achieve greater harmonisation worldwide to ensure that safe, effective, and high quality medicines are developed and registered in the most resource-efficient manner. *Ref: https://www.ich.org/about/mission.html).* The comments in this document are the consolidated opinion from the EBF member companies mentioned above and are very

Stakeholder number	General comment (if any)
	similar to the EFPIA comments which were provided as a separated file via EFPIA. The background is that virtually all EFPIA member companies are also a member of the EBF and the comments were gathered through identical surveys amongst the EBF and EFPIA members, from discussions as part of EBF and AAPS workshops on ICH M10, held in Barcelona (May 2019) and Silver Spring (June 2019) respectively. A few comments were given specifically by the EBF, which are added in this document. In order to facilitate triage, they are highlighted with red line numbers in the column "line N°". For a few comments, the EBF refined the view or comment as given by EFPIA. In those cases, the line numbers are highlighted as 'strike through' (e.g. ~~714-722~~) and should not be considered. All other comments with line numbers in black are identical to the comments given by EFPIA General remarks (refinements as proposed in our comments further in the document are of significant importance for industry): • "Scope" is generally perceived as too broad and ambiguous. If unchanged, all studies, all matrices and all analytes are at risk of becoming in scope. • Some parts of stability assessment are perceived as too broad. Example given is co-med stability assessment. • Consider harmonised decision-based acceptance criteria rather than technology-based ones (LCMS vs LBA). (*Ref: Bioanalysis (2018) 10(16), 1255–1259*). Also, this would prepare the Guideline for future technologies entering the regulatory BMV space. • "Table 1: Documentation" and "Paragraph 2.1: Method Development" carry the risk of becoming overinterpreted and are increasing the resource requirements for industry, whilst stifling scientific freedom required in the method development arena (and not aligned with the mission of ICH). ➢ For "Documentation" we suggest to limit the requirements in table 1 to BA/BE-studies, and allow reporting of other studies to be less detailed (i.e. less in reports but allow documentation to be available at the analytical site) ➢ For "Method Development," we suggest to limit to scope to changes to already validated methods in later stages of development. • 3Rs: EFPIA feels that a sustainable and science based guideline should consider animal welfare and not require unnecessary use of animals. (*Ref: https://www.nc3rs.org.uk/the-3rs*)

Stakeholder number	General comment (if any)
	➢ Replace = allow surrogate matrix used when proven valid (e.g. sample dilutions, calibrators,..) ➢ Reduce = using smaller volumes/less replicates of sample or matrix in preclinical assays ➢ Refine = facilitate micro-sampling assays More details on above general comments are provided below. Suggested changes are given in red. Where available, the justification is provided in blue
5	The ORBIS project members are pleased to have been given the opportunity to comment on the draft ICH guideline under public consultations by the EMA. ORBIS consortium, supported by the European Union under Horizon 2020, comprises universities, pharmaceutical companies and R&D enterprises. The team involved in this public consultation included experts from the Łukasiewicz Research Network - Pharmaceutical Research Institute (Warsaw, Poland) and Poznan University of Medical Sciences (Poznań, Poland). Numerous recommendations are greatly appreciated, including: (1) extrapolating stability at -20°C to lower temperatures for small molecules, (2) detailed description for endogenous compounds, (3) Table 1 specifying reporting. We hope that our specific comments – especially regarding matrix effect and incurred sample reanalysis – as well as our previous paper on comparison of EMA and FDA recommendations (M. Kaza et al. doi: 10.1016/j.jpba.2018.12.030) will inspire further improvement of the guideline. The ICH guideline on bioanalytical method validation is a great and long awaited step towards global unification of bioanalytical regulatory recommendations. It will allow to avoid confusing differences in terminology as well as the unnecessary effort of being compliant with two or more guidelines. Thus, we would like to thank all parties involved for their commitment to develop draft guideline. *Note: ORBIS project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 778051.* We have found the text in the draft guideline as too long. Thus, we would like to suggest more concise style: without general statements, unnecessary (out of scope) information and repeated sentences. Table 1 specifying reporting is a greatly appreciated. Adding other tables, similar to tables presented in the appendix to the FDA guidance on Bioanalytical Method Validation (2018) will be greatly appreciated too. We support European Bioanalysis Forum proposal to replace current technology-based acceptance criteria with decision-based ones

Stakeholder number	General comment (if any)
	(P. Timmerman et al. http://dx.doi.org/10.4155/bio-2018-0131)
6	Overall the document is well written. However, AstraZeneca is providing suggested changes to ensure that the final ICH M10 is a harmonised guideline for Bioanalytical Method Validation (BMV) that also supports method development, validation and study sample analysis for all phases of pharmaceutical research and drug development. In keeping with the mission of ICH to provide safe and effective medicines to patients in the most resource efficient manner, items which increase requirements have been noted. Additionally, in some ICH regions the ICH M10 guideline will become law (e.g. ANVISA). This creates a challenge for some parts of the guideline that include ambiguity and may be interpreted differently, particularly the scope. For industry, it may not be evident early on in drug development that a study would be used to make a regulatory decision and form part of a submission. Therefore, it would be advantageous to clarify, as much as possible, in the guideline rather than solely relying on training activities. Overall general comments: 1. As a guidance document on how to conduct bioanalytical method validation, it should be clearly stated that other approaches may be taken when scientifically justified. At present there is no flexibility to allow alternatives in the case of 'free' PK assays when the use of a surrogate matrix may be more scientifically appropriate. 2. The scope of the guideline is broad and is open to being interpreted differently by both industry and regulators in the ICH regions. If not revised, the consequence will be that all studies are brought into scope by industry as at the time of conduct it may not be known whether a study may be used to support a regulatory decision at a later stage. This will in turn result in increased resources and potentially delay the delivery of safe and effective medicines to patients. 3. Consider harmonised decision-based acceptance criteria rather than technology-based ones (LCMS vs LBA) as proposed in Bioanalysis (2018) 10(16), 1255–1259). This will also provide the advantage of preparing the guideline for future technologies entering the regulatory BMV space. 4. Table 1 "Documentation" will result in requirements that are higher than those recommended in current guidance and result in extensive reporting. 5. For "Method Development" it is suggested to limit to scope to changes to already validated methods and focus on life-cycle management of validated methods. 6. The concept of the 3Rs (reduction, replacement and refinement) and the use of animal samples should be considered in the ICH M10 guideline. There should be provision made to allow for the use of fewer samples when conducting preclinical assessments

Stakeholder number	General comment (if any)
	especially given these are inbred animals and lack variability. Apart from the ethical considerations of using more animals when there is no additional scientific value, reducing animal usage delivers on sustainability goals. 7. For validation assessments the term "QC" should be replaced with "VC" (validation control). "QC" should be used when conducting sample analysis. 8. The term(s) "sources/lots" is confusing and could mean different suppliers rather than from different animal or human individuals. Suggest that the term be changed to "individuals". 9. Training will be an important element of the guideline and it is suggested that a Q&A or addendum is provided as soon as possible to help in interpretation if certain areas of the guideline remain ambiguous. Proposed changes have been made as follows: • Deletion is text shown as strikethrough • Additional text shown as bold

2. Specific comments on text

Line no.	Stakeholder no.	Comment and rationale; proposed changes
112-113 and 135-136	5	**Comments:** Selectivity and Specificity may be combined in one paragraph "Selectivity & Specificity". Sequence of points Selectivity and Specificity should be the same for chromatography and LBAs. **Proposed change:** Combining Selectivity and Specificity in one paragraph.
176	2	**Comments:** Exact objective of the guideline is not clearly described, a statement in the objective that the guideline only covers "total drug" quantification would be helpful. **Proposed change:** Line 176: Please add that this guideline covers total drug assays
176-180	3	**Comments:** refine **Proposed change:** This guideline is intended to provide recommendations for the validation of bioanalytical methods for chemical and biological drug quantification and their application in the analysis of study samples. Adherence to the principles presented in this guideline will ensure the quality and consistency of the bioanalytical data in support of the development and market approval of both chemical and biological drugs.
176-180	4	**Comments:** refine

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: This guideline is intended to provide recommendations for the validation of bioanalytical methods for chemical and biological drug quantification and their application in the analysis of study samples. Adherence to the principles presented in this guideline will ensure the quality and consistency of the bioanalytical data in support of the development and market approval of both chemical and biological drugs.
181-185	3	**Comments:** refine **Proposed change:** The objective of the validation of a bioanalytical method is to demonstrate that it is appropriate for its intended purpose. Changes from the recommendations in this guideline may be acceptable if appropriate scientific justification is documented and provided upon request to regulatory authorities. Applicants are encouraged to consult the regulatory authority(ies) regarding significant changes in method validation approaches when an alternate approach is proposed or taken
181-185	4	**Comments:** refine **Proposed change:** The objective of the validation of a bioanalytical method is to demonstrate that it is appropriate for its intended purpose. Changes from the recommendations in this guideline may be acceptable if appropriate scientific justification is documented and provided upon request to regulatory authorities. Applicants are encouraged to consult the regulatory authority(ies) regarding significant changes in method validation approaches when an alternate approach is proposed or taken
182	2	**Comments:** Changes from the recommendations in this guideline may be acceptable, e.g. for the quantification of free drug, or in any other case if appropriate scientific justification is provided.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
187	2	**Comments:** Exact description required which assays are meant. This guideline does e.g. not cover special requirements for free drug assays. **Proposed change:** Please clarify.
187-193	3	**Comments:** add - (proposal deconvolutes background from scope) **Proposed change:** Concentration measurements of chemical and biological drug(s) and their metabolite(s) in biological matrices are an important aspect of drug development. The results of studies employing such methods contribute to regulatory decisions regarding the safety and efficacy of drug products. It is therefore critical that the bioanalytical methods used are well characterised, appropriately validated and documented in order to ensure reliable data to support regulatory decisions.
187-193	4	**Comments:** add - (proposal deconvolutes background from scope) **Proposed change:** Concentration measurements of chemical and biological drug(s) and their metabolite(s) in biological matrices are an important aspect of drug development. The results of studies employing such methods contribute to regulatory decisions regarding the safety and efficacy of drug products. It is therefore critical that the bioanalytical methods used are well characterised, appropriately validated and documented in order to ensure reliable data to support regulatory decisions.
188	2	**Comments:** How is pivotal defined?

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: Remove "pivotal" for nonclinical toxicokinetic /pharmacokinetic studies, or explain exactly what "pivotal" means (which type of study)
195-204	3	**Comments:** Suggested changes of or additions to the paragraph in red **Proposed change:** This guideline describes the method validation that is expected for bioanalytical assays that are submitted to support regulatory submissions. The guideline is applicable to the validation of bioanalytical methods used to measure concentrations of chemical and biological drug(s) and their metabolite(s) in biological samples (e.g., blood, plasma, serum, other body fluids ~~or tissues~~) obtained in nonclinical TK studies falling under the scope of the GLPs that are used to make regulatory decisions, nonclinical PK studies that are conducted as surrogates for clinical studies, and all phases of clinical trials in regulatory submissions for which a primary objective of the study is to assess, compare or characterize drug exposure. Full method validation is expected for the primary matrix(ces) intended to support regulatory submissions. Primary matrix(ces) are identified based on the objective(s) of individual studies and these should be indicated in the study protocol or sample analysis plan. For non-primary analytes/matrices validation should be performed in line with the anticipated use of the data, using the appropriate/applicable principles (i.e. partial validation or alternative approaches). ~~The analytes that should be measured in nonclinical and clinical studies and the types of studies necessary to support a regulatory submission are described in other ICH and regional regulatory documents.~~ *(proposal to delete last sentence as it creates more confusion than clarity and opens ICH M10 to become dependent on (future) regional regulations)*
195-204	4	**Comments:** Suggested changes of or additions to the paragraph in red **Proposed change:** This guideline describes the method validation that is expected for bioanalytical assays that are submitted to support regulatory submissions. The guideline is applicable to the validation of bioanalytical methods used to measure concentrations of chemical and biological drug(s) and their metabolite(s) in biological samples (e.g., blood, plasma, serum, other body fluids ~~or tissues~~) obtained

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		in nonclinical TK studies falling under the scope of the GLPs that are used to make regulatory decisions, nonclinical PK studies that are conducted as surrogates for clinical studies, and all phases of clinical trials in regulatory submissions for which a primary objective of the study is to assess, compare or characterize drug exposure. Full method validation is expected for the primary matrix(ces) intended to support regulatory submissions. Primary matrix(ces) are identified based on the objective(s) of individual studies and these should be indicated in the study protocol or sample analysis plan. For non-primary analytes/matrices validation should be performed in line with the anticipated use of the data, using the appropriate/applicable principles (i.e. partial validation or alternative approaches). ~~The analytes that should be measured in nonclinical and clinical studies and the types of studies necessary to support a regulatory submission are described in other ICH and regional regulatory documents.~~ *(proposal to delete last sentence as it creates more confusion than clarity and opens ICH M10 to become dependent on (future) regional regulations)*
195-204	5	**Comments:** Please consider more concise style **Proposed change:** This guideline describes the validation of bioanalytical methods - used to measure concentrations of chemical and biological drug(s) and their metabolite(s) in biological samples (e.g., blood, plasma, serum, other body fluids or tissues) - submitted to support regulatory submissions. The guideline is applicable to pivotal nonclinical TK/PK studies and all phases of clinical trials. Full method validation is expected for the primary matrix(-ces), additional matrices should be partially validated. The analytes that should be measured are described in other ICH and regional regulatory documents.
195-207	6	**Comments:** The scope is not clear and has the potential to be interpreted differently. It is not always clear in the early stages of development that a study will be considered pivotal or becomes part of a regulatory submission. Therefore, this will result in all studies being fully validated, rather than applying scientific validation, that consider the context of use, in early stages of development. Scope and background are potentially conflicting; it is not clear whether only pivotal studies, or all studies that are part of a regulatory submission, are within scope. **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Define "pivotal" in the glossary. Clarify the types of study that would be expected to be pivotal in a Q&A/Addendum or as part of an ICH training package.
205-207	3	**Comments:** delete - *(one does not know a priori if a study will be considered for regulatory decisions, thus in practice, only limiting to studies not included in submissions is possible)* **Proposed change:** For studies that are not submitted for regulatory approval ~~or not considered for regulatory decisions regarding safety, efficacy or labelling (e.g., exploratory investigations)~~, applicants may decide on the level of qualification that supports their own internal decision making.
205-207	4	**Comments:** delete - *(one does not know a priori if a study will be considered for regulatory decisions, thus in practice, only limiting to studies not included in submissions is possible)* **Proposed change:** For studies that are not submitted for regulatory approval ~~or not considered for regulatory decisions regarding safety, efficacy or labelling (e.g., exploratory investigations)~~, applicants may decide on the level of qualification that supports their own internal decision making.
205-207	5	**Comments:** The studies not submitted for regulatory approval are out of scope of the guideline. **Proposed change:** delete lines 205-207
202-204	6	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		The inclusion of regional regulatory documents allows for additional requirements to be added by a Health Authority. This goes against the concept of harmonisation and is contrary to the mission of ICH; to conduct activities in the most resource efficient manner providing the safety and efficacy of patients is not compromised. Additionally, it should be considered that in some ICH regions this guideline will become law rather than guidance and therefore interpretation cannot be applied differently across the ICH regions. **Proposed change:** "The analytes that should be measured in nonclinical and clinical studies and the types of studies necessary to support a regulatory submission are described in other ICH ~~and regional regulatory~~ documents."
208-209	5	**Comments:** LC should be mentioned before LBAs to be in line with table of contents **Proposed change:** LC mentioned before LBAs
208-211	2	**Comments:** Is it necessary to name specific bioanalytical technologies? These are the most common ones, however the guideline should also apply if (for whatever reason) another, not mentioned technology is used. **Proposed change:** Remove mentioned bioanalytical platforms, this keeps the guideline more flexible.
208-215	5	**Proposed change:** combine in one paragraph
210-211	5	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Phrase "and occasionally with other detectors" is unnecessary. **Proposed change:** delete "and occasionally with other detectors"
212-213	3	**Comments:** rephrase **Proposed change:** For studies that are subject to Good Laboratory Practice (GLP) the bioanalysis of study samples must also conform to its requirements. In accordance with Good Clinical Practice (GCP), the bioanalysis of clinical study samples must be conducted as described by the study protocol and within the limits of the informed consent agreed to by study participants
212-213	4	**Comments:** rephrase **Proposed change:** For studies that are subject to Good Laboratory Practice (GLP) the bioanalysis of study samples must also conform to its requirements. In accordance with Good Clinical Practice (GCP), the bioanalysis of clinical study samples must be conducted as described by the study protocol and within the limits of the informed consent agreed to by study participants
212-213	6	**Comments:** Further clarification on the scope with relation to GLP and GCP regulations and whether this is applicable to studies and phases. Not all regions have GCP requirements for Bioanalysis and this should be considered. **Proposed change:** Change to "For studies **and phases** that are subject to Good Laboratory Practice (GLP) or Good Clinical Practice (GCP) the

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		bioanalysis of study samples should also **follow these principles**."
217	3	**Comments:** General comment – if comment to delete the section is not considered, more editorial comments are provided from line 224 onwards, albeit this would be our second choice. As first suggestion, we don't provide alterative text since the proposal is to delete this section: The method development is a previous work that only concerns the laboratories and their organization and knowhow to obtain robust, accurate and precise assays. Nevertheless, once fully validated, it is considered relevant to provide information on the method changes, the evolution of methods and reasons, as it is commented in a very correct way is section 8.1 for the CTD **Proposed change:** delete this section
217	4	**Comments:** General comment – if comment to delete the section is not considered, more editorial comments are provided from line 224 onwards, albeit this would be our second choice. As first suggestion, we don't provide alterative text since the proposal is to delete this section: The method development is a previous work that only concerns the laboratories and their organization and knowhow to obtain robust, accurate and precise assays. Nevertheless, once fully validated, it is considered relevant to provide information on the method changes, the evolution of methods and reasons, as it is commented in a very correct way is section 8.1 for the CTD **Proposed change:** delete this section
217-243	5	**Comments:** Whole paragraph is out of scope of the guideline. If paragraph 2.1 is deleted then there is no use for subtitle 2.2.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: delete whole paragraph 2.1 and subtitle 2.2.
224-226	3	**Comments:** There should not be an expectation that all parameters are fine tuned to an **optimum**.
224-226	3	**Comments:** Recommendation to rephrase verbiage around method development (MD) activities such as "MD can/may involve assessments of the following" or delete list as it becomes a risk of being a mandated requirement **Proposed change:** delete bullets
224-226	4	**Comments:** There should not be an expectation that all parameters are fine tuned to an **optimum**.
224-226	4	**Comments:** Recommendation to rephrase verbiage around method development (MD) activities such as "MD can/may involve assessments of the following" or delete list as it becomes a risk of being a mandated requirement **Proposed change:** delete bullets
227-237	6	**Comments:** This is guidance for method validation and not method development. The list of typical parameters to include in method development does not add value.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: Delete the list of parameters listed as bullet points
236	2	**Comments:** Stability of the analyte should also be proven in working solutions, if necessary. **Proposed change:** Remove "in matrix"
238	2	**Comments:** Ambiguous wording: Method development. What is meant is probably the history of validated methods used for a program **Proposed change:** Replace "Method development" by "Method evolution"
238	6	**Comments:** Whilst extensive recording may not be a requirement, it should be appropriately recorded. **Proposed change:** "Bioanalytical method development should be **appropriately recorded** and does not require formal reporting"
238-240	3	**Comments:** add **Proposed change:** However, the applicant should record the changes to procedures as well as any issues and their resolutions to provide a rationale for any changes made to validated methods (i.e. Method Evolution) immediately prior to or in the course of analysing study

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		samples for pivotal studies.
238-240	3	**Comments:** add **Proposed change:** Formal reporting is not required, however a proper use of the CTD detailing rationale for any changes is encouraged.
238-240	4	**Comments:** add **Proposed change:** However, the applicant should record the changes to procedures as well as any issues and their resolutions to provide a rationale for any changes made to validated methods (i.e. Method Evolution) immediately prior to or in the course of analysing study samples for pivotal studies.
238-240	4	**Comments:** add **Proposed change:** Formal reporting is not required, however a proper use of the CTD detailing rationale for any changes is encouraged.
241-242	3	**Comments:** Rephrase **Proposed change:** Once the method has been developed, the method is ready for validation to generate a validated method which is suited for

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		sample analysis
241-242	4	**Comments:** Rephrase **Proposed change:** Once the method has been developed, the method is ready for validation to generate a validated method which is suited for sample analysis
244, 270, 275	5	**Comments:** subtitles are misleading as partial and cross validation are described in paragraph 6. **Proposed change:** delete subtitles
245-254	5	**Comments:** More concise style is advised. **Proposed change:** A full validation of a bioanalytical method should be performed when: (1) establishing a new bioanalytical method, (2) implementing an analytical method that is reported in the literature and (3) a commercial kit is repurposed for bioanalytical use in drug development. Usually one analyte has to be determined. If more than one analytes are measured (i.e. different drugs, a parent drug with its metabolites or the enantiomers or isomers of a drug), the principles of validation and analysis apply to all analytes of interest.
249	2	**Comments:** to discuss - it is not clear what the pivotal nonclinical studies are

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: Change to "… and nonclinical studies which are performed under the regulations of GLP"
249	6	**Comments:** The text states that all clinical and pivotal nonclinical studies are in scope. However, in the scope (lines 205-207), "applicants can decide on the level of qualification that supports their own internal decision making". Therefore, this appears to be contradictory. **Proposed change:** Change language to "A full validation of a bioanalytical method should be performed when establishing a bioanalytical method for the quantification of an analyte in **pivotal** clinical and in pivotal nonclinical studies."
255, 259, 273	5	**Comments:** Words "elements" and "items" should be replaced with "parameters". **Proposed change:** elements (and items) => parameters
256	5	**Comments:** Is studying matrix effect necessary for assays based on UV-vis detection? **Proposed change:** matrix effect => matrix effect (in case of LC-MS methods)
256-258	5	**Comments:** repeating definition of range is not necessary here. Is reinjection reproducibility mandatory? Recovery should be mentioned. **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		calibration curve and range, accuracy, precision, carry-over, dilution integrity, stability and reinjection reproducibility (if necessary), recovery (if method employs sample extraction).
259-261	2	**Comments:** Assessment of matrix effect is missing for LBAs **Proposed change:** Please add
262-266	2	**Comments:** Requirements for free drug assays are not considered, use of surrogate matrix is limited to rare matrices only. **Proposed change:** Include the possibility to use surrogate matrix also for the use in free drug assays
262-266	3	**Comments:** Suggest specifying that a change in counterion is considered the same anticoagulant. We suggest editing the text to read: **Proposed change:** The matrix used for analytical method validation should be the same as the matrix of the study samples, including anticoagulants and additives. A different counterion is considered the same anticoagulant (e.g., Na/Li-heparin; K_2/K_3 EDTA). In some cases where rare matrices are considered primary matrix, it may be difficult to obtain an identical matrix to that of the study samples. In such cases, surrogate matrices may be acceptable for analytical method validation. The alternative matrix should be selected and justified scientifically for use in the analytical method.
262-266	4	**Comments:** Suggest specifying that a change in counterion is considered the same anticoagulant. We suggest editing the text to read:

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: where rare matrices are considered primary matrix, it may be difficult to obtain an identical matrix to that of the study samples. In such cases, surrogate matrices may be acceptable for analytical method validation. The alternative matrix should be selected and justified scientifically for use in the analytical method.
265-266	5	**Proposed change:** analytical => bioanalytical
267-269	6	**Comments:** These lines are related to method development and would be better suited in the method development section. **Proposed change:** Move to the end of the method development section
273-275	1	**Comment:** The matrix effect should be evaluated by analysing at least 3 replicates of low and high QCs, each prepared using matrix from at least 6 different sources/lots. The accuracy should be within ±15% of the nominal concentration and the precision (per cent coefficient of variation (%CV) should not be greater than 15% in all individual matrix sources/lots. Use of fewer sources/lots may be acceptable in the case of rare matrices. **Proposed change:** As per this statement, matrix effect should evaluate by using calibration standards: are the calibration standards required to evaluate matrix effect? We are proposing that for evaluation of matrix effect calibration curve is not required. Area ratio method can be adopted as per EMEA Guideline on bioanalytical method validation "For each analyte and the IS, the matrix factor (MF) should be calculated for each lot of matrix, by calculating the ratio of the peak area in the presence of matrix (measured by analysing blank matrix spiked after extraction with analyte), to the peak area in absence of matrix (pure solution of the analyte). The IS normalised MF should also be calculated by dividing the MF of the analyte by the MF of the IS. The CV of the IS-normalised MF calculated from the 6 lots

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		of matrix should not be greater than 15 %. This determination should be done at a low and at a high level of concentration (maximum of 3 times the LLOQ and close to the ULOQ)".
276-279	2	**Comments:** It is not clear what is meant by "different methods". Are 2 fully validated methods with the same characteristics (sensitivity, calibration range, …) established at 2 different labs already different methods? Is a change in the calibration range considered as a different method? The given examples should not trigger a cross-validation. **Proposed change:** 1. Please clarify and delete "across" in line 276, or mention that x-validation across studies is only required if data should be compared. 2. Cross-validation of different methods (e.g. LBA vs LC-MS/MS) detecting/quantifying different fractions of the same analyte (e.g. total/free/target binding competent) would not make sense, this should also be stated. 3. Replace "comparison of those data is needed by "combination of those data is needed"
276-279	3	**Comments:** Propose to use identical text as 6.2 to prevent confusion: **Proposed change:** Cross validation is required (i) when data are obtained from different fully validated methods within a study, (ii) data are obtained from different fully validated methods across studies that are going to be combined or compared to support special dosing regimens, or regulatory decisions regarding safety, efficacy and labelling, or (iii) Data are obtained within a study from different laboratories with the same bioanalytical method. (Refer to Section 6.2)
276-279	4	**Comments:** Propose to use identical text as 6.2 to prevent confusion:

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: Cross validation is required (i) when data are obtained from different fully validated methods within a study, (ii) data are obtained from different fully validated methods across studies that are going to be combined or compared to support special dosing regimens, or regulatory decisions regarding safety, efficacy and labelling, or (iii) Data are obtained within a study from different laboratories with the same bioanalytical method. (Refer to Section 6.2)
281	3	**Comments:** general comment: Acknowledge the molecular diversity (which includes peptide and proteins) in chromatography and the impact on reference standards. Current section 3.1 is written around NCE.
281	4	**Comments:** general comment: Acknowledge the molecular diversity (which includes peptide and proteins) in chromatography and the impact on reference standards. Current section 3.1 is written around NCE.
286	5	**Comments:** testing (especially selection of theoretical value) and criteria for accuracy of stock solution should be specified
288	5	**Proposed change:** The absence of an IS should be ~~technically~~ justified.
286-288	3	**Comments:** Proposed change: It is recommended to add a suitable internal standard (IS) ~~should be added~~ to all calibration standards, QCs and study samples during sample processing.
286-288	4	**Comments:** delete, The validation data justifies the absence of the IS.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: ~~The absence of an IS should be technically justified~~
286-288	4	**Comments:** rephrase **Proposed change:** It is recommended to add a suitable internal standard (IS) ~~should be added~~ to all calibration standards, QCs and study samples during sample processing.
289	6	**Comments:** Strength can also mean potency and the terms can be used inter-changeably. It should also be considered that the chromatography section will also apply to large molecule analysis via chromatographic methods and potency may be interpreted differently for large molecules. **Proposed change:** Please define the terms "strength" and "purity" in the glossary
289-291	6	**Comments:** The sentence for reference standard and IS should be separated. **Proposed change:** "It is important that the reference standard is well characterised and the quality (purity, strength, identity) defined. The suitability of the IS should be ensured, as the quality will affect the outcome of the analysis and, therefore, the study data."
292-294	4	**Comments:** delete

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: ….study sample analysis should be obtained from an authentic and traceable source. ~~The reference standard should be identical to the analyte. If this is not possible, an established form (e.g., salt or hydrate) of known quality may be used.~~
293-294	5	**Comments:** The requirement of using reference standard identical to the analyte is questionable. Using salt or hydrate may improve dissolution in solution containing more water than organic solvent. This helps to avoid protein precipitation during spiking and allows to produce QC and calibration standards mimicking more closely real samples. **Proposed change:** Reference standard should be identical to the analyte, but salts or hydrates are acceptable.
293-294	6	**Comments:** It should be noted that large molecules may also by analysed using chromatographic methods. In these cases, it may not be possible to have exactly the same material as mentioned in Section 4.1.1. (Reference Standards for LBA). **Proposed change:** "If this is not possible, an established 294 form (e.g., salt or hydrate) of known quality may be used. **In the case of large molecules, it is recommended that the manufacturing batch of the reference standard used for the preparation of calibration standards and QCs is derived from the same batch of drug substance as that used for dosing in the nonclinical and clinical studies whenever possible. If the reference standard batch used for bioanalysis is changed, bioanalytical evaluation should be carried out prior to use to ensure that the performance characteristics of the method are within the acceptance criteria**."
295-296	4	**Comments:** delete

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: Suitable reference standards include compendial standards, commercially available standards or sufficiently characterised standards prepared in-house or by an external ~~non-commercial~~ organisation.
295-297	6	**Comments:** The potential sources of standards is not helpful. It has already been stated that the reference standard is suitably characterised. An external organisation is likely to be a commercial vendor, therefore the use of "non-commercial" is not appropriate. It is suggested that the whole sentence is removed. **Proposed change:** Remove the sentence: ~~"Suitable reference standards include compendial standards, commercially available standards or sufficiently characterised standards prepared in-house or by an external non-commercial organisation~~."
297-299	3	**Comments:** add **Proposed change:** A certificate of analysis (CoA) or an equivalent alternative is required to ensure quality and to provide information on the purity, storage conditions, retest or expiration date, batch or lot number and manufacturer or source of the reference standard
298-299	6	**Comments:** Add identity to the list of information for CoA. Strength is not included but a listed requirement for line 289. The requirements in lines 289 and 298 could be combined and presented as a simple list. **Proposed change:** "A certificate of analysis (CoA) or an equivalent alternative is required to ensure quality and to provide information on the purity,

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		storage conditions, **strength**, retest/expiration date and batch number of the reference standard." The requirements in lines 289 and 298 could be combined and presented as a simple list.
297-299	4	**Comments:** add **Proposed change:** A certificate of analysis (CoA) or an equivalent alternative is required to ensure quality and to provide information on the purity, storage conditions, retest or expiration date, batch or lot number and manufacturer or source of the reference standard
303-304	5	**Comments:** High isotope purity is not essential because the IS is added in a constant amount and a constant addition of the analyte does not influence quantitation (see A. Tan et al. doi: 10.1016/j.jchromb.2011.05.027). Instead of isotope purity, appropriate mass shift seems to be more important factor for the reliable results of bioanalysis. If the mass shift is not large enough, then variable analyte concentrations contribute to the IS signal and influence the quality of results. **Proposed change:** It is essential that mass shift between the analyte and the IS is large enough and that no exchange reaction occurs.
304-306	3	**Comments:** add **Proposed change:** The presence of unlabelled analyte should be checked and if unlabelled analyte is detected, the potential influence should be evaluated and/or reduced to an acceptable level during method validation.
304-306	4	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		add **Proposed change:** The presence of unlabelled analyte should be checked and if unlabelled analyte is detected, the potential influence should be evaluated and/or reduced to an acceptable level during method validation.
307-309	3	**Comments:** delete **Proposed change:** Stock and working solutions can only be prepared from reference standards that are within the stability period as documented in the CoA (either expiration date or the retest date) ~~in early development phase).~~
307-309	4	**Comments:** delete **Proposed change:** Stock and working solutions can only be prepared from reference standards that are within the stability period as documented in the CoA (either expiration date or the retest date) ~~in early development phase).~~
307-309	6	**Comments:** If the reference standard or stock solution is within stability then it should be possible to create a stock solution working stock if stability is demonstrated. Limiting this to only early development adds no value. **Proposed change:** "Stock ~~and working~~ solutions can only be prepared from reference standards that are within the stability period as documented in the CoA (either expiration date or the retest date~~ in early development phase~~)."

Line no.	Stakeholder no.	Comment and rationale; proposed changes
314-317	3	**Comments:** Suggested acceptance criteria: add **Proposed change:** Selectivity is evaluated using blank samples (matrix samples processed without addition of an analyte or IS) obtained from at least 6 individual sources/lots (non-haemolysed and non-lipaemic). At least 5 out of 6 should pass. Use of fewer sources may be acceptable in the case of rare matrices. Selectivity for the IS should also be evaluated.
314-317	3	**Comments:** The number of individual matrix sources required for selectivity and matrix effect assessment should take into account the diversity of the study population. An assessment in a single lot may be satisfactory for a study in a non-diverse nonclinical population - add **Proposed change:** Selectivity is evaluated using blank samples (matrix samples processed without addition of an analyte or IS) obtained from at least 6 individual sources/lots (non-haemolysed and non-lipaemic). Use of fewer sources may be acceptable in the case of rare matrices and when scientifically justified for non-clinical matrices. Selectivity for the IS should also be evaluated.
314-317	4	**Comments:** Suggested acceptance criteria: add **Proposed change:** Selectivity is evaluated using blank samples (matrix samples processed without addition of an analyte or IS) obtained from at least 6 individual sources/lots (non-haemolysed and non-lipaemic). At least 5 out of 6 should pass. Use of fewer sources may be acceptable in the case of rare matrices. Selectivity for the IS should also be evaluated.
314-317	4	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		The number of individual matrix sources required for selectivity and matrix effect assessment should take into account the diversity of the study population. An assessment in a single lot may be satisfactory for a study in a non-diverse nonclinical population - add **Proposed change:** Selectivity is evaluated using blank samples (matrix samples processed without addition of an analyte or IS) obtained from at least 6 individual sources/lots (non-haemolysed and non-lipaemic). Use of fewer sources may be acceptable in the case of rare matrices and when scientifically justified for non-clinical matrices. Selectivity for the IS should also be evaluated.
315	5	**Comments:** In most cases when serum and plasma are used, the haemolysed and lipaemic samples are expected during bioanalysis. Thus haemolysed and lipaemic sources should be included within 6 lots tested for selectivity. **Proposed change:** … at least 6 individual sources/lots including haemolysed and lipaemic ones if applicable.
320	5	**Comments:** Are undetected responses possible? **Proposed change:** Responses ~~detected and~~ attributable…
321	5	**Comments:** If the comment on a constant presence of analyte in the IS is accepted (lines 303-304) then criteria for LLOQ should be adapted accordingly. **Proposed change:** In case constant amount of analyte is present in the IS, then criteria for selectivity should be based on amount of analyte spiked

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		(after deduction of analyte added with the IS).
323-324	3	**Comments:** Instead of routine, this should be included as a for cause experiment: **Proposed change:** If required, ~~For the~~ the investigation of selectivity in lipaemic matrices at least one source of matrix should be used
323-324	4	**Comments:** Instead of routine, this should be included as a for cause experiment: **Proposed change:** If required, ~~For the~~ the investigation of selectivity in lipaemic matrices at least one source of matrix should be used
323-331	6	**Comments:** Suggest that the lipaemia assessment is only performed as a for cause approach rather than a routine validation parameter. **Proposed change:** "**The investigation of selectivity in lipaemic matrices should be performed as a for cause activity. When required, at least one source of matrix should be used. To be scientifically meaningful, the matrix used for these tests should be representative as much as possible of the expected study samples**."
324-325	5	**Comments:** Selectivity is tested during bioanalytical method validation, usually before clinical samples are collected. Thus, it is not possible to use matrix "representative as much as possible of the expected study samples" **Proposed change:** delete "To be scientifically meaningful, the matrix used for these tests should be representative as much as possible of the

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		expected study samples.”
332-334	3	**Comments:** rephrase **Proposed change:** If required ~~For the~~ investigation of selectivity in haemolysed matrices at least one source of matrix should be used. Haemolysed matrices are obtained by spiking matrix with haemolysed whole blood (at least 2% V/V) ~~to generate a visibly detectable haemolysed sample.~~
332-334	4	**Comments:** rephrase **Proposed change:** If required ~~For the~~ investigation of selectivity in haemolysed matrices at least one source of matrix should be used. Haemolysed matrices are obtained by spiking matrix with haemolysed whole blood (at least 2% V/V) ~~to generate a visibly detectable haemolysed sample.~~
332-334	6	**Comments:** Flexibility should be added to allow the use of a truly haemolysed sample rather than only samples spiked with whole blood. **Proposed change:** “For the investigation of selectivity in haemolysed matrices at least one source of matrix should be used. Haemolysed **matrices may be prepared** ~~are obtained~~ by spiking matrix with haemolysed whole blood (at least 2% V/V) to generate a visibly detectable haemolysed sample.”
338	5	**Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		isomer => isomers
341-345	6	**Comments:** This section may be interpreted differently within the community. Standard industry practice is to restrict the evaluation of comedications to only those that have been dosed in combination. Line 342 change "should" to "may". **Proposed change:** "If the presence of related substances is anticipated in the biological matrix of interest, the impact of such substances ~~should~~ **may** be evaluated during method validation, or alternatively, in the pre-dose study samples. In the case of LC-MS based methods, to assess the impact of such substances, the evaluation may include comparing the molecular weight of a potential interfering related substance with the analyte and chromatographic separation of the related substance from the analyte."
343-345	3	**Comments:** rephrase **Proposed change:** In the case of ~~LC~~-MS based methods, to assess the impact of such substances, the evaluation may ~~include~~ be done by comparing the molecular weight…
343-345	4	**Comments:** rephrase **Proposed change:** In the case of ~~LC~~-MS based methods, to assess the impact of such substances, the evaluation may ~~include~~ be done by comparing the molecular weight…
355-356	3	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		rephrase **Proposed change:** The extent of back-conversion should be established, controlled where possible. If present, the impact on the study results should be discussed in the Bioanalytical Report.
355-356	4	**Comments:** rephrase **Proposed change:** The extent of back-conversion should be established, controlled where possible. If present, the impact on the study results should be discussed in the Bioanalytical Report.
357	3	**Comments:** 2 levels x 3 replicates x 6 sources (+ haemolysed + lipaemic) = excessive for ALL validations at all stages of development… also, consider 3Rs (purpose bred – 1 per species should be sufficient).
357	4	**Comments:** 2 levels x 3 replicates x 6 sources (+ haemolysed + lipaemic) = excessive for ALL validations at all stages of development… also, consider 3Rs (purpose bred – 1 per species should be sufficient).
357-370	5	**Comments:** This is one of the weakest sections of the guideline. The experimental design do not allow to asses influence of ME, because 3 replicates for each lot and evaluation based on individual lots may mask the between lot variability. **Comments:** Estimation of matrix effect based on the QCs accuracy and precision values only is not sufficient. It seems that the matrix factor (MF) or IS-normalised matrix factor better describe a possible influence of the matrix components on ionisation of analytes (see

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		EMA bioanalytical method validation guideline). Instead of matrix factors or IS-normalised matrix factors other approaches may be also considered for recommendation (Matuszewski BK et al. Anal Chem. 2003;75(13):3019-30; Matuszewski BK. J Chromatogr B 2006;830(2):293-300; Bienvenu JF doi: 10.1021/acs.analchem.7b01383; Adamowicz and Wrzesien doi: 10.1134/S1061934816080025). IS normalized matrix factors and IS normalised relative matrix effect do not show relevant differences (Rudzki PJ et al. doi: 10.1016/j.jpba.2018.03.052). IS normalized matrix factors are influenced by the variability of response in neat solutions thus IS normalised relative matrix effect may be more fit-for-purpose approach. Matrix effect evaluation should be dedicated to LC-MS methods only, as for other detectors "invisible compounds" do not influence quantification. Haemolysed and lipaemic matrix – due to its different composition - should be mandatory among 6 lots tested (if applicable). Clear description of the text including formulas for calculation should be added. Proposed change: The matrix effect should be evaluated by analysing at least low and high QCs, each prepared using different source/lot of matrix (at least 6 different sources/lots should be used). The matrix factor (MF) should be estimated for each analyte and the IS by calculating the ratio of the peak area of the analyte added to blank matrix after extraction, to the peak area of the analyte in pure solution. The IS normalised MF should be estimated by dividing the MF of the analyte by the MF of the IS. The CV of the IS-normalised MF calculated from the 6 different sources of matrix should not be greater than 15 %. Use of fewer sources/lots may be acceptable in the case of rare matrices.
357-370	6	**Comments:** The absence of Matrix Factor in the document is welcomed.
359-360	5	**Comments:** Phrase "During method validation it is necessary to evaluate the matrix effect between different independent sources/lots." is unnecessary. Proposed change: delete phrase "During method validation it is necessary to evaluate the matrix effect between different independent sources/lots."
361-365	6	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Sources/lots is a confusing term; change to the term "individuals" rather than sources/lots. Fewer lots should be possible for preclinical assessments given that there is no variability seen from purpose bred animals. Propose to align with stability assessments and use mean accuracy. **Proposed change:** "The matrix effect should be evaluated by analysing at least 3 replicates of low and high QCs, each prepared using matrix from at least 6 different **individuals** ~~sources/lots~~. The **mean** accuracy should be within ±15% of the nominal concentration and the precision (per cent coefficient of variation (%CV)) should not be greater than 15% in all individual matrix sources/lots. Use of fewer **individuals** ~~sources/lots~~ may be acceptable in the case of rare matrices and preclinical methods."
364	5	**Comments:** Main matrix effect influence on reliability of bioanalysis is due to variability between matrix sources not within individual matrix. Proposed change: individual => all combined
366-370	3	**Comments:** refine **Proposed change:** The matrix effect should also be evaluated in haemolysed and hyperlipidaemic control matrix, where applicable, and in relevant patient populations or special populations (e.g., hepatically impaired or renally impaired) when available. QC samples should be prepared in at least a single source/lot of this control matrix, at LOW and HIGH concentrations and should be extracted and analysed. The acceptance criteria (RE and CV) is the same as for the assessment of intra-batch accuracy and precision. The evaluation of lipaemic matrices is not necessary for preclinical studies unless the drug impacts lipid metabolism or is administered in a particular animal strain that is hyperlipidaemic'
366-370	4	**Comments:** refine

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: The matrix effect should also be evaluated in haemolysed and hyperlipidaemic control matrix, where applicable, and in relevant patient populations or special populations (e.g., hepatically impaired or renally impaired) when available. QC samples should be prepared in at least a single source/lot of this control matrix, at LOW and HIGH concentrations and should be extracted and analysed. The acceptance criteria (RE and CV) is the same as for the assessment of intra-batch accuracy and precision. The evaluation of lipaemic matrices is not necessary for preclinical studies unless the drug impacts lipid metabolism or is administered in a particular animal strain that is hyperlipidaemic'
367	5	**Proposed change:** when available => when applicable
371	5	**Proposed change:** Calibration Curve ~~and Range~~
371-407	5	**Comments:** Number of calibration curves to define model should be recommended. There are no acceptance criteria specified for blank sample and zero sample. **Proposed change:** Six calibration curves to define model should be used.
375	6	**Comments:** The requirement of the same biological matrix may be restrictive and will not be possible for endogenous proteins using a chromatography approach **Proposed change:** Add an additional sentence to state that alternative matrices may be used if appropriately justified. Add "**If standards cannot be**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		prepared in the same biological matrix due to the presence of endogenous counterpart, please refer to Section 7.1"
375-378	3	**Comments:** add **Proposed change:** ….Calibration standards should be prepared in the same biological matrix as the study samples. (delete: The calibration range is defined by the LLOQ, which is the lowest calibration standard, and the ULOQ, which is the highest calibration standard.). For rare and difficult to obtain matrices, tissues and endogenous methods, the use an appropriate surrogate matrix is allowed. The calibration range should be appropriate for the analysis of samples. In the event that a significant number of samples require diluting into the calibration range, partial validation of an overlapping or non-overlapping higher calibration range is recommended. There should be one calibration curve for each analyte studied during method validation and for each analytical run
375-378	4	**Comments:** add **Proposed change:** ….Calibration standards should be prepared in the same biological matrix as the study samples. (delete: The calibration range is defined by the LLOQ, which is the lowest calibration standard, and the ULOQ, which is the highest calibration standard.). For rare and difficult to obtain matrices, tissues and endogenous methods, the use an appropriate surrogate matrix is allowed. The calibration range should be appropriate for the analysis of samples. In the event that a significant number of samples require diluting into the calibration range, partial validation of an overlapping or non-overlapping higher calibration range is recommended. There should be one calibration curve for each analyte studied during method validation and for each analytical run
379	6	**Comments:** This sentence can be misleading **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Reword to "A calibration curve should consist of at least 6 concentration levels of calibration standards, between and including the LLOQ and the ULOQ. Also add a sentence: "**A blank sample and a zero sample (blank sample spiked with IS) should also be included in the bioanalytical run**."
379-381	3	**Comments:** add **Proposed change:** Additional concentration levels are required when non-linear e.g. quadratic, regression analysis is used.
379-381	4	**Comments:** rephrase **Proposed change:** A calibration curve should be generated with ~~a blank sample, a zero sample (blank sample spiked with IS), and~~ at least 6 concentration levels of calibration standards, including the LLOQ and the ULOQ. A blank sample and zero sample should be included in the run.
379-381	4	**Comments:** add **Proposed change:** Additional concentration levels are required when non-linear e.g. quadratic, regression analysis is used.
382	5	**Proposed change:** A simple => The simplest
382	6	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Intention is likely that the simplest regression model rather than a simple regression model would be used **Proposed change:** "~~A simple~~ **The simplest** regression model that adequately describes the concentration-response relationship should be used."
382-388	3	**Comments:** Regression model selection needs to be documented (SOP) isn't needed. Suggest to remove.
382-388	4	**Comments:** Regression model selection needs to be documented (SOP) isn't needed. Suggest to remove.
391-392	2	**Comments:** What does several days mean? **Proposed change:** Rephrase to "All acceptable curves obtained during validation, based on a minimum of 3 independent runs over at least 2 days, should be reported" (see also line 428-429).
393-394	5	**Comments:** guideline defines the small molecules acceptance criteria for LLOQ and ULOQ at 20% and 15%, respectively. These two points on a calibration curve are determined with higher uncertainty than others. It is caused by possible variation of slope between sequences. This is why one may observe wider confidence interval for calibration curve at LLOQ and ULOQ than in the middle of the concentration range in the case of y = ax + b model. (see M. Kaza et al. doi: 10.1016/j.jpba.2018.12.030) **Proposed change:** accuracy of the back-calculated concentrations of each calibration standard should be within ±20% of the nominal concentration at the LLOQ and ULOQ, and within ±15% at all the other levels

Line no.	Stakeholder no.	Comment and rationale; proposed changes
394-403	6	**Comments:** Lines 394-396 and lines 397-403 appear to be contradictory. Please clarify, if calibration standards are run in multiple replicates, do you include all the individual replicates in the 75% acceptance criteria? **Proposed change:** "At least 75% of the calibration standards with a minimum of 6 calibration standard levels **including LLOQ and ULOQ** should meet the above criteria"
397	6	**Comments:** Appears that LLOQ could be 15% or 20% therefore suggest adding a comma and remove the word "or" **Proposed change:** "(within +/-15% ~~or~~**,** 20% for LLOQ)"
397-404	3	**Comments:** There is a clear discrepancy in criteria when a single CAL or replicates are used. When a single CAL is used, it is accepted to fail in one conc. level if at least 6 con. levels remain. However, when 2 CALs/level are used, and two CALs/level fail (not the 50%, as stated), but there are also 6 levels remaining, the calibration curve is not accepted. (this is also in contradiction with the section "3.3.2 Acceptance Criteria for an Analytical Run" when it is stated that "If replicate calibration standards are used and only one of the LLOQ or ULOQ standards fails, the calibration range is unchanged". **Proposed change:** delete: ~~In the case that replicates are used, the criteria (within ±15% or ±20% for LLOQ) should also be fulfilled for at least 50% of the calibration standards tested per concentration level.~~
397-404	4	**Comments:** There is a clear discrepancy in criteria when a single CAL or replicates are used. When a single CAL is used, it is accepted to fail in

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		one conc. level if at least 6 con. levels remain. However, when 2 CALs/level are used, and two CALs/level fail (not the 50%, as stated), but there are also 6 levels remaining, the calibration curve is not accepted. (this is also in contradiction with the section "3.3.2 Acceptance Criteria for an Analytical Run" when it is stated that "If replicate calibration standards are used and only one of the LLOQ or ULOQ standards fails, the calibration range is unchanged". **Proposed change:** delete: ~~In the case that replicates are used, the criteria (within ±15% or ±20% for LLOQ) should also be fulfilled for at least 50% of the calibration standards tested per concentration level.~~
402	5	**Proposed change:** the sentence should end after the phrase "then the run should be rejected".
403-404	4	**Comments:** rephrase **Proposed change:** delete: … should be rejected, the possible source of the failure should be determined ~~and the method revised if necessary. If the next validation run also fails, then the method should be revised before restarting validation.~~
403-404	6	**Comments:** Failure of two consecutive runs may not be for the same reason and this language does allow for failures due to different reasons **Proposed change:** "If the next validation run also fails **for the same reason**, then the method should be revised before restarting validation."
405-407	6	**Comments:** Allow flexibly for previously prepared and stored (i.e. frozen or refrigerated) calibration standards

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: Change to "Subsequently, ~~frozen~~ **previously prepared and stored** calibration standards can be used within their defined period of stability"
408-414	1	**Comment:** The stability of the stock and working solutions of the analyte and IS should be determined under the storage conditions used during the analysis of study samples by using the lowest and the highest concentrations of these solutions. If the stability varies with concentration, then the stability of all concentrations of the stock and working solutions needs to be assessed. As per this statement stock solution stability should be performed at lowest (LLOQ) and Highest (ULLOQ) levels? **Proposed change:** We are proposing that stock solution stability can be evaluated at LQC (3 times of the LLOQ) and HQC concentration levels. Since at LLOQ concentration high variations are expected and at ULLOQ the concentration may fall above the concentration range.
410-412	5	**Comments:** It is not always possible to estimate exact number of QCs for the whole study to meet recommendation "storing them under the conditions anticipated for study samples" . Thus using freshly prepared QCs should be acceptable. Validating of storage is done by the long-term stability test. **Proposed change:** Add sentence: "Using freshly prepared QC is acceptable."
414-421	1	**Comment:** "The routine practice of making stock and working solutions from reference standards solely for extending the expiry date for the use of the reference standard is not acceptable." The sentence is ambiguous, it could be understood in two ways: • The described practice, which is routine in the industry, is completely unacceptable.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		• It is acceptable to follow the described practice occasionally, but not routinely. The wording of lines 417-419 ("If the reference standard expires, or it is past the retest date, the stability of the stock solutions made previously with this lot of reference standard are defined by the expiration or retest date established for the stock solution.") would suggest the latter is the correct interpretation. However, in such case, the meaning of the word "routinely" is left out for interpretation. Finally, it is unclear why this "routine practice" is not allowed, since the stability of stock and working solutions would have been demonstrated anyway. **Proposed change:** **Remove the sentence**
415-416	5	**Comments:** unnecessary repeated lines 285-286. **Proposed change:** Delete repeated information
415-416	6	**Comments:** Stock stability is described later in the document. Stability of the stock solutions adds no value to this section; the key parameter is accuracy of the stock solution. Remove "and stability" **Proposed change:** "However, calibration standards and the QCs may be prepared from the same stock solution, provided the accuracy ~~and stability~~ of the stock solution have been verified"
417	2	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		it is impossible to prepare Cals and QCs using matrix-effect free matrix, at least for LBAs. **Proposed change:** Delete "or matrix effects"
417	5	**Comments:** Blank matrix does not need to be free of matrix effect if the IS is compensating matrix effect. **Proposed change:** which should be free of interference ~~or matrix effects, as described in Section 3.2.3~~.
419-421	3	**Comments:** change **Proposed change:** During method validation the QCs should be prepared at a minimum of 4 concentration levels within the calibration curve range: the LLOQ, within three times of the LLOQ (low QC), around geometric mean of the calibration curve range (medium QC) and at least 75% of the ULOQ (high QC).
419-421	4	**Comments:** change **Proposed change:** During method validation the QCs should be prepared at a minimum of 4 concentration levels within the calibration curve range: the LLOQ, within three times of the LLOQ (low QC), around geometric mean of the calibration curve range (medium QC) and at least 75% of the ULOQ (high QC).
419-421	6	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		It should be permissible to prepare QCs using a geometric mean rather then 30-50% of the calibration curve range. **Proposed change:** "During method validation the QCs should be prepared at a minimum of 4 concentration levels within the calibration curve range: the LLOQ, within three times of the LLOQ (low QC), around ~~30 – 50%~~ **the geometric mean** of the calibration curve range (medium QC) and at least 75% of the ULOQ (high QC)."
421	5	**Comments:** To avoid problems with high QC being over ULOQ, HQC should be lower than ULOQ. **Proposed change:** "at least 75% of the ULOQ (high QC)" => "75%-85% of the ULOQ (high QC)"
434-437	5	**Comments:** What is the difference between "overall estimate of within-run accuracy and precision for each QC level" and "Between-run (intermediate) precision and accuracy"? Please provide formulas for calculation. Consistent use of "accuracy and precision" instead of "precision and accuracy" is suggested.
436-437	3	**Comments:** remove "(intermediate)" **Proposed change:** Between-run ~~(intermediate)~~ precision and accuracy should be calculated by combining the data from all runs.
436-437	4	**Comments:** remove "(intermediate)" **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Between-run ~~(intermediate)~~ precision and accuracy should be calculated by combining the data from all runs.
438-440	5	**Comments:** unnecessary repeated lines 405-407. **Proposed change:** Delete repeated information
241	2	**Comments:** It is not clear if the medium QC conc. should be based on arithmetic or geometric mean
448-449	5	**Comments:** How many blank samples are recommended? How many pairs ULOQ-blank-sample should be analysed? **Proposed change:** ~~Carry-over should be assessed and minimised during method development.~~ ~~During validation~~ carry-over should be assessed by analysing blank samples after the calibration standard at the ULOQ.
458-461	3	**Comments:** in consideration of 3Rs, delete **Proposed change:** Dilution integrity …… The same matrix ~~from~~ ~~the same species~~ used for preparation of ……..should not exceed 15%.
458-461	4	**Comments:** in consideration of 3Rs, delete **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Dilution integrity …… The same matrix ~~from the same species~~ used for preparation of ……..should not exceed 15%.
462-468	3	**Comments:** It is difficult to determine in validation which exact dilution ratio will be needed during sample analysis. Currently, one dilution ratio is performed during validation. At the time of sample analysis, QCs prepared above the ULOQ are diluted at the same ratio as study samples and included in the run which is a sufficient evaluation of dilution integrity. Delete **Proposed change:** ~~The dilution ratio(s) applied during study sample analysis should be within the range of the dilution rations evaluated during validation. The mean accuracy of the dilution QCs should be within ±15% of the nominal concentration and the precision (%CV) should not exceed 15%.~~
462-468	4	**Comments:** It is difficult to determine in validation which exact dilution ratio will be needed during sample analysis. Currently, one dilution ratio is performed during validation. At the time of sample analysis, QCs prepared above the ULOQ are diluted at the same ratio as study samples and included in the run which is a sufficient evaluation of dilution integrity. Delete **Proposed change:** ~~The dilution ratio(s) applied during study sample analysis should be within the range of the dilution rations evaluated during validation. The mean accuracy of the dilution QCs should be within ±15% of the nominal concentration and the precision (%CV) should not exceed 15%.~~
465-466	5	**Comments:** This part of guideline is about validation not study sample analysis, thus the sentence could be rephrased. **Proposed change:** The dilution ratio(s) evaluated during validation should be within the range of the dilution ratios expected during study sample

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		analysis.
469-470	3	**Comments:** rephrase **Proposed change:** ~~In~~ The ~~cases of rare matrices~~ use of a surrogate matrix for dilution may be acceptable, as long as it has been demonstrated that this does not affect precision and accuracy
469-470	4	**Comments:** rephrase **Proposed change:** ~~In~~ The ~~cases of rare matrices~~ use of a surrogate matrix for dilution may be acceptable, as long as it has been demonstrated that this does not affect precision and accuracy
471-493	5	**Comments:** To avoid differences in handling storage temperature issues the paragraph on stability could be supplemented with standard naming terminology for four main storage conditions: room temperature, refrigerator, freezer, and ultra-freezer (see Table 1 in M.J. Redrup et al. http://dx.doi.org/10.1208/s12248-016-9869-2). **Proposed change:** add standard naming terminology for main storage conditions
480-481	5	**Comments:** are concentrations in low and high stability QCs corresponding to concentrations in low and high QCs? How does "stability QC" differ from QC? Is the term "stability QC" necessary?

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: use QC instead of stability QC
480-483	3	**Comments:** industry remains unclear on the requirements to evaluate in triplicate/QC level
480-483	3	**Comments:** rephrase **Proposed change:** Stability of the analyte in the studied matrix is evaluated using low and high concentration stability QCs. Aliquots of the low and high stability QCs are analysed ~~at time zero~~ and after the applied storage conditions that are to be evaluated. Analysis of the stability QCs prior to storage (e.g. at t=0) may be informative with respect to confirming that they have been correctly prepared, but is not required.
480-483	4	**Comments:** industry remains unclear on the requirements to evaluate in triplicate/QC level
480-483	4	**Comments:** rephrase **Proposed change:** Stability of the analyte in the studied matrix is evaluated using low and high concentration stability QCs. Aliquots of the low and high stability QCs are analysed ~~at time zero~~ and after the applied storage conditions that are to be evaluated. Analysis of the stability QCs prior to storage (e.g. at t=0) may be informative with respect to confirming that they have been correctly prepared, but is not required.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
480-483	6	**Comments:** The language is not particularly clear and could mean 3 aliquots of a single preparation or 3 independent preparations of each QC concentration level/storage condition/time point. It is recommended that a single preparation is used for this assessment rather than 3 independent spikes which only serves to test pipetting technique. Therefore, further clarification is needed as to what is required. **Proposed change:** "A minimum of ~~three~~ **one stability** ~~QCs~~ **QC** should be prepared**, divided into at least 3 aliquots** ~~and analysed~~ per concentration level/storage condition/timepoint **and analysed**."
481	2	**Comments:** Unclear how many aliquots should be analysed per QC sample **Proposed change:** Change to "**At least 3 aliquots** of the low and high …"
481-482	5	**Comments:** is it acceptable to use freshly prepared samples (at the day of analysis of samples after storage) as reference samples?
482-483	2	**Comments:** It is not clear what "prepared means". QCs should be prepared in bulk. **Proposed change:** Rephrase the last sentence so that it reads: "A minimum of three stability QC sample aliquots should be stored and analysed per concentration level/storage condition/timepoint"
484	6	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		For the cases where rare matrix is used, the amount of matrix required for regularly preparing fresh calibrators can be restrictive and not feasible. Once stability has been demonstrated then it should be possible to use frozen calibration curves. It should also be considered that in some cases such as ADCs there may be a safety reason why a frozen curve may be used. It is recognised that there needs to be an element of either a fresh calibration curve or fresh QCs. There should be flexibility that either the curve or the QCs can be fresh rather than only allowing a fresh calibration curve. **Proposed change:** Add "**Alternative experimental design for stability assessments may be used if scientifically justified**."
484-486	2	**Comments:** It is unclear for which purpose the freshly prepared QCs or QCs for which stability has been proven should be used. For analytical run acceptance? **Comments:** Please rephrase to " …with analytical run acceptance QCs (freshly prepared or for which stability has been proven).
489-490	3	**Comments:** delete **Proposed change:** …unless it is recognised that this may not be possible ~~in nonclinical studies~~ due to solubility limitations.
489-490	4	**Comments:** delete **Proposed change:** …unless it is recognised that this may not be possible ~~in nonclinical studies~~ due to solubility limitations.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
484-490	5	**Comments:** Acceptance criteria for comparison of stability QCs and reference QCs (freshly prepared or with proven stability) concentrations are missing. Using described criteria +14.9% for reference samples and -14.9% for stability samples is acceptable. This extreme example shows that difference of 29.8% loss of analyte during stability testing is acceptable. **Proposed change:** use 90% confidence intervals for comparison of tested to reference samples (see Rudzki and Leś, Acta Pol. Pharm 2008;65(6):743-7).
487-489	5	**Comments:** Validation should be performed before analysis of study samples. Thus, concentrations of the study samples are unknown during method validation. Results for adjusted high stability QC may be presented in Bioanalytical Report. **Proposed change:** Add "Results for adjusted high stability QC should be presented in Bioanalytical Report"
489-490	6	**Comments:** Solubility challenges may not be limited to preclinical studies and can also be seen in clinical studies. Delete "in nonclinical studies." **Proposed change:** "It is recognised that this may not be possible ~~in nonclinical studies~~ due to solubility limitations."
491-493	3	**Comments:** rephrase **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		If multiple analytes are present in the study samples (e.g., studies with a fixed combination, or due to a specific drug regimen) the stability test of an analyte in matrix containing all dosed compounds should be considered. In the case of a fixed combination stability information of the combination dosage form may be considered. In the case of a drug regimen, the known chemistry and stabilities of the individually dosed drugs should be used as a basis for determining whether additional stability studies are needed. DDI studies are not is scope of this requirement.
491-493	4	**Comments:** rephrase **Proposed change:** If multiple analytes are present in the study samples (e.g., studies with a fixed combination, or due to a specific drug regimen) the stability test of an analyte in matrix containing all dosed compounds should be considered. In the case of a fixed combination stability information of the combination dosage form may be considered. In the case of a drug regimen, the known chemistry and stabilities of the individually dosed drugs should be used as a basis for determining whether additional stability studies are needed. DDI studies are not is scope of this requirement.
491-493	6	**Comments:** The stability assessment in this case would be a retrospective evaluation and this needs to be clear. The cases where there is an impact are rare and can generally be predicted ahead of time. **Proposed change:** Add language that this should be a for cause assessment rather than a routine validation parameter. However, if this is not acceptable then the language should be changed to: "If multiple ~~analytes~~ **drugs or expected drug related materials** are present in the study samples (e.g. **where known metabolites are present,** studies with a fixed combination or due to a specific drug regimen) the stability ~~test of an analyte~~ in matrix should be conducted with the matrix containing all ~~the analytes~~ **dosed drug molecules**."

Line no.	Stakeholder no.	Comment and rationale; proposed changes
496-509	3	**Comments:** delete **Proposed change:** 1) Stability of stock and working solutions The stability of the stock and working solutions of the analyte and IS should be determined under the storage conditions used during the analysis of study samples by using the lowest and the highest concentrations of these solutions. ~~They are assessed using the response of the detector. Stability of the stock and working solutions should be tested with an appropriate dilution, taking into consideration the linearity and measuring range of the detector.~~ If the stability …… solution. ~~The routine practice of making stock and working solutions from reference standards solely for extending the expiry date for the use of the reference standard is not acceptable.~~
496-509	4	**Comments:** delete **Proposed change:** 1) Stability of stock and working solutions The stability of the stock and working solutions of the analyte and IS should be determined under the storage conditions used during the analysis of study samples by using the lowest and the highest concentrations of these solutions. ~~They are assessed using the response of the detector. Stability of the stock and working solutions should be tested with an appropriate dilution, taking into consideration the linearity and measuring range of the detector.~~ If the stability …… solution. ~~The routine practice of making stock and working solutions from reference standards solely for extending the expiry date for the use of the reference standard is not acceptable.~~
498	5	**Comments:** Stock and working solutions are sometimes prepared in different solvents. **Proposed change:** … the highest concentrations of these solutions. =>… the highest concentrations of these solutions in particular solvent.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
500-502	6	**Comments:** It should be considered that for macromolecules assessed by chromatography, the reference standard will usually be in liquid form and not powder form. Therefore, if the same formulation buffer for a working stock is created then further stability assessments are not needed.
507-509	2	**Comments:** Stability of stock and working solutions **Proposed change:** Stability of stock or working solutions may not exceed the stability of the used reference standard
510, 519, 528, 535, 546	5	**Comments:** Subtitles are misleading, because stability of the analyte (not matrix / sample/ whole blood) is investigated **Proposed change:** Freeze-thaw stability in matrix, Bench top (short-term) stability in matrix, Stability in processed sample, long-term stability in matrix, stability in whole blood
516-518	3	**Comments:** delete **Proposed change:** …… cycles undergone by the study samples~~, but a minimum of three cycles should be conducted~~
516-518	4	**Comments:** delete

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: …… cycles undergone by the study samples~~, but a minimum of three cycles should be conducted~~
516-518	6	**Comments:** The number of samples should be appropriate to cover the study samples, a minimum of 3 cycles should not be needed. **Proposed change:** "The number of freeze-thaw cycles validated should equal or exceed that of the freeze-thaw cycles undergone by the study samples~~, but a minimum of three cycles should be conducted~~."
522-524	3	**Comments:** delete **Proposed change:** ~~Low and high stability QCs should be thawed in the same manner as the study samples and kept on the bench~~
522-524	4	**Comments:** delete **Proposed change:** ~~Low and high stability QCs should be thawed in the same manner as the study samples and kept on the bench~~
525	6	**Comments:** Real-life situation does not reflect this statement; samples will go in and out of the freezer. Actual handling conditions should be reflected in the stability experiment. **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Remove "~~The total time on the bench top should be concurrent~~"
528-534	3	**Comments:** rephrase **Proposed change:** 4) Processed sample stability: The stability of processed samples, including the time until completion of analysis (in the autosampler/instrument), should be determined. ~~For example (i) Stability of the processed sample at the storage conditions to be used during the analysis of study samples (dry extract or in the injection phase) (ii) On-instrument/ autosampler stability of the processed sample at injector or autosampler temperature.~~
528-534	4	**Comments:** rephrase **Proposed change:** 4) Processed sample stability: The stability of processed samples, including the time until completion of analysis (in the autosampler/instrument), should be determined. ~~For example (i) Stability of the processed sample at the storage conditions to be used during the analysis of study samples (dry extract or in the injection phase) (ii) On-instrument/ autosampler stability of the processed sample at injector or autosampler temperature.~~
528-534	6	**Comments:** For all other experiments fresh against frozen is assessed. This assessment is for sample stability following processing and could lead to poor practices being encouraged. Move to Section 3.2.9 and call processed sample viability. **Proposed change:** Move to Section 3.2.9 and name "Processed sample ~~stability~~ **viability**"
535-544	3	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		delete **Proposed change:** 5) Long-term matrix stability: The long-term stability of the analyte in matrix stored in the freezer should be established. Low and high stability QCs should be stored in the freezer under the same storage conditions and at least for the same duration as the study samples. ~~For chemical drugs,~~ It is considered acceptable to extrapolate the stability at one temperature (e.g., -20°C) to lower temperatures (e.g., -70°C). ~~For biological drugs, it is acceptable to apply a bracketing approach, e.g., in the case that the stability has been demonstrated at -70°C and at -20°C, then it is not necessary to investigate the stability at temperatures in between those two points at which study samples will be stored.~~
535-544	4	**Comments:** delete **Proposed change:** 5) Long-term matrix stability: The long-term stability of the analyte in matrix stored in the freezer should be established. Low and high stability QCs should be stored in the freezer under the same storage conditions and at least for the same duration as the study samples. ~~For chemical drugs,~~ It is considered acceptable to extrapolate the stability at one temperature (e.g., -20°C) to lower temperatures (e.g., -70°C). ~~For biological drugs, it is acceptable to apply a bracketing approach, e.g., in the case that the stability has been demonstrated at -70°C and at -20°C, then it is not necessary to investigate the stability at temperatures in between those two points at which study samples will be stored.~~
539-544	6	**Comments:** There is no industry or known literature examples that require macromolecules to be treated differently from chemical drugs. Delete the requirement that macromolecules must be tested at two temperatures. **Proposed change:** "~~For chemical drugs,~~ It is considered acceptable to extrapolate the stability at one temperature (e.g., -20°C) to lower temperatures (e.g., -70°C).

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		~~For biological drugs, it is acceptable to apply a bracketing approach, e.g., in the case that the stability has been demonstrated at -70°C and at -20°C, then it is not necessary to investigate the stability at temperatures in between those two points at which study samples will be stored."~~
546-553	3	**Comments:** rephrase **Proposed change:** ~~1) Whole blood stability:~~ Sample collection integrity: Sufficient attention should be paid to the ~~stability~~ integrity of the analyte in the sampled matrix ~~(blood)~~ directly after collection from subjects and prior to preparation for storage to ensure that the concentrations obtained by the analytical method reflect the concentrations of the analyte in the subject's ~~blood~~ sample at the time of sample collection. Conditions for sample collection should be identified during method development or validation. If the matrix used is plasma ~~or serum~~, the stability of the analyte in blood should be evaluated considered during method development ~~(e.g., using an exploratory method in blood)~~ , and, in the case of molecules that are, based on their structure, potentially unstable, assessed during method validation. The results of such assessments or, in the event they are not conducted, rationale for their absence, should be provided in the Validation Report.
546-553	4	**Comments:** rephrase **Proposed change:** ~~1) Whole blood stability:~~ Sample collection integrity: Sufficient attention should be paid to the ~~stability~~ integrity of the analyte in the sampled matrix ~~(blood)~~ directly after collection from subjects and prior to preparation for storage to ensure that the concentrations obtained by the analytical method reflect the concentrations of the analyte in the subject's ~~blood~~ sample at the time of sample collection. Conditions for sample collection should be identified during method development or validation. If the matrix used is plasma ~~or serum~~, the stability of the analyte in blood should be evaluated considered during method development ~~(e.g., using an exploratory method in blood)~~ , and, in the case of molecules that are, based on their structure,

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		potentially unstable, assessed during method validation. The results of such assessments or, in the event they are not conducted, rationale for their absence, should be provided in the Validation Report.
547-553	6	**Comments:** Whole blood stability is not relevant for serum as you cannot perform the assessment in the same matrix. Flexibility should be added to allow whole blood stability to be on a case by case approach. Remove "or serum" in line 551 and start line 547 with "Where relevant, sufficient …" **Proposed change:** **"Where relevant,** sufficient attention should be paid to the stability of the analyte in the sampled matrix (blood) directly after collection from subjects and prior to preparation for storage to ensure that the concentrations obtained by the analytical method reflect the concentrations of the analyte in the subject's blood at the time of sample collection. If the matrix used is plasma ~~or serum~~, the stability of the analyte in blood should be evaluated during method development (e.g., using an exploratory method in blood) or during method validation. The results should be provided in the Validation Report."
553	6	**Comments:** Whole blood stability can be performed in method development and not necessarily during validation. Therefore, the last sentence in the whole blood stability section should be removed. **Proposed change:** Remove "~~The results should be provided in the Validation Report~~"
554	3	**Comments:** change title **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		3.2.9. Processed sample Viability
554	4	**Comments:** change title **Proposed change:** 3.2.9. Processed sample Viability
555-559	3	**Comments:** replace **Proposed change:** The viability of processed samples supporting the storage of processed samples before analysis and re-analysis in the event of an analytical run failing to complete or the entire run needing to be reinjected due to technical error, including the time until completion of analysis (in the autosampler/instrument), should be determined. Re-inject a stored run, comprising of calibration and QC samples, if sufficient processed sample volume permits. The re-injected run should include a minimum n=5 replicates of the low and high QCs. Calculate the QC results from the re-injected calibration curve regression and assess the assay accuracy and precision criteria.
555-559	4	**Comments:** replace **Proposed change:** The viability of processed samples supporting the storage of processed samples before analysis and re-analysis in the event of an analytical run failing to complete or the entire run needing to be reinjected due to technical error, including the time until completion of analysis (in the autosampler/instrument), should be determined. Re-inject a stored run, comprising of calibration and QC samples, if sufficient processed sample volume permits. The re-injected run should include a minimum n=5 replicates of the low and high QCs. Calculate the QC results from the re-injected calibration curve regression and assess the assay accuracy

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		and precision criteria.
555-559	5	**Comments:** Reinjection reproducibility should not be mandatory if bioanalytical laboratory do not use reinjection. QC concentrations and number of samples to be analysed are not specified. Time difference between original and repeated injection during the study should not exceed time difference between original and repeated injection during validation. **Proposed change:** Test should be conducted using low and high QC samples, minimum of 5 samples per concentration. Time difference between original and repeated injection during sample analysis should not exceed time difference between original and repeated injection during validation.
555-559	6	**Comments:** Rename this section to "Processed Sample Viability" **Proposed change:** Rename this section to "Processed Sample ~~Stability~~ **Viability**" "**The viability of processed samples supporting the storage of processed samples before analysis and re-analysis in the event of an analytical run failing to complete or the entire run needing to be reinjected due to technical error, including the time until completion of analysis (in the autosampler/instrument), should be determined. Re-inject a stored run, comprising of calibration and QC samples, if sufficient processed sample volume permits. The re-injected run should include a minimum n=5 replicates of the low and high QCs. Calculate the QC results from the re-injected calibration curve regression and assess the assay accuracy and precision criteria**."
577	6	**Comments:** Whilst it is accepted that samples are bracketed by QCs, there is no mention what happens if the middle set of QCs fail on large batches, please clarify in the document.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
578-579	5	**Comments:** repeated lines 415-416 **Proposed change:** delete lines 578-579
587-590	3	**Comments:** It is suggested to eliminate batch vs. run acceptance approach
587-590	4	**Comments:** It is suggested to eliminate batch vs. run acceptance approach
591-595	3	**Comments:** Some information is redundant with section 3.2.6. Consider reducing redundancy
591-595	4	**Comments:** Some information is redundant with section 3.2.6. Consider reducing redundancy
591-595	4	**Comments:** delete **Proposed change:** … samples, injection of blank samples after samples with an expected high concentration) or the validity of the reported concentrations should be justified ~~in the Bioanalytical Report.~~
598-600	1	**Comment:** In cases where the value needs to be confirmed (e.g., pre-dose sample with measurable concentrations) replicate determinations

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		are required if sample volume allows. If analysis is performed in singlet what is the rationale of performing repeat analysis in replicate? In case of replicate analysis, defining the acceptance criteria will lead to bias of the results. **Proposed change:** we are proposing that all the repeat analysis should be performed in singlet and repeat value should be accepted.
602-636	6	**Comments:** Suggest considering the European Bioanalysis Forum (EBF) paper on "A call for decision-based acceptance criteria to allow broader acceptance criteria for chromatography methods" (Bioanalysis (2018) 10(16), 1255–1259)
608-615	3	**Comments:** Consider changing acceptance criteria for the new lower limit calibration standard (after the LLOQ was rejected) to ±20%
608-615	4	**Comments:** Consider changing acceptance criteria for the new lower limit calibration standard (after the LLOQ was rejected) to ±20%
616-620	3	**Comments:** Consider adding a similar statement in the LBA section
616-620	4	**Comments:** Consider adding a similar statement in the LBA section
621-622	2	**Comments:** Dilution QCs should be used only if dilution linearity has not been demonstrated during validation **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Please add that dilution QCs have only to be measured if the applied sample dilution factor was not covered by dilution linearity assessment during assay validation. Lines 623-626 could thus be deleted.
621-622	6	**Comments:** Dilution QCs may not be required if the dilution concentrations have been tested in validation. Additionally, it should be possible that a bracketing approach could be used. **Proposed change:** "Analytical runs containing samples that are diluted **outside the existing validated maximal dilution factor,** and reanalysed should include dilution QCs to verify the accuracy and precision of the dilution method during study sample analysis. **If dilutions are assessed in validation, a bracketing approach is acceptable.** The concentration of the dilution QCs should exceed that of the study samples being diluted (or of the ULOQ) and they should be diluted using the same dilution factor. The within-run acceptance criteria of the dilution QC(s) will only affect the acceptance of the diluted study samples and not the outcome of the analytical run."
621-626	3	**Comments:** Clarify how many replicates of dilution QCs are expected (2?).
621-626	4	**Comments:** Clarify how many replicates of dilution QCs are expected (2?).
621-626	5	**Comments:** Introducing this novel recommendation seems not to be necessary. Dilution is checked during method validation. If calibration range is set properly, number of diluted samples during the study is very small. If number of diluted samples is large then calibration range should be changed. **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		delete lines 621-626
627-628	5	**Comments:** is one curve containing all analytes acceptable?
627-630	3	**Comments:** Clarify that calibration curve for a given analyte can be prepared as part of a cocktail with other analytes when more than one analyte is quantified
627-630	4	**Comments:** Clarify that calibration curve for a given analyte can be prepared as part of a cocktail with other analytes when more than one analyte is quantified
627-630	6	**Comments:** Add a clarifying statement to ensure that multiple datasets are not reported in the case of reanalysis. **Proposed change:** "When several analytes are assayed simultaneously, there should be one calibration curve for each analyte studied. If an analytical run is acceptable for one analyte but has to be rejected for another analyte, the data for the accepted analyte should be used. The determination of the rejected analyte requires a reextracted analytical batch and analysis. **The reanalysis of multiple analytes will only calculate concentrations for the individual analytes included in the reanalysis**."
631	5	**Proposed change:** ~~passed and~~ accepted
631-636	3	**Comments:** Consider removing the "(between-run)" in the "overall (between-run) accuracy and precision". Between run is the residual

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		variability between the runs. Overall is the sample population variability.
631-636	3	**Comments:** Consider adding similar level of detail to the LBA section
631-636	4	**Comments:** Consider removing the "(between-run)" in the "overall (between-run) accuracy and precision". Between run is the residual variability between the runs. Overall is the sample population variability.
631-636	4	**Comments:** Consider adding similar level of detail to the LBA section
632-635	5	Comment: Should "overall mean accuracy or precision" include outlying results of QCs? Such a result may strongly influence mean value, especially for small studies. **Proposed change:** "The overall (between-run) accuracy and precision of the QCs of all accepted runs should be calculated at each concentration level…" => "The overall (between-run) accuracy and precision of the QCs (excluding outliers using predefined statistical methods) of all accepted runs should be calculated at each concentration level…"
638-639	6	**Comments:** During validation the range has been proven as acceptable and therefore the narrowing of the calibration range does not add value. **Proposed change:** Delete "~~it is recommended to either narrow the calibration curve range~~"
638-641	3	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		The assay quantification range has been validated during assay validation phase. Please consider removing this requirement or clarifying what QC concentration would satisfy the requirement to adequately reflect study samples concentrations.
638-641	4	**Comments:** The assay quantification range has been validated during assay validation phase. Please consider removing this requirement or clarifying what QC concentration would satisfy the requirement to adequately reflect study samples concentrations.
644-646	6	**Comments:** The range has been validated **Proposed change:** Change to adapt the concentrations of the QCs and remove the requirement to narrow the calibration curve. "At the intended therapeutic dose(s), if an unanticipated clustering of study samples at one end of the calibration curve is encountered after the start of sample analysis, the analysis should be stopped and either ~~the standard calibration range narrowed (i.e., partial validation),~~ existing QC concentrations revised, or QCs at additional concentrations added to the original curve within the observed range before continuing with study sample analysis.
648-652	3	**Comments:** Consider defining "large number of the analyte concentrations..", for example, by providing % of samples. Alternatively, allow for a sample dilution option
648-652	4	**Comments:** Consider defining "large number of the analyte concentrations..", for example, by providing % of samples. Alternatively, allow for a sample dilution option
648-652	4	**Comments:** Difficult to manage in an early development setting. Requirement should be limited to BA/BE studies

Line no.	Stakeholder no.	Comment and rationale; proposed changes
653-655	3	**Comments:** Please clarify the need to have 2 QC levels within the range of sample concentrations if method was already validated for a given range of quantitation and sample concentrations are derived from assay calibration curve, not QCs.
653-655	4	**Comments:** Please clarify the need to have 2 QC levels within the range of sample concentrations if method was already validated for a given range of quantitation and sample concentrations are derived from assay calibration curve, not QCs.
656	3	**Comments:** Clarify that this includes re-injection of same run and re-extraction of same run. - add in paragraph **Proposed change:** For study samples involving multiple analytes, a valid result for one analyte should not be rejected because of another analyte failing the acceptance criteria. Additionally if a sample is re-analysed because one of the analytes failed to meet acceptance criteria the data for the analyte(s) that previously met acceptance criteria need not be regressed.
656	3	**Comments:** Many comments came in asking for detailed clarification. An overarching theme was to consider clarifying that reanalysis is related to samples that produced valid results (e.g. >ULOQ or <LLOQ). Reanalysis of samples from failed or rejected runs that did not produce acceptable results should not be viewed as "reanalysis" in the context of this paragraph. Hence we suggest to clearly separated the examples to reflect both cases • Reanalysis of a sample which didn't give a reportable concentration, is not reanalyses per se but generates a 1st reportable result • Reanalysis of a sample for which the 1st reportable result is 'unexpected' (positive placebo, unexpected PK,…), is reanalysis. It should be performed in replicate and compared to the original result with the aim to confirm or disprove this original result.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		An SOP /decision tree should be in place to guide reanalysis and reporting
656	4	**Comments:** Clarify that this includes re-injection of same run and re-extraction of same run. - add in paragraph **Proposed change:** For study samples involving multiple analytes, a valid result for one analyte should not be rejected because of another analyte failing the acceptance criteria. Additionally if a sample is re-analysed because one of the analytes failed to meet acceptance criteria the data for the analyte(s) that previously met acceptance criteria need not be regressed.
656	4	**Comments:** Many comments came in asking for detailed clarification. An overarching theme was to consider clarifying that reanalysis is related to samples that produced valid results (e.g. >ULOQ or <LLOQ). Reanalysis of samples from failed or rejected runs that did not produce acceptable results should not be viewed as "reanalysis" in the context of this paragraph. Hence we suggest to clearly separated the examples to reflect both cases • Reanalysis of a sample which didn't give a reportable concentration, is not reanalyses per se but generates a 1st reportable result • Reanalysis of a sample for which the 1st reportable result is 'unexpected' (positive placebo, unexpected PK,…), is reanalysis. It should be performed in replicate and compared to the original result with the aim to confirm or disprove this original result. An SOP /decision tree should be in place to guide reanalysis and reporting
665-666	5	**Comments:** Calibrations standards and QC samples do not always exactly mimic study samples (see lines 992-993). Thus, IS response may vary for Calibrations standards and QC samples vs. study samples. IS response significantly differing from other study samples should also be stated as reason for reanalysis.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: IS response significantly different from the response for the calibration standards, QCs or study samples (as pre-defined in an SOP)
670-680	1	**Comment:** The purpose of specificity evaluation is to demonstrate the ability of assay method to distinguish and detect the target analyte even in the presence of interference related molecules. Thus, specificity assessment at ULOQ is not fit for the purpose. We suggest to consider removing ULOQ from the testing parameters. **Proposed change (if any):** N/A
673-674	2	**Comments:** Bullet point is ambiguous, you mean pre-dose samples collected prior to the first drug administration (applicable in case of repeated dosing). Any other later collected pre-dose sample can contain drug at trough level. **Proposed change:** … analyte levels in pre-dose samples collected prior to the first administration of drug, …
673-674	6	**Comments:** Multiple dose studies will have multiple pre-dose samples and therefore may contain quantifiable drug concentrations. Change pre-dose to "naïve pre-dose". **Proposed change:** "Identification of quantifiable analyte levels in **naïve** pre-dose samples, control or placebo samples"
685	2	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		See comment and proposed change for lines 673-674
691-695	6	Proposed change: "Reinjection of processed samples can be made in the case of equipment failure **if processed sample viability** has been demonstrated during validation or provided in the Bioanalytical Report where it was conducted. Reinjection of a full analytical run or of individual calibration standards or QCs simply because the calibration standards or QCs failed, without any identified analytical cause, is not acceptable."
697-702	3	**Comments:** rephrase **Proposed change:** Chromatogram integration and reintegration should be described in a study plan, protocol or SOP. Any deviation from the procedures described a priori should be discussed in the Bioanalytical Report. Chromatogram integration parameters and in case of re-integration, initial and the final integration data should be documented at the laboratory and should be available upon request
697-702	3	**Comments:** The term "re-integration" needs to be defined. For example, consider: "integration that occurs after the initial save of the results table."
697-702	4	**Comments:** rephrase Proposed change: **Proposed change:** Chromatogram integration and reintegration should be described in a study plan, protocol or SOP. Any deviation from the procedures described a priori should be discussed in the Bioanalytical Report. Chromatogram integration parameters and in case of re-integration, initial and the final integration data should be documented at the laboratory and should be available upon request

Line no.	Stakeholder no.	Comment and rationale; proposed changes
697-702	4	**Comments:** The term "re-integration" needs to be defined. For example, consider: "integration that occurs after the initial save of the results table."
698-699	1	**Comment:** In the corresponding lines, it is stated that selectivity for lipaemia/haemolysis samples can be evaluated using a single source of matrix. The definition of "a single source" is unclear; an individual serum or a lot of pooled serum of representative population. **Proposed change:** For lipaemic and haemolysed samples, test can be evaluated once using **at least one individual source.**
706-713	3	**Comments:** add **Proposed change:** …. possible, or from a batch which has shown analytical comparability. If the reference standard batch used for bioanalysis is changed, bioanalytical evaluation should be carried out prior to use to ensure that the performance characteristics of the method are within the acceptance criteria and to ensure consistency of results between batches in case of change during bioanalysis of samples from a given nonclinical or clinical study
706-713	4	**Comments:** add **Proposed change:** …. possible, or from a batch which has shown analytical comparability. If the reference standard batch used for bioanalysis is changed, bioanalytical evaluation should be carried out prior to use to ensure that the performance characteristics of the method are within the acceptance criteria and to ensure consistency of results between batches in case of change during bioanalysis of

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		samples from a given nonclinical or clinical study
706-713	6	**Comments:** Chemical drugs and not only biotherapeutics may also be measured using LBA. There needs to be language added to allow for chemical drugs analysed by LBA and a sentence should be added to the paragraph. **Proposed change:** "The biological reference standard should be …. **Refer to Section 3.1 for chemical reference standards.**"
708-711	2	**Comments:** This sentence is too strong and in most cases not applicable. The reference standard is often defined at an earlier stage and each new drug batch is released against this standard. Thus dosing is almost always done with new batches (after release testing against the reference standard) but not with the reference standard itself. Furthermore, at later stages drug product batches might consist of several drug substance batches. **Proposed change:** Maybe add"....is derived from the same batch of drug substance as that used for dosing in the nonclinical and clinical studies or from a batch which has shown comparability in the used bioanalytical method"...
715-719	6	**Comments:** The definition of critical reagents is not comprehensive and should be broader. There are occasions where it is not only limited to the binding agents. Additionally, there are more moieties than just enzymatic ones. **Proposed change:** "Critical reagents, including **but not limited to,** binding reagents (e.g., binding proteins, aptamers, antibodies or conjugated antibodies) and those containing enzymatic **or labelled** moieties, have direct impact on the results of the assay and, therefore, their quality should be assured. ~~Critical reagents bind the analyte and, upon interaction, lead to an instrument signal~~

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		~~corresponding to the analyte concentration~~. The critical reagents should be identified and defined in the assay method."
715-721	3	**Comments:** rephrase **Proposed change:** ~~Critical reagents bind the analyte and, upon interaction, lead to an instrument signal corresponding to the analyte concentration.~~ Critical reagents, including binding reagents (e.g. binding proteins, aptamers, antibodies or conjugated antibodies), have direct impact on the results of the assay and therefore their quality must be assured. The critical reagents should be identified and defined in the assay method~~. Reliable procurement of critical reagents, whether manufactured in-house or purchased commercially, should be considered early in method development.~~
715-721	4	**Comments:** rephrase **Proposed change:** ~~Critical reagents bind the analyte and, upon interaction, lead to an instrument signal corresponding to the analyte concentration.~~ Critical reagents, including binding reagents (e.g. binding proteins, aptamers, antibodies or conjugated antibodies), have direct impact on the results of the assay and therefore their quality must be assured. The critical reagents should be identified and defined in the assay method~~. Reliable procurement of critical reagents, whether manufactured in-house or purchased commercially, should be considered early in method development.~~
720-721	6	**Comments:** This sentence is best practice; reliable procurement may not always be possible in early development. **Proposed change:** Remove sentence "~~Reliable procurement of critical reagents, whether manufactured in-house or purchased commercially, should~~

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		~~be considered early in method development~~.”
725-734	3	**Comments:** add/delete – *Justification: There is ambiguity with respect to minor vs major. What is e.g. the difference between minor (source of reagent is changed) vs major (change in production method, new supplier for antibody). Therefore, the suggestion is to bring one clear example for minor, 1 clear example for major (as proposed above), and leave it a scientific decision to define what is minor vs major.* **Proposed change:** ….for characterisation, with proper documentation kept at the analytical site. If the change is major (e.g. switch from antigen-based detection molecule to an antibody-based detection molecule), then additional validation experiments are necessary. Ideally, assessment of changes will compare the assay with the new reagents to the assay with the old reagents directly. ~~Major changes include, but are not limited to, change in production method of antibodies, additional blood collection from animals for polyclonal antibodies and new clones or new supplier for monoclonal antibody production.~~
725-734	4	**Comments:** rephrase – *justification There is ambiguity with respect to minor vs major. What is e.g. the difference between minor (source of reagent is changed) vs major (change in production method, new supplier for antibody). Therefore, the suggestion is to bring one clear example for minor, 1 clear example for major (as proposed above), and leave it a scientific decision to define what is minor vs major.* **Proposed change:** ….for characterisation, with proper documentation kept at the analytical site. If the change is major (e.g. switch from antigen-based detection molecule to an antibody-based detection molecule), then additional validation experiments are necessary. Ideally, assessment of changes will compare the assay with the new reagents to the assay with the old reagents directly. ~~Major changes include, but are not limited to, change in production method of antibodies, additional blood collection from animals for polyclonal antibodies and new clones or new supplier for monoclonal antibody production.~~

Line no.	Stakeholder no.	Comment and rationale; proposed changes
728-730	6	**Comments:** The example given for the minor change is not optimal and may depend on the reagent. **Proposed change:** Reference the King *et al.* (2014) doi: 10.1208/s12248-014-9583-x paper that discusses minor and major changes. Add "**If the change is major rather than minor more extensive assessment will be required**". Remove "~~If the change is minor (e.g. the source of one reagent is changed), a single comparative accuracy and precision assessment is sufficient for characterisation. If the change is major, then additional validation experiments are necessary~~." Move the sentence starting in line 732 up to the sentence that ends in line 728
730-732	1	**Comment:** It is understood that QC samples must be prepared/stored under the same conditions as anticipated study samples (i.e. frozen QC if study samples is frozen); however, stability data is generally not available during A&P assessment thus it is more commonly recommended to use fresh QC samples for A&P assessment. It needs further clarification whether suggested QC preparation guideline is specific for A&P runs or for other method validation runs. If latter, 4.2.4.1 section can be a separate section, following '4.2.3 Calibration Curve and Range', instead of a subsection under '4.2.4 Accuracy and Precision'. Then, further clarification on QC preparation instruction (i.e. fresh or frozen) for A&P runs would be helpful. **Proposed change (if any):** N/A
738-740	3	**Comments:** add **Proposed change:** …The performance parameters should be documented at the analytical site in order to support the extension or replacement of the

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		critical reagent.
738-740	4	**Comments:** add **Proposed change:** …The performance parameters should be documented at the analytical site in order to support the extension or replacement of the critical reagent.
742-743	6	Proposed change: Remove "assay format". Change to "When using LBA, study samples can be analysed using an assay format of 1 or more well(s) per sample. The ~~assay format~~ **method description** should be specified in the protocol, study plan or SOP."
742-749	3	**Comments:** add **Proposed change:** … the replicate wells or by averaging the concentrations calculated from each response. Acceptance criteria regarding the mean of the response or concentrations values should be predefined. Data evaluation should be performed on reportable concentration values.
742-749	4	**Comments:** add **Proposed change:** … the replicate wells or by averaging the concentrations calculated from each response. Acceptance criteria regarding the mean of the response or concentrations values should be predefined. Data evaluation should be performed on reportable concentration values.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
742–749 and 985	2	**Comments:** 4. The text refers to "1 or more well(s) per sample". There are LBA platforms that do not necessarily perform the testing using wells as done in classical microtitration plate assays. 5. Please clarify that singlicate analysis is acceptable also for cals and QCs. **Proposed change:** suggest to phrase more generally, e.g. "1 or more replicate(s) per calibration standard, QC sample and validation or study sample". Clearly emphasize that the idea is to allow singlicate measurements if validation data can demonstrate the appropriateness of the singlicate testing approach.
744-745	6	**Comments:** The use of singlicate analysis is welcomed. Rephrase to align the expectation that validation should be conducted in the same manner as that intended in sample analysis. **Proposed change:** "If ~~method development and~~ assay validation is performed using 1 or more well(s) per sample, then study sample analysis should also be performed using **the same number of wells** ~~1 or more well(s) per sample, respectively~~."
744-746	1	**Comments:** The term 'replicates' used in the corresponding line needs further clarification. It is unclear how replicates of QC samples for A&P runs can be prepared; whether samples can be prepared as a single bulk stock or samples should be independently prepared. Throughout the guideline in other parameters except this section, the term 'replicate' is understood as the exact same samples that are prepared together but tested separately. **Proposed change:** Accuracy and precision should be determined by analysing at least 3 ~~replicates~~ **independently prepared samples** per run at

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		each QC concentration level (LLOQ, low, medium, high, ULOQ) in at least 6 runs over 2 or more days.
750	3	**Comments:** consider adding a definition, as e.g. done with Selectivity
750	4	**Comments:** consider adding a definition, as e.g. done with Selectivity
751-752	2	**Comments:** 6. What exactly is "related molecule"? 7. There is no discrimination between unwanted (total assays) and wanted interference (necessary for free analyte assays) **Proposed change:** Please clarify and consider free analyte assays
751-752	3	**Comments:** Add definition of what consists a 'structurally related molecule'
751-752	4	**Comments:** Add definition of what consists a 'structurally related molecule'
751-754	6	**Comments:** The sentence reads awkwardly please re-phrase. Additionally, there is no value spiking at ULOQ. If a high spike is needed this should be at HQC although it is proposed to remove lines 753-754. **Proposed change:** "Specificity is evaluated by spiking pooled or individual blank matrix samples, and samples at LLOQ ~~and ULOQ~~ at the maximal

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		concentrations of structurally related molecules anticipated in the study. ~~The accuracy of the target analyte at the LLOQ and at the ULOQ should be investigated in the 753 presence of related molecules at the maximal concentration(s) anticipated in study samples~~.”
753-757	3	**Comments:** rephrase **Proposed change:** The accuracy of the target analyte at the LLOQ and at the ~~ULOQ~~ High QC should be investigated in the presence of related molecules at the maximal concentration(s) anticipated in study samples. The response of blank samples spiked with related molecules should be below the LLOQ. The accuracy of the target analyte in presence of related molecules should be within ±25% and ±20% of the nominal values for the LLOQ and High QC spike respectively.
~~753-757~~	4	**Comments:** add/delete **Proposed change:** The accuracy of the target analyte at the LLOQ and at the ~~ULOQ~~ High QC should be investigated in the presence of related molecules at the maximal concentration(s) anticipated in study samples. The response of blank samples spiked with related molecules should be below the LLOQ. The accuracy of the target analyte in presence of related molecules should be within ±25% and ±20% of the nominal values for the LLOQ and High QC spike respectively.
753-757	4	**Comments:** delete – data driven discussions in EBF challenge the scientific need for including the ULOQ Or even a High QC **Proposed change:** The accuracy of the target analyte at the LLOQ ~~and at the ULOQ~~ should be investigated in the presence of related molecules at the maximal concentration(s) anticipated in study samples. The response of blank samples spiked with related molecules should be

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		below the LLOQ. The accuracy of the target analyte in presence of related molecules should be within ±25% of the nominal values..
758-763	3	**Comments:** rephrase **Proposed change:** In the event of non-specificity, the impact on the method should be evaluated by spiking increasing concentrations of interfering molecules in blank matrix and measuring the accuracy of the target analyte at the LLOQ and ~~ULOQ~~ High QC/at the maximal expected sample concentration of the target analyte. It is essential to …
758-763	4	**Comments:** rephrase **Proposed change:** In the event of non-specificity, the impact on the method should be evaluated by spiking increasing concentrations of interfering molecules in blank matrix and measuring the accuracy of the target analyte at the LLOQ and ~~ULOQ~~ High QC/at the maximal expected sample concentration of the target analyte. It is essential to …
758-763	6	**Comments:** Assessment of molecules that may bind the analyte are not well described and should be added to this section. Additionally, the sentence in line 759-760 does not read well and accuracy of the target analyte should be removed. A high spike requirement adds no value. Remove "measuring the accuracy of the target analyte". **Proposed change:** "In the event of non-specificity, the impact on the method should be evaluated by spiking increasing concentrations of interfering molecules in blank matrix ~~and measuring the accuracy of the target analyte~~ at the LLOQ ~~and ULOQ~~. It is essential to determine the minimum concentration of the related molecule where interference occurs. Appropriate mitigation during sample analysis

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		should be employed, e.g., it may be necessary to adjust the LLOQ~~/ULOQ~~ accordingly or consider a new method."
768-771	3	**Comments:** delete **Proposed change:** Selectivity is the ability of the method to detect and differentiate the analyte of interest in the presence of ~~other~~ "unrelated compounds" (non-specific interference) in the sample matrix.
768-771	4	**Comments:** delete **Proposed change:** Selectivity is the ability of the method to detect and differentiate the analyte of interest in the presence of ~~other~~ "unrelated compounds" (non-specific interference) in the sample matrix.
772-776	4	**Comments:** delete **Proposed change:** … is evaluated using blank samples obtained from at least 10 individual sources and by spiking the individual blank matrices at the LLOQ ~~and at the high QC level~~. The response of the blank samples should be below the LLOQ in at least 80% of the individual sources.
777	1	**Comments:** In line 777, it is stated that at least 3 runs should be performed. However, the purpose and value of performing multiple runs for dilution linearity assessment is unclear. Running multiple plates would only be able to evaluate plate-to-plate variability, which is not the scope of dilution linearity. We suggest to evaluate multiple independently prepared sets of samples on a single plate to

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		properly assess the dilution linearity of assay method. **Proposed change:** For each dilution factor tested, at least 3 independently prepared sets should be analysed.
777-778	1	**Comments:** In the corresponding line, it is stated that dilution linearity runs should be performed using the number of replicates that will be used in sample analysis. Further clarification on 'the number of replicates' is necessary; whether 'replicate' means the number of wells used for a sample (i.e. singlicate vs. duplicate) or it means the number of dilution QCs to be used during the sample analysis. **Proposed change (if any):** N/A
779	2	**Comments:** Are lipemic and/orhemolyzed samples included in the 10 sources? **Proposed change:** Please clarify if this assessment should be done in addition to the 10 individual sources which are used for selectivity assessment (10+x) or if it is included (8 or 9 + x)
779-782	3	**Comments:** Add text from 3.2.1 rather than a reference, including suggested edits to the original draft
779-782	3	**Comments:** rephrase **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Selectivity should be evaluated in lipaemic samples and haemolysed samples if relevant. For lipaemic and haemolysed samples, tests can be evaluated once using a single source of matrix as part of the 10 individuals……In the case of relevant patient populations, when available, there should be at least five individual patients.
779-782	4	**Comments:** Add text from 3.2.1 rather than a reference, including suggested edits to the original draft
779-782	4	**Comments:** rephrase **Proposed change:** Selectivity should be evaluated in lipaemic samples and haemolysed samples if relevant. For lipaemic and haemolysed samples, tests can be evaluated once using a single source of matrix as part of the 10 individuals……In the case of relevant patient populations, when available, there should be at least five individual patients.
780	2	**Comments:** 1. Lipemic sample testing is usually not needed for nonclinical studies, due to controlled food uptake 2. What is a "single source of matrix"? Is it individual or pooled? In order to investigate interference, pooled matrix would be sufficient. **Proposed change:** Please clarify
781-782	2	**Comments:** 1. Text refers to "relevant patient populations". What is considered a "relevant patient population"? Any patient population for which the drug is intended to be used?

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		2. Acceptance rate for selectivity testing in patient samples is missing. Is it 80% as for standard selectivity testing? **Proposed change:** 1. Please clarify 2. Please add acceptance criterion
780-782	6	**Comments:** Further clarity is needed for patient populations. It is suggested that training be given to industry and regulatory inspectors and regulatory reviewers on the expectation of what is a relevant patient population and performing lipaemic and haemolysis assessments on a case by case basis. For example, haemolysis rarely impacts LBA methods and would only be warranted should the biology require such an assessment. Remove cross reference to Section 3.2.1. **Proposed change:** "**When required,** ~~For~~ lipaemic and haemolysed samples, tests can be evaluated once using a single source of matrix. Selectivity should be assessed in samples from relevant patient populations. In the case of relevant patient populations at least five individual patients should be used to assess the impact of lipaemia and haemolysis."
781-783	1	**Comments:** The scope of 'all the dilutions' is understood as all the dilutions applied, not necessarily the valid dilutions to be used. In addition, it is unclear how precision would be assessed for multiple replicate runs whether it is calculated for each individual or all replicates averaged. **Proposed change:** The calculated concentration for each dilution should be within ± 20% of nominal concentration after correction for dilution and the precision of the final concentrations **from valid dilutions of** all **(or individual) replicates** should not exceed 20%.
781-783	1	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		It is understood from this corresponding line that acceptable relative error rate is ±20%, regardless of the level of measured concentrations after dilution. However, the acceptance criteria evaluating A&P allows up to ±25% relative error for the level at LLOQ and ULOQ (lines 752-756). We propose to adjust the criteria for dilutional linearity for samples at LLOQ/ULOQ levels to be within 25% of nominal concentration. **Proposed change:** The calculated concentration for each dilution should be within ±20% **(±25% if diluted concentration is at LLOQ or ULOQ)** of the nominal concentration after correction for dilution and the precision of the final concentrations across all the dilutions should not exceed 20%.
784-790	2	**Comments:** Please make clear that the guideline covers total drug quantification only and add a statement which gives more flexibility to scientific correct free/active drug quantification (as discussed in the white paper Gupta et al. Bioanalysis. 2017 Dec;9(24):1967-1996). The FDA statement could be a good guidance ("When surrogate matrices are necessary, the sponsor should justify and validate the calibration curves") or use the identical wording as used in line 1255-1256: "..calibration standards and QCs prepared in a matrix different from the study samples should be justified and appropriate experiments should be performed". This topic is important not only for free drug quantification but also use of high sensitive target/anti-id capture assays when the blank matrix contains relevant amount of soluble target. The increasing use of MABEL approaches in the drug development while also increase the importance of having clarity in this field.
784-790	3	**Comments:** add **Proposed change:** …Calibration standards and QCs prepared in a matrix different from the study samples should be justified and appropriate experiments should be performed"

Line no.	Stakeholder no.	Comment and rationale; proposed changes
784-790	4	**Comments:** add **Proposed change:** …Calibration standards and QCs prepared in a matrix different from the study samples should be justified and appropriate experiments should be performed"
787-788	2	**Comments:** Calibration standards should be prepared in the same matrix as the study samples **Proposed change:** QCs should be prepared in the same matrix as the study samples, the calibration standard can be prepared in a surrogate matrix, or buffer only can be used with justification
787-789	6	**Comments:** Flexibility for rare matrices, cases of a free PK assay or endogenous counterpart needs to be added. **Proposed change:** Add "**There are times when the use of the exact biological matrix may not be scientifically relevant. Therefore, the use of a suitable surrogate matrix may be necessary for example when using a free assay, a rare matrix or in the case of an endogenous counterpart**."
791	2	**Comments:** Why has the calibration curve to be generated with at least 6 concentration levels, what is the scientific rationale? The number of required calibration standrads is dependent on different factors (platform, regression model, analytical method, etc). **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Please consider that the number of concentration levels to generate the calibration curve can be reduced to a minimum as long as the sponsor can prove validity of the method.
791-792	6	**Comments:** 6 concentration levels are expected in the range and should exclude anchor points. This needs to be clearer in the document. **Proposed change:** "A calibration curve should be generated with at least 6 concentration levels **within the expected validated range** of calibration standards, including LLOQ and ULOQ standards, plus a blank sample."
791-797	3	**Comments:** consider deleting blank matrix (blank sample), as there is no added value on having this in each and every analytical run → delete **Proposed change:** A calibration curve should be generated with at least 6 concentration levels of calibration standards, including LLOQ and ULOQ standards~~, plus a blank sample. The blank sample should not be included in the calculation of calibration curve parameters.~~ Anchor point samples at concentrations below the LLOQ and above the ULOQ of the calibration curve may also be used to improve curve fitting. The relationship between response and concentration for a calibration curve is most often fitted by a 4- or 5-parameter logistic model if there are data points near the lower and upper asymptotes, although other models may be used with suitable justification.
791-797	4	**Comments:** consider deleting blank matrix (blank sample), as there is no added value on having this in each and every analytical run → delete **Proposed change:** A calibration curve should be generated with at least 6 concentration levels of calibration standards, including LLOQ and ULOQ standards~~, plus a blank sample. The blank sample should not be included in the calculation of calibration curve parameters.~~ Anchor point samples at concentrations below the LLOQ and above the ULOQ of the calibration curve may also be used to improve

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		curve fitting. The relationship between response and concentration for a calibration curve is most often fitted by a 4- or 5-parameter logistic model if there are data points near the lower and upper asymptotes, although other models may be used with suitable justification.
798-799	3	**Comments:** propose to delete - Justification: suggest deleting this sentence as it might create ambiguity. These factors are considered anyway during A&P runs. In the way it is written now, you can interpret that you have to do these 6 runs, followed by at least A&P runs.
798-799	4	**Comments:** propose to delete - Justification: suggest deleting this sentence as it might create ambiguity. These factors are considered anyway during A&P runs. In the way it is written now, you can interpret that you have to do these 6 runs, followed by at least A&P runs.
798-799	6	**Comments:** The factors contributing to between-run variability is vague, suggest that this is removed, or it should be further defined. Several days can be ambiguous and propose that the language is changed to "over at least 2 days". This language should be used throughout and would be consistent with examples such as line 825. **Proposed change:** "A minimum of 6 independent runs should be evaluated over ~~several~~ **2 or more days**". Remove "~~considering the factors that may contribute to between-run variability~~"
800-805	3	**Comments:** precision never can be negative **Proposed change:** The precision should be within 20%, except for LLOQ and ULOQ where precision should be within 25%.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
800-805	4	**Comments:** precision never can be negative **Proposed change:** The precision should be within 20%, except for LLOQ and ULOQ where precision should be within 25%.
806-808	3	**Comments:** rephrase **Proposed change:** … If freshly spiked calibration standards are not used, the frozen calibration standards can be used ~~within their defined period of stability~~. under the condition that freeze-thaw stability and a defined period of stability has been proven for the calibrator standards.
806-808	4	**Comments:** rephrase **Proposed change:** … If freshly spiked calibration standards are not used, the frozen calibration standards can be used ~~within their defined period of stability~~. under the condition that freeze-thaw stability and a defined period of stability has been proven for the calibrator standards.
806-809	1	**Comments:** It is understood that the concentrations of QC samples for the stability test needs to be adjusted (to be at a higher concentration (e.g. C_{trough}, C_{max}) requiring additional dilutions), if additional dilution would be performed during sample analysis. However, the stability evaluation covering the detection range (LQC-HQC) is rather relevant and more critical for the validation of assay method. We suggest to remove lines 806-809.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		In case we need to use adjusted stability QC samples (at higher concentrations), it is not clear whether fresh prepared QC (for the stability run) is also needed to be adjusted. **Proposed change (if any):** N/A
807	2	**Comments:** typographical error **Proposed change:** …. spiked calibration standards are not used, then frozen calibration standards can be used….
811-812	2	**Comments:** Free analyte QC concept not considered **Proposed change:** 1. QC samples should be prepared by spiking matrix with a known quantity of the analyte (and binding partners in case of free analyte assays) 2. Use of surrogate matrix (ligand-free) should be allowed for free analyte assa
811-813	3	**Comments:** add **Proposed change:** Calibration standards and QCs prepared in a matrix different from the study samples should be justified and appropriate experiments should be performed"

Line no.	Stakeholder no.	Comment and rationale; proposed changes
811-813	4	**Comments:** add **Proposed change:** Calibration standards and QCs prepared in a matrix different from the study samples should be justified and appropriate experiments should be performed"
813	6	**Comments:** Endogenous QC samples plus or minus a spike may also be a viable approach. **Proposed change:** Add "**The use of an alternative matrix that differs from the study samples or the use of endogenous samples can be justified**."
814-819	3	**Comments:** delete – justification: the reference standard is usually a single liquid stock **Proposed change:** The dilution series for the preparation of the QCs should be completely independent from the dilution series for the preparation of calibration standard samples. ~~They may be prepared from a single stock provided that its accuracy has been verified or is known.~~ The QCs should be prepared at a minimum of 5 concentration levels within the calibration curve range: The analyte should be spiked at the LLOQ, within three times of the LLOQ (low QC), around the geometric mean of the calibration curve range (medium QC), and at least at 75% of the ULOQ (high QC) and at the ULOQ
814-819	4	**Comments:** delete – justification: the reference standard is usually a single liquid stock

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: The dilution series for the preparation of the QCs should be completely independent from the dilution series for the preparation of calibration standard samples. ~~They may be prepared from a single stock provided that its accuracy has been verified or is known.~~ The QCs should be prepared at a minimum of 5 concentration levels within the calibration curve range: The analyte should be spiked at the LLOQ, within three times of the LLOQ (low QC), around the geometric mean of the calibration curve range (medium QC), and at least at 75% of the ULOQ (high QC) and at the ULOQ
815-817	1	**Comments:** In the corresponding lines, ICH M10 guidance suggests to evaluate stability of biological drugs at two different temperatures to ensure stability in between these two points. However, it is possible to apply the extrapolation of stability toward lower temperatures as for chemical drugs (lines 813-814). We suggest to apply the same extrapolation approach for biological drug stability. **Proposed change (if any):** N/A
816-817	6	**Comments:** Clarify that this excludes anchor points **Proposed change:** "The QCs should be prepared at a minimum of 5 concentration levels within the calibration curve range **excluding anchor points**"
818	6	**Comments:** The use of geometric for the placement of the mid QC is a welcomed addition to the guideline for LBA and propose that it is also used for chromatographic methods.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
824-831	3	**Comments:** rephrase **Proposed change:** … Within-run accuracy and precision data should be reported for each run, unless there was an obvious documented error to justify the rejection of a run(s). An overall calculation of within-run accuracy and precision for each QC level should be determined. Within-run accuracy or precision criteria do not need to be met in all runs for the assessment to be successful. Between-run precision and accuracy should be calculated by combining the data from all runs.
824-831	4	**Comments:** rephrase **Proposed change:** … Within-run accuracy and precision data should be reported for each run, unless there was an obvious documented error to justify the rejection of a run(s). An overall calculation of within-run accuracy and precision for each QC level should be determined. Within-run accuracy or precision criteria do not need to be met in all runs for the assessment to be successful. Between-run precision and accuracy should be calculated by combining the data from all runs.
826-828	6	**Comments:** The language is ambiguous, and all runs should be listed but not necessarily included in the statistics. **Proposed change:** "Reported method validation data and the determination of accuracy and precision should include all results obtained~~, except those cases where errors are obvious and documented~~. **In those cases where errors are due to a documented assignable cause, the runs should be listed but the data should be excluded from assessment**."
836-837	6	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		The value of Total Error is questioned, suggest removing these lines. Delete lines 836 and 867 **Proposed change:** ~~Furthermore, the total error (i.e., sum of absolute value of the errors in accuracy (%) and precision (%)) should be evaluated. The total error should not exceed 30% (40% at LLOQ and ULOQ).~~
839-841	6	**Comments:** An alternative approach to using a blank sample is to add a LLOQ sample after the ULOQ. **Proposed change:** "**If the assay platform is prone to carry-over**, the potential of carry-over should be investigated by placing blank samples after the calibration standard at the ULOQ. The response of blank samples should be at a minimum below the LLOQ. **An alternative approach to using a blank sample is to add a LLOQ sample after the ULOQ. The LLOQ should demonstrate acceptable accuracy, and precision is measured in greater than singlicate analysis.**"
843	6	**Comments:** The term narrow range adds no value, propose to remove. **Proposed change:** "~~Due to the narrow assay range in many LBAs,~~ Study samples may require dilution in order to achieve analyte concentrations within the range of the assay."
843-848	2	**Comments:** 1. The statement "due to the narrow assay range" is not fully correct, since a) there are plenty of LBA technology with calibration ranges of >3 orders of magnitude and b) dilution is more often needed due to the high sensitivity of the LBA assays and the lack of the possibility to shift the calibration range, as it is possible for chromatographic methods. 2. Hook effect: One step immunoassays are prone to hook effect. This format might be required in many cases and thus in

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		some cases a hook effect cannot be excluded. **Proposed change:** 1. The guideline should state that a. hook effect needs to be evaluated 2. If a hook effect is observed measures should be implemented to exclude any erroneous result (e.g. by analysis of multiple dilutions)
843-848	3	**Comments:** rephrase **Proposed change:** Due to ~~the narrow assay range~~ high analyte concentrations in study samples in many LBAs, study samples may require dilution in order to achieve analyte concentrations within the range of the assay. Dilution linearity is assessed to confirm: (i) that measured concentrations are not affected by dilution within the calibration range and (ii) the absence or presence of a hook effect. ~~that sample concentrations above the ULOQ of a calibration curve are not impacted by hook effect (i.e., a signal suppression caused by high concentrations of the analyte), whereby yielding an erroneous result.~~ Add: Calibration standards and QCs prepared in a matrix different from the study samples should be justified and appropriate experiments should be performed"
843-848	4	**Comments:** rephrase **Proposed change:** Due to ~~the narrow assay range~~ high analyte concentrations in study samples in many LBAs, study samples may require dilution in order to achieve analyte concentrations within the range of the assay. Dilution linearity is assessed to confirm: (i) that measured concentrations are not affected by dilution within the calibration range and (ii) the absence or presence of a hook effect. ~~that sample concentrations above the ULOQ of a calibration curve are not impacted by hook effect (i.e., a signal suppression caused by~~

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		~~high concentrations of the analyte), whereby yielding an erroneous result.~~ Add: Calibration standards and QCs prepared in a matrix different from the study samples should be justified and appropriate experiments should be performed"
849	2	**Comments:** Dilution with target containing matrix can result in erroneous results if a target capture assay format is used. **Proposed change:** It should be made clear that the given statement is only true for total drug assays and that alternative matrices might be used for QC preparation and dilution of samples (Staack et al., Bioanalysis. 2014 Feb;6(4):485-96.).
849	3	**Comments:** add **Proposed change:** The same matrix as that of the study sample should be used for preparation of the QCs for dilution. Calibration standards and QCs prepared in a matrix different from the study samples should be justified and appropriate experiments should be performed.
849	4	**Comments:** add **Proposed change:** The same matrix as that of the study sample should be used for preparation of the QCs for dilution. Calibration standards and QCs prepared in a matrix different from the study samples should be justified and appropriate experiments should be performed.
850-853	2	**Comments:** Specifies dilution linearity to be assessed by diluting sample with blank matrix. Will result in high consumption of matrix for tox

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		studies, high dose clinical studies and assessment of rare matrices. Better to dilute first with assay diluent at MRD and following with assay diluent with matrix concentration of MRD. **Proposed change:** **Please add the possibility to dilute with buffer to MRD, all following dilution in assay buffer containing matrix to maintain a stable matrix concentration**
850-856	3	**Comments:** add **Proposed change:** ….with blank matrix (or matrix diluted with buffer) to a concentration within the calibration range. For each dilution ….
850-856	4	**Comments:** add **Proposed change:** ….with blank matrix (or matrix diluted with buffer) to a concentration within the calibration range. For each dilution ….
850-856	4	**Comments:** reason for above additional text (or matrix diluted with buffer) is to prevent erroneous results from diluting with matrix containing endogenous compounds
850-856	6	**Comments:** Linearity of dilution is performed to assess the accuracy of dilution and therefore the value of performing this assessment in 3 separate runs is questioned. If 3 runs are required, the text does not allow for any failures and it is therefore recommended that 2/3 should fulfil acceptance criteria.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: "Dilution linearity should be demonstrated by generating a dilution QC, i.e., spiking the matrix with an analyte concentration above the ULOQ, analysed undiluted (for hook effect) and diluting this sample (to at least 3 different dilution factors) with blank matrix to a concentration within the calibration range. For each dilution factor tested, at least ~~3 runs~~ **1 run** should be performed using the number of replicates that will be used in sample analysis. **The absence of hook effect is demonstrated if samples spiked at concentrations above the ULOQ have responses that are above the ULOQ**. ~~The absence or presence of response reduction (hook effect) is checked in the dilution QCs and,~~ If observed, measures should be taken to ~~eliminate response reduction~~ **mitigate** during the analysis of study samples. The calculated concentration for each dilution should be within ±20% of the nominal concentration after correction for dilution and the precision of the final concentrations across all the dilutions should not exceed 20%."
855	2	**Comments:** See above: please add a statement that enables use of one-step LBA assay format (which always have a hook effect at a certain concentration). **Proposed change:** Line 855: ...if observed, measures should be taken to eliminate the response reduction during analysis of study samples or measures are taken to exclude reporting of erroneous results due to the hook effect (e.g. analysis at multiple dilutions)
857-859	3	**Comments:** add **Proposed change:** The calculated concentration for each dilution within the calibration range should be within ±20% of the nominal …..

Line no.	Stakeholder no.	Comment and rationale; proposed changes
857-859	4	**Comments:** add **Proposed change:** The calculated concentration for each dilution within the calibration range should be within ±20% of the nominal …..
860 - 861	2	**Comments:** if samples during study sample analysis require a dilution factor that is higher than the highest dilution factor tested during assay validation, a dilution QC sample should be included in the study sample analysis to demonstrate that the corresponding high concentrations can be adequately measured.
863	3	**Comments:** Include a statement that WBS is not required for LBA assays
863	4	**Comments:** Include a statement that WBS is not required for LBA assays
866-870	3	**Comments:** add **Proposed change:** The storage and analytical conditions applied to the stability tests, such as the sample storage times (e.g. maximum time between sample collection and sample analysis) and temperatures, ….
866-870	4	**Comments:** add

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: The storage and analytical conditions applied to the stability tests, such as the sample storage times (e.g. maximum time between sample collection and sample analysis) and temperatures, ….
866-870	6	**Comments:** Container materials implies that the exact same containers would be required in this assessment. Additionally, in regions where M10 will be law, this will potentially increase the number of assessments needed. **Proposed change:** "The storage and analytical conditions applied to the stability tests~~, such as the sample storage times and temperatures, sample matrix, anticoagulant, and container materials~~ should reflect those used for the study samples. Reference to data published in the literature is not considered sufficient. Validation of storage periods should be performed on stability QCs that have been stored for a time that is equal to or longer than the study sample storage periods."
873-874	3	**Comments:** rephrase … A minimum of three replicates ~~stability QCs~~ should be prepared and analysed per concentration level/storage condition/timepoint.
873-874	4	**Comments:** rephrase … A minimum of three replicates ~~stability QCs~~ should be prepared and analysed per concentration level/storage condition/timepoint.
873-874	6	**Comments:** The language is not particularly clear and could mean 3 aliquots of a single preparation or 3 independent preparations of each QC

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		concentration level/storage condition/time point. It is recommended that a single preparation is used for this assessment rather than 3 independent spikes which only serves to test pipetting technique. Therefore, further clarification is needed as to what is required. **Proposed change:** "A minimum of ~~three~~ **one stability** ~~QCs~~ **QC** should be prepared**, divided into at least 3 aliquots** ~~and analysed~~ per concentration level/storage condition/timepoint **and analysed**."
875-877	2	**Comments:** See comment on lines 484-486 and proposed change
875-881	6	**Comments:** A freshly spiked calibration curve is only essential for long-term stability. Scientific justification for whether T=0 or nominal should be used to prevent the acceptance of stability when large shifts are seen between the preparation and the stability result. The control should be the same batch of controls that is not treated for parameter parameters except long-term stability. **Proposed change:** "The stability QCs are analysed against a calibration curve, obtained from freshly spiked calibration standards in a run with its corresponding freshly prepared QCs or QCs for which stability has been proven. While the use of freshly prepared calibration standards and QCs is the preferred approach, it is recognised that in some cases, for macromolecules, it may be necessary to freeze them overnight. In such cases, valid justification should be provided, and freeze-thaw stability demonstrated. The mean concentration at each level should be within ±20% of the nominal concentration **or the overall mean of the control sample for at least 2/3 of the stability samples at each concentration**."
878	2	**Comments:** What is meant by "macromolecules" **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Please clarify and rephrase
880-881	3	**Comments:** add **Proposed change:** …The mean concentration of the three replicates at each level should be within ±20% of the nominal concentration.
880-881	4	**Comments:** add **Proposed change:** …The mean concentration of the three replicates at each level should be within ±20% of the nominal concentration.
882-885	3	**Comments:** delete
882-885	4	**Comments:** delete
882-885	6	**Comments:** Stability is analyte dependent rather than concentration dependent; typically, the low concentrations show the greatest change over time. The impact of adding this requirement (it is not currently not in any other guidance document), will be the assessment of high concentration stability QCs for all toxicology studies. This would be against the principles of the 3Rs. Delete the paragraph lines 882-885. **Proposed change:** ~~"Since sample dilution may be required for many LBA assays due to a narrow calibration range, the concentrations of the study~~

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		~~samples may be consistently higher than the ULOQ of the calibration curve. If this is the case, the concentration of the stability QCs should be adjusted, considering the applied sample dilution, to represent the actual sample concentration range."~~
884	2	**Comments:** "If this is the case, the concentration of the stability QCs should be adjusted,…." Is EMA recommending to use a high concentrated QC above ULOQ instead of the High QC? **Proposed change:** Please delete this requirement, there is no scientific rationale if stability is proven for LQC and HQC. **Proposed change:** **Even better: Delete lines 882-885 completely.**
889-890	3	**Comments:** add **Proposed change:** For both chemical and biological drugs, it is considered acceptable to extrapolate the stability at one temperature (e.g., -20°C) to lower temperatures (e.g., -70°C).
889-890	4	**Comments:** add **Proposed change:** For both chemical and biological drugs, it is considered acceptable to extrapolate the stability at one temperature (e.g., -20°C) to lower temperatures (e.g., -70°C).
889-893	6	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		There is no industry or known literature examples that require macromolecules to be treated differently from chemical drugs. Delete the requirement that macromolecules must be tested at two temperatures. **Proposed change:** "~~For chemical drugs,~~ It is considered acceptable to extrapolate the stability at one temperature (e.g., -20°C) to lower temperatures (e.g., -70°C). ~~For biological drugs, it is acceptable to apply a bracketing approach, e.g., in the case that the stability has been demonstrated at -70°C and at -20°C, then it is not necessary to investigate the stability at temperatures in between those two points at which study samples will be stored~~."
891-893		**Comments:** There is no scientific rationale to differ from the procedure for chemical drugs **Proposed change:** Please stick to the FDA recommendation: "determination of stability at -20 would cover stability at colder temperatures".
895-896	6	**Comments:** Pre-study would be a better term to distinguish between pre-study and in-study validation activities for the first sentence. **Proposed change:** "The analysis of study samples can be carried out after **pre-study** validation has been completed however it is understood that some parameters may be completed at a later stage (e.g., long-term stability)."
902	2	**Comments:** Why does an analytical run need to contain a blank sample? What are the acceptance criteria of that sample? **Proposed change:** An analytical run **may** contain a blank sample, which can be used as an anchor point for calibration curve fitting.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
902-907	3	**Comments:** delete **Proposed change:** An analytical run consists of a ~~blank sample~~, calibration standards at a minimum of 6 concentration levels, at least 3 levels of QCs (low, medium and high) applied as two sets (or at least 5% of the number of study samples, whichever is higher) and the study samples to be analysed. ~~The blank sample should not be included in the calculation of calibration curve parameters. The QCs should be placed in the run in such a way that the accuracy and precision of the whole run is ensured taking into account that study samples should always be bracketed by QCs.~~ *Justification: refer to comments for line 791-797 wrt blank sample. Delete last sentence, as bracketing only makes sense for instruments on which samples are read out in a linear mode or the test system is influenced by a prolonged read time.*
902-907	4	**Comments:** delete **Proposed change:** An analytical run consists of a ~~blank sample~~, calibration standards at a minimum of 6 concentration levels, at least 3 levels of QCs (low, medium and high) applied as two sets (or at least 5% of the number of study samples, whichever is higher) and the study samples to be analysed. ~~The blank sample should not be included in the calculation of calibration curve parameters. The QCs should be placed in the run in such a way that the accuracy and precision of the whole run is ensured taking into account that study samples should always be bracketed by QCs.~~ *Justification: refer to comments for line 791-797 wrt blank sample. Delete last sentence, as bracketing only makes sense for instruments on which samples are read out in a linear mode or the test system is influenced by a prolonged read time.*
902-907	6	**Comments:** The term blank is ambiguous, and it is suggested that the term blank matrix is used.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: "An analytical run consists of a blank **matrix** sample, calibration standards at a minimum of 6 concentration levels, at least 3 levels of QCs (low, medium and high) applied as two sets (or at least 5% of the number of study samples, whichever is higher) and the study samples to be analysed. The blank **matrix** sample should not be included in the calculation of calibration curve parameters. The QCs should be placed in the run in such a way that the accuracy and precision of the whole run is ensured taking into account that study samples should always be bracketed by QCs."
907	2	**Comments:** "Study samples should be bracketed by QCs" difficult on a microplate, may depend on processing direction?
908-909	6	**Comments:** Multiple batches should be demonstrated in validation and if acceptable, multiple standard curves should not be required during sample testing for more than one batch in a run. This would align with the language used for batches and runs in chromatography. **Proposed change:** Change to: ~~"Most often microtitre plates are used for LBAs~~. An analytical run may comprise of one or more ~~plate(s)~~ **batches (e.g., plate, CD etc.).** Typically, each ~~plate~~ **batch** contains an individual set of calibration standards and QCs. If each ~~plate~~ **batch** contains its own calibration standards and QCs then each plate should be assessed on its own. However, for some platforms the sample capacity may be limited. In this case, sets of calibration standards may be placed ~~on~~ **in** the first and the last ~~plate~~ **batch**, but QCs should be placed ~~on~~ **in** every single plate **batch**. QCs should be placed at least at the beginning (before) and at the end (after) of the study samples of each ~~plate~~ **batch**. The QCs ~~on~~ **in** each ~~plate~~ **batch** and each calibration curve should fulfil the acceptance criteria (Refer to Section 4.3.2). For the calculation of concentrations, the calibration standards should be combined to conduct one regression analysis. If the combined calibration curve does not pass the acceptance criteria the whole run fails."
919-923	6	**Comments:** The language in Section 4.3.1 compared with Section 4.3.2 does not align. If the QCs pass in a batch, there should be no

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		requirement to compare all QCs across the whole run. The use of a run would apply to cases where 2 curves have been used for application to multiple batches. **Proposed change:** "Criteria for the acceptance or rejection of an analytical run should be defined in the protocol, in the study plan or in an SOP. ~~In the case that a run contains multiple batches, acceptance criteria should be applied to the whole run and to the individual batches.~~ **Run acceptance is based on the performance of the calibration curves and plate acceptance is based on the performance of the QC samples on each batch (plate, CD etc.)**. It is possible for the run to meet acceptance criteria, even if a batch within that run is rejected for failing to meet the batch acceptance criteria."
924-930	3	**Comments:** Harmonise wording in whole document around anchor points vs anchor calibrators *vs* anchor calibration standard
924-930	4	**Comments:** Harmonise wording in whole document around anchor points vs anchor calibrators *vs* anchor calibration standard
924-930	4	**Comments:** Harmonise wording in whole document around anchor points vs anchor calibrators *vs* anchor calibration standard Proposed change: EBF suggests "Anchor Points"
928-930	6	**Comments:** Please clarify masking procedures as current guidance does not expect unacceptable calibration points to be removed from the curve fit; is it acceptable to keep points outside of acceptance criteria in the curve fit if 75% of the standards and at least 6 standards pass? A priori procedures for rejection/masking of calibration standards that have failed acceptance criteria should be documented in a SOP.
932-933	6	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		It may not be the highest standard (if anchor points are used), the use of the term "ULOQ" would be more appropriate. **Proposed change:** "If the ~~highest~~ **ULOQ** calibration standard is rejected, the new upper limit for the analytical …."
934	6	**Comments:** Use LLOQ and ULOQ **Proposed change:** "The new ~~lower and upper limit~~ **LLOQ and ULOQ** calibration standard will retain their original acceptance criteria (i.e., ±20%). The revised calibration range should cover all QCs (low, medium and high)."
938-940	6	**Proposed change:** Delete first sentence. Move the 2nd sentence to 4.3.1 and start the paragraph with "At least 2/3 of the QCs and 50%"
949-955	2	**Comments:** Please define "clustering". **Proposed change:** We do not see the sense if a fully validated method is used and quantification is done within the validated calibration range, please remove requirements to modify QC concentrations, to add additional QCs or to narrow the calibration range or delete lines 948-955 completely, since this is not applicable to LBAs.
949-955	3	**Comments:** delete paragraph *Justification*: *During method validation it is demonstrated that samples can be diluted into the validated range. Assay range is narrow, fixed and validated for LBA For chromatography methods no change of method is needed when adjusting calibration range, however, for LBA it would mean a new method needs to be established (e.g. titration of reagents, change of*

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		reagents etc **Proposed change:** delete paragraph
949-955	4	**Comments:** delete paragraph *Justification*: *During method validation it is demonstrated that samples can be diluted into the validated range. Assay range is narrow, fixed and validated for LBA For chromatography methods no change of method is needed when adjusting calibration range, however, for LBA it would mean a new method needs to be established (e.g. titration of reagents, change of reagents etc* **Proposed change:** delete paragraph
949-955	6	**Comments:** New requirement for LBA. During validation the range has been proven as acceptable and therefore the narrowing of the calibration range does not add value. Adding of QCs is best practice and not guidance. **Proposed change:** Delete the paragraph
956	3	**Comments:** Many comments came in asking for detailed clarification. An overarching theme was to consider clarifying that reanalysis is related to samples that produced valid results (e.g. >ULOQ or <LLOQ). Reanalysis of samples from failed or rejected runs that did not produce acceptable results should not be viewed as "reanalysis" in the context of this paragraph. Hence we suggest to clearly separated the examples to reflect both cases

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		• Reanalysis of a sample which didn't give a reportable concentration, is not reanalyses per se but generates a 1st reportable result • Reanalysis of a sample for which the 1st reportable result is 'unexpected' (positive placebo, unexpected PK,…), is reanalysis. It should be performed in replicate and compared to the original result with the aim to confirm or disprove this original result. An SOP /decision tree should be in place to guide reanalysis and reporting
956	4	**Comments:** Many comments came in asking for detailed clarification. An overarching theme was to consider clarifying that reanalysis is related to samples that produced valid results (e.g. >ULOQ or <LLOQ). Reanalysis of samples from failed or rejected runs that did not produce acceptable results should not be viewed as "reanalysis" in the context of this paragraph. Hence we suggest to clearly separated the examples to reflect both cases • Reanalysis of a sample which didn't give a reportable concentration, is not reanalyses per se but generates a 1st reportable result • Reanalysis of a sample for which the 1st reportable result is 'unexpected' (positive placebo, unexpected PK,…), is reanalysis. It should be performed in replicate and compared to the original result with the aim to confirm or disprove this original result. An SOP /decision tree should be in place to guide reanalysis and reporting
960-961	6	**Comments:** The number of samples and percentage of total number of samples that have been reanalysed adds no value. More important data is presented in the re-assay table. **Proposed change:** Delete

Line no.	Stakeholder no.	Comment and rationale; proposed changes
963-969	1	**Comments:** Examples of trends that are of concern include: • All samples from one subject fail • All of samples from one run fail All aspects of ISR evaluations should be documented to allow reconstruction of the study and any investigations. Individual samples that are quite different from the original value (e.g., > 50%, "flyers") should not trigger reanalysis of the original sample and do not need to be investigated. ISR sample data should not replace the original study sample data. **Proposed change:** suggest to include If all samples of one subject fail, we can repeat the subject upon investigation and repeated value can be considered for PK and stats evaluations
971-972	6	**Comments:** Pre-dose is also a possibility for multiple dose studies and trough levels may be present. Either use the term naïve pre-dose or add where a concentration is expected. **Proposed change:** "Identification of quantifiable analyte levels in **naïve** pre-dose samples **(where a concentration is not expected)**, control or placebo samples."
973-975	2	**Comments:** Please add the requirement for an acceptance criterion if samples are analysed in replicates (e.g. max 20% CV). If the precision exceeds this limit, the mean signal/concentration values should not be further processed. **Proposed change:** Please clarify

Line no.	Stakeholder no.	Comment and rationale; proposed changes
981	6	**Proposed change:** Delete "~~Further, a summary table of the total number of samples that have been reanalysed due to each reason should be provided of samples that have been reanalysed due to each reason should be provided.~~"
986-987	2	**Comments:** ".. (e.g., pre-dose sample with measurable concentrations) multiple determinations are required where sample volume allows." How many multiple determinations can be performed - needs to be defined? **Proposed change:** Proposal: Two replicate determinations should be performed where sample volume allows.
991	3	**Comments:** ISR = post validation = OK. During production however, ISR something else, i.e. process control, and for this you have QCs. Don't need to have multiple process controls (QC, ISR, Dil QC…) in all studies
991	4	**Comments:** ISR = post validation = OK. During production however, ISR something else, i.e. process control, and for this you have QCs. Don't need to have multiple process controls (QC, ISR, Dil QC…) in all stu
992-996	2	**Comments:** ISR will not allow to identify the descriped effects/differences between study sample and calibrators/QCs. If the study sample shows any of the described effects which is responsible for a bias wrt calibrators/QCs, reanalysis will give the identical (biased) result. **Proposed change:** Proposal: Please focus on the fact that ISR provides information of reproducibility of the assay using real samples.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
992-996	3	**Comments:** If the study sample shows any of the described effects which is responsible for a bias, ISR reanalysis may give the identical (biased) result. hence, ISR is related to reproducibility rather than verification of reliability of data, and accuracy and precision of the analysis. Please consider to rephrase 992-996 and focus on the fact that ISR provides information of reproducibility of the assay using real samples.
992-996	4	**Comments:** If the study sample shows any of the described effects which is responsible for a bias, ISR reanalysis may give the identical (biased) result. hence, ISR is related to reproducibility rather than verification of reliability of data, and accuracy and precision of the analysis. Please consider to rephrase 992-996 and focus on the fact that ISR provides information of reproducibility of the assay using real samples.
1001	2	**Comments:** main nonclinical TK studies **Proposed change:** Please replace by "GLP Tox studies"
1001-1004	3	**Comments:** rephrase **Proposed change:** For preclinical studies, ISR should, in general, be performed for the GLP-regulated toxicokinetic studies once per species.
1001-1004	4	**Comments:** rephrase

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: For preclinical studies, ISR should, in general, be performed for the GLP-regulated toxicokinetic studies once per species.
1005	3	**Comments:** Avoid the term "Pivotal"
1005	4	**Comments:** Avoid the term "Pivotal"
1006-1007	6	**Comments:** Industry have data as published via the European Bioanalysis Forum (EBF) which demonstrates that ISR rarely fails (frequency 1.4%) mostly due by human error (Bioanalysis (2018) 10(21), 1723–1732). Further information published by AstraZeneca: Arfvidsson *et al*. (2018) 10(21), 1733-1745 https://doi.org/10.4155/bio-2018-0162). This should be considered and the requirements for ISR to be reduced. This is ambiguity in what defines a different patient population and needs to be further defined or clarified via training, e.g. is the intention for age, sex, ethnicity, differences in cancer populations to be used in this assessment. The first use of the assay in each lab would be more appropriate as selectivity parameters have been tested in validation. It is proposed that ISR is limited to the first time the method is used in a pivotal PK or TK study and the first time in humans. Selectivity has been assessed in validation for changed populations and therefore testing in ISR adds no scientific value as shown in the EBF and AZ papers. Proposed change: See below • For preclinical studies, ISR should, in general, be performed for the main nonclinical TK ~~studies~~ **study** once per species. However, ISR in a PK study instead of a TK study might also be acceptable, as long as the respective study has been conducted as a pivotal study, used to make regulatory decisions.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		• All pivotal comparative BA/BE studies • First clinical trial in subjects • ~~Pivotal early patient trial(s), once per patient population~~ • First or pivotal trial in patients with impaired hepatic and/or renal function
1007	3	**Comments:** Please avoid the term "pivotal" and clarify if/which Phase III studies and non-PK studies are excluded from the list.
1007	4	**Comments:** Please avoid the term "pivotal" and clarify if/which Phase III studies and non-PK studies are excluded from the list.
1008	6	**Comments:** For LBA methods, the need for conducting ISR is questioned in hepatic and/or renal function. Proposed change: "First or pivotal trial in patients with impaired hepatic and/or renal function (**where scientifically appropriate**)"
1009-1010	3	**Comments:** ISR analysis should be able to be conducted on the same day for methods with stability related issues. We suggest adding text to discuss the ability to conduct analysis on the same day when appropriate.
1009-1010	4	**Comments:** ISR analysis should be able to be conducted on the same day for methods with stability related issues. We suggest adding text to discuss the ability to conduct analysis on the same day when appropriate.
1009-1010	6	**Comments:** The value of running on a separate day is questioned. Some platforms can generate a result within <1 hour, therefore it is

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		suggested that an element of future proofing for new technology is considered in the guideline. **Proposed change:** "ISR is conducted by repeating the analysis of a subset of samples from a given study in separate (i.e., different to the original) runs ~~on different days~~ using the same bioanalytical method. **This may be conducted on the same day if a valid reportable result has been verified**."
1010	2	**Comments:** It should be possible to perform ISR on the same day in an independent analytical run.
1011	6	**Comments:** Move this sentence to the introductory paragraphs. **Proposed change:** Move this sentence to the introductory paragraphs.
1011-1015 1024-1027	5	**Comments:** Proposed sample size and acceptance criteria do not match (see Rudzki P.J. et al. doi: 10.1208/s12248-019-0293-2; and Rudzki P.J. et al. doi: 10.4155/bio-2017-0142). Proposed fixed ratio of clinical samples to be analysed as ISRs results in clinical-study-size-dependent probability of passing ISR test acceptance criteria. Also numerous analyses are conducted after it is known that overall ISR test criteria passed (or failed). Consider replacing "overall ISR result" with "%ISR" (see Table 1 in Rudzki P.J. doi: 10.4155/bio-2017-0142). Thus, scientific background of the test in its current version seems to be questionable. **Proposed change:** ISR methodology and implement a more statistically rationalized and risk-controlled approach using hypergeometric distribution (see Rudzki P.J. et al. doi: 10.1208/s12248-019-0293-2). Consider fixed number of samples to be tested (e.g. 30 samples) as well as possibility of adaptive design (Rudzki P.J. et al. doi: 10.4155/bio-2017-0142) or combination of both.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
1011-1020	3	**Comments:** Please consider to provide a cap, i.e. a maximum for sample number to be analysed as part of ISR. There are strong scientific data suggesting that reanalyses of large portions of samples do not add scientific value. Literature suggests that 30 samples should be sufficient power in any study size. A consensus proposal could be: For ISR, reanalyse 10% of the study of samples, with a minimum of 20 and a maximum of 100 samples **Proposed change:** remove % and move to min/max number of samples
1011-1020	4	**Comments:** Please consider to provide a cap, i.e. a maximum for sample number to be analysed as part of ISR. There are strong scientific data suggesting that reanalyses of large portions of samples do not add scientific value. Literature suggests that 30 samples should be sufficient power in any study size. A consensus proposal could be: For ISR, reanalyse 10% of the study of samples, with a minimum of 20 and a maximum of 100 samples **Proposed change:** remove % and move to min/max number of samples
1012-1015	6	**Comments:** The current requirement for ISR is excessive. Therefore, it is suggested that the total number is reduced and that a maximum number of samples is recommended. Industry have data as published via the European Bioanalysis Forum (EBF) which demonstrates that ISR fails only in 1.4% of case (reference: Bioanalysis (2018) 10(21), 1723–1732). This should be considered and the requirements for ISR to be reduced. **Proposed change:** Delete the current sentences and replace with: "**At least 5% of the number of samples should be assessed up to a suggested maximum of 100 samples**."

Line no.	Stakeholder no.	Comment and rationale; proposed changes
1021-1023	3	**Comments:** ISR should be able to be conducted on the same day, especially if there are stability concerns. Please remove the second part of the sentence to allow ISR on the same day. → rephrase **Proposed change:** Samples should not be pooled, as pooling may limit anomalous findings. ISR samples and QCs should be prepared in the same manner as in the original analysis. ISR should be performed within the stability window of the analyte ~~but not on the same day as the~~ but in a separate run to the original analysis.
1021-1023	4	**Comments:** ISR should be able to be conducted on the same day, especially if there are stability concerns. Please remove the second part of the sentence to allow ISR on the same day. → rephrase **Proposed change:** Samples should not be pooled, as pooling may limit anomalous findings. ISR samples and QCs should be prepared in the same manner as in the original analysis. ISR should be performed within the stability window of the analyte ~~but not on the same day as the~~ but in a separate run to the original analysis.
1023	6	**Comments:** The value of running on a separate day is questioned. Some platforms can generate a result within <1 hour, therefore it is suggested that an element of future proofing for new technology is considered in the guideline. **Proposed change:** Remove: but not on the same day as the original analysis so that the sentence reads: "ISR should be performed within the stability window of the analyte~~., but not on the same day as the original analysis.~~"
1028-1033	3	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		This is vague and ether needs removal or clarification – suggested rephrasing **Proposed change:** If the overall ISR results fail the acceptance criteria, an investigation should be conducted and the causes remediated. There should be an SOP that directs how any investigations are triggered and conducted. If ISR meets the acceptance criteria yet shows large or systematic differences between results for multiple samples, this may indicate analytical issues and it is advisable to investigate this further. The potential impact of an ISR investigation on study validity should be provided in the bioanalytical report.
1028-1033	4	**Comments:** This is vague and ether needs removal or clarification – suggested rephrasing **Proposed change:** If the overall ISR results fail the acceptance criteria, an investigation should be conducted and the causes remediated. There should be an SOP that directs how any investigations are triggered and conducted. If ISR meets the acceptance criteria yet shows large or systematic differences between results for multiple samples, this may indicate analytical issues and it is advisable to investigate this further. The potential impact of an ISR investigation on study validity should be provided in the bioanalytical report.
1031-1040	6	**Comments:** Remove lines 1031-1036; if criteria are met, it has passed acceptance. The language is ambiguous and is open to misinterpretation. **Proposed change:** Remove lines 1031-1036 to read: ~~"If ISR meets the acceptance criteria yet shows large or systemic differences between results for multiple samples, this may indicate analytical issues and it is advisable to investigate this further.~~

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		~~Examples of trends that are of concern include:~~ • ~~All samples from one subject fail~~ • ~~All of samples from one run fail~~ If an investigation does not identify the cause of the failure, the potential impact of an ISR failure on study validity should also be provided in the Bioanalytical Report. All aspects of ISR evaluations should be documented to allow reconstruction of the study and any investigations. Individual samples that are quite different from the original value (e.g., > 50%, "flyers") should not trigger reanalysis of the original sample. ISR sample data should not replace the original study sample data."
1034	3	**Comments:** add **Proposed change:** Examples of trends that are of concern may include:
1034	4	**Comment;** add **Proposed change:** Examples of trends that are of concern may include:
1034-1036	5	**Comments:** There can be also time- and concentration-dependent trends (see Rudzki PJ et al. doi: 10.4155/bio-2017-0210). **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Add: time- and concentration-dependent trends.
1040	5	**Comments:** To facilitate at-a-glance evaluation of ISR data, the combination of modified Bland-Altman plot and Cumulative ISR plot could be recommended for ISR reporting (see Rudzki P.J. et al. doi: 10.4155/bio-2017-0038). Recommending standard for graphical presentation of ISR data would facilitate dossier evaluation and comparison of methods between laboratories. **Proposed change:** It is recommend to support ISR reporting with visual data presentation, e.g. using Bland-Altman plot with fixed limits and Cumulative ISR plot.
1047-1070	6	**Comments:** Combine the chromatography and LBA examples for partial validation rather than separating them. Proposed change: "For chromatographic **and LBA methods**, typical bioanalytical method modifications or changes that fall into this category include, but are not limited to, the following situations: • A change in analytical methodology (e.g., change in detection systems, platform) • A change in sample processing procedures • A change in sample volume (e.g., the smaller volume of paediatric samples) • Changes to the calibration concentration range • A change in anticoagulant (but not changes in the counter-ion) in biological fluids (e.g., heparin to ethylenediaminetetraacetic acid (EDTA)) • Change from one matrix within a species to another (e.g., switching from human plasma to serum or cerebrospinal fluid) or changes to the species within the matrix (e.g., switching from rat plasma to mouse plasma) • A change in storage conditions

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		For LBAs, additional typical bioanalytical method modifications or changes may also include changes in MRD or use of a different binding reagent (e.g. change in monoclonal clone). ~~For LBAs, typical bioanalytical method modifications or changes that fall into this category include, but are not limited to, the following situations:~~ ~~☐ Changes in LBA critical reagents (e.g., lot-to-lot changes)~~ ~~☐ Changes in MRD~~ ~~☐ A change in storage conditions~~ ~~☐ Changes to the calibration concentration range~~ ~~☐ A change in analytical methodology (e.g., change in detection systems, platform)~~ ~~☐ Analytical site change using same method (i.e., bioanalytical method transfers between laboratories)~~ ~~☐ A change in sample preparation~~"
1049-1050	3	**Comments:** remove – this is not a change of a method.
1049-1050	4	**Comments:** remove – this is not a change of a method.
1054	3	**Comments:** Rephrase Proposed change: Extension of calibration range below LLOQ or above ULOQ
1054	4	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Rephrase Proposed change: Extension of calibration range below LLOQ or above ULOQ
1060	3	**Comments:** Add Proposed change: A change in sample storage conditions
1060	4	**Comments:** Add Proposed change: A change in sample storage conditions
1063	4	**Comments:** delete Proposed change: ~~Changes in LBA critical reagents (e.g., lot-to-lot changes)~~
1063	6	**Comments:** Critical reagents do not need to be part of partial validation and may be managed as a reagent bridging activity. It should be clear that the documentation for bridging critical reagents does not need to be a formal validation activity. **Proposed change:** Remove line 1063 "~~Changes in LBA critical reagents (e.g., lot-to-lot changes)~~"
1065	3	**Comments:** Add

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: A change in sample storage conditions
1065	4	**Comments:** Add **Proposed change:** A change in sample storage conditions
1066	3	**Comments:** Rephrase **Proposed change:** Extension of calibration range below LLOQ or above ULOQ
1066	4	**Comments:** Rephrase **Proposed change:** Extension of calibration range below LLOQ or above ULOQ
1068-1069	3	**Comments:** remove – this is not a change of a method.
1068-1069	4	**Comments:** remove – this is not a change of a method.
1073	3	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Guideline needs to better explain why cross validation is needed. Propose to add **Proposed change:** Cross validation is conducted to evaluate the bias between methods (or laboratories) such that the results from studies using them can be appropriately interpreted. Cross validation allows the comparison of two methods (labs) and informs us how they are related.
1073	4	**Comments:** Guideline needs to better explain why cross validation is needed. Propose to add **Proposed change:** Cross validation is conducted to evaluate the bias between methods (or laboratories) such that the results from studies using them can be appropriately interpreted. Cross validation allows the comparison of two methods (labs) and informs us how they are related.
1076-1078	2	**Comments:** See comment for lines 276-279
1076-1078 and 1081-1082	6	**Comments:** These lines contradict each other; 1076-1078 requires cross validation when data are combined whereas 1081-1082 does not require cross validation across studies when different laboratories have used the same method, but each lab has solely analysed a study. **Proposed change:** Remove 1076-1078 or define exactly which studies where this would apply
1081-1086	6	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		The flexibility to use QCs and/or study samples should be permissible. Additionally, line 1083 should be removed. **Proposed change:** "Cross validation is not generally required to compare data obtained across studies from different laboratories using the same validated method at each site. Cross validation should be assessed by measuring the same set of QCs (low, medium and high) in triplicate and**/or** study samples that span the study sample concentration range (if available n≥30) with both assays or in both laboratories."
1083	3	**Comments:** Although this is the same wording included in the EMA, it is too specific. It is just a company risk. Doing it sooner or later will not affect the quality of the data if the final cross-validation result is accepted **Proposed change:** ~~Cross validation should be performed in advance of study samples being analysed, if possible.~~
1083	4	**Comments:** Although this is the same wording included in the EMA, it is too specific. It is just a company risk. Doing it sooner or later will not affect the quality of the data if the final cross-validation result is accepted **Proposed change:** ~~Cross validation should be performed in advance of study samples being analysed, if possible.~~
1084-1086	2	**Comments:** triplicate analysis of QC samples **Proposed change:** Please change to "… ***at least*** in triplicate …"

Line no.	Stakeholder no.	Comment and rationale; proposed changes
1084-1086	3	**Comments:** rephrase **Proposed change:** Cross validation should be assessed by measuring the same set of QCs (low, medium and high) in triplicate or study samples that span the study sample concentration range (if available n≥30) with both assays or in both laboratories
1084-1086	4	**Comments:** rephrase **Proposed change:** Cross validation should be assessed by measuring the same set of QCs (low, medium and high) in triplicate or study samples that span the study sample concentration range (if available n≥30) with both assays or in both laboratories
1085	5	**Comments:** Cross validation is a similar problem to ISR and basing on hypergeometric distribution n = 30 seems to be sufficient number of samples to be evaluated (see Rudzki P.J. et al. doi: 10.1208/s12248-019-0293-2) **Proposed change:** (if available n≥30) => (if available n=30)
1087-1091	3	**Comments:** More discussion will be needed to understand the intention and the practical implementation of this new requirement. Not having general acceptance criteria is OK, but is new to the BA community. Hence, here needs to be systematic education all involved (industry, regulators), E.g. Who owns the decision/impact/application of correct factor?
1087-1091	4	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		More discussion will be needed to understand the intention and the practical implementation of this new requirement. Not having general acceptance criteria is OK, but is new to the BA community. Hence, here needs to be systematic education all involved (industry, regulators), E.g. Who owns the decision/impact/application of correct factor?
1090 - 1093	6	**Comments:** Disproportional bias needs to be defined *a priori*. Language is required to distinguish between individual analytes; multiple methods may be used to define different components of molecules (e.g. ADCs). **Proposed change:** "If disproportionate bias is observed between methods **(as defined *a priori* in an SOP or validation plan)**, the impact on the clinical data interpretation should be assessed. The use of multiple bioanalytical methods per analyte or individual analyte in the conduct of one comparative BA/BE study is strongly discouraged."
1094	2	**Comments:** A separate section with requirements for free analyte assays could be added
1094	6	**Comments:** The purpose of this section could be clearer. Additionally, it would benefit from an introduction to explain that these are not mandatory requirements, especially considering ICH regions that will use this as a law rather than guidance. **Proposed change:** Rename section to "**Method Development and** additional considerations"
1095	3	**Comments:** change title **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Endogenous homologue compounds
1095	4	**Comments:** change title **Proposed change:** Endogenous homologue compounds
1095-1137	2	**Comments:** This is a great section! However, this topic is more relevant for biomarker analysis (which is out of scope) and only relevant in some cases of replacement therapy. Typically at least slightly modified versions of endogenous compounds are developed as drugs, thus specific bioanalysis is possible. Given the relevance of this as compared to the relevance of challenges we observe for accurate free drug quantification/calibration of target capture LBAs at low concentrations, the paragraph is too long.....and the more important challenges to the BA community should at least be a bit more valued. **Proposed change:** Shorten significantly.
1095-1198	3	**Comments:** Industry consolidated comment = suggest rewriting this section as it is too detailed and prescriptive.
1095-1198	4	**Comments:** Industry consolidated comment = suggest rewriting this section as it is too detailed and prescriptive.
1095-1198	4	**Comments:** in addition to above comment, EBF suggests that the re-written section at least contains the "background addition approach"
1095-1198	6	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		This section is overly prescriptive. Propose that this section is re-written with examples only listed (but not limited to) and the "how to" details removed. The uses of alternate matrices or approaches should be justified. The background addition approach is missing. **Proposed change:** Section is re-written with examples (with background addition added to those examples) but the detail removed.
1099-1105	2	**Comments:** See comment for line 417
1104	5	**Comments:** The same comment as line 417: Blank matrix does not need to be free of interference if the IS is compensating matrix effect. Proposed change: should be free of ~~matrix effect and~~ endogenous analyte at the level that causes interference
1124	2	**Comments:** Please define "authentic analyte" and "authentic biological matrix", is "actual" meant?
1131	2	**Comments:** Please consider also use of surrogate matrix without the endogenous compound to enable preparation of QCs with defined concentrations.
1199	2	**Comments:** Use of blank matrix containing endogenous binding protein (e.g. soluble target) will automatically result in failed parallelism (due to formation of complexes) in case of target capture/free drug assays **Proposed change:** Please see proposed change in line 849 and add it here as well.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
1200-1215	3	**Comments:** rephrase **Proposed change:** …is suspected during study sample analysis. ~~Parallelism investigation or the justification for its absence should be included in the Bioanalytical Report.~~ Where assessed, parallelism investigation should be reported. As parallelism assessments ….. should be defined a priori.
1200-1215	4	**Comments:** rephrase Proposed change: …is suspected during study sample analysis. ~~Parallelism investigation or the justification for its absence should be included in the Bioanalytical Report.~~ Where assessed, parallelism investigation should be reported. As parallelism assessments ….. should be defined a priori.
1200-1215	6	**Comments:** The assessment of parallelism as a for cause activity is welcomed. Delete lines 1204 to 1215. Proposed change: "Parallelism is defined as a parallel relationship between the calibration curve and serially diluted study samples to detect any influence of dilution on analyte measurement. Although lack of parallelism is a rare occurrence for PK assays, parallelism of LBA should be evaluated on a case-by-case basis, e.g., where interference caused by a matrix component (e.g., presence of endogenous binding protein) is suspected during study sample analysis. Parallelism investigations should be included in the Bioanalytical Report when performed. ~~Parallelism investigation or the justification for its absence should be included in the Bioanalytical Report. As parallelism assessments are rarely possible during method development and method validation due to the unavailability of study samples and parallelism is strictly linked to the study samples (i.e., an assay may have perfectly suitable parallelism for a certain population of samples, yet lack it for another population), these experiments should be conducted during the analysis of the study samples. A high concentration study sample (preferably close to Cmax) should be diluted to at least three concentrations with blank matrix. The precision between samples in a dilution series should not exceed 30%. However, when applying the 30% criterion, data should be carefully monitored as results that pass this criterion may still reveal trends of~~

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		~~non-parallelism. In the case that the sample does not dilute linearly (i.e., in a non-parallel manner), a procedure for reporting a result should be defined *a priori*.~~"
1216-1225	5	**Comments:** Placing this paragraph in the section "Additional considerations" makes recovery a bit hidden. Moreover, recovery is not mentioned in the previous paragraphs, excluding "method development". **Proposed change:** Move the paragraph to the section 3.2. with set out information that it should be evaluated only for methods using extraction.
1217-1225	3	**Comments:** add **Proposed change:** For methods that employ sample extraction, the recovery (extraction efficiency) should be evaluated during method development.
1217-1225	4	**Comments:** add **Proposed change:** For methods that employ sample extraction, the recovery (extraction efficiency) should be evaluated during method development.
1219-1222	5	**Comments:** The procedure of the recovery estimation should be more precise. **Proposed change:** Recovery is determined by comparing the analyte response in a biological sample that is spiked with the analyte and processed,

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		with the response in a biological blank sample that is processed and then spiked with the analyte post-extraction.
1223-1225	5	**Comments:** Adaptive design is advised – if bracketing (low and high QC concentrations) gives consistent results then no further experiments are necessary. If not then use also medium QC. **Proposed change:** Recovery experiments are recommended to be performed by comparing the analytical results for extracted samples at low and high QC concentrations. If the data is not consistent then medium QC concentration should be tested.
1226	2	**Comments:** More flexibility would be desirable. If absence of any matrix effect is shown during validation e.g. overlapping calibration curves at different matrix concentrations, varying sample dilutions during sample analysis could be made possible. Example if a sample is BLQ in an assay runing in 10% of matrix, reanalysis at a lower MRD (higher % of matrix), e.g. in 50 or 100% of matrix, would be possible to increase the sensitivity. This would be highly valuable for dose escalation studies, particularly when MABEL approach is applied.
1238	6	**Comments:** Allow use of kits rather than just repurposing a kit (e.g. generic IgG kits). Add "uses a kit". **Proposed change:** "If an applicant **uses a kit,** repurposes a kit (instead of developing a new assay) or utilises "research use only" kits to measure chemical or biological drug concentrations during the development of a novel drug, the applicant should assess the kit validation to ensure that it conforms to the drug development standards described in this guideline."
1238-1241	3	**Comments:** rephrase

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: If an applicant uses a kit, repurposes a kit (instead of developing a new assay) or utilises "research use only" kits to measure chemical or biological drug concentrations during the development of a novel drug, the applicant should ~~assess the kit~~ perform a validation to ensure …
1238-1241	4	**Comments:** rephrase **Proposed change:** If an applicant uses a kit, repurposes a kit (instead of developing a new assay) or utilises "research use only" kits to measure chemical or biological drug concentrations during the development of a novel drug, the applicant should ~~assess the kit~~ perform a validation to ensure …
1242	3	**Comments:** rephrase **Proposed change:** Validation specific considerations for kit assays include, but are not limited to, the following:
1242	4	**Comments:** rephrase **Proposed change:** Validation specific considerations for kit assays include, but are not limited to, the following:
1242-1261	6	**Comments:** The bullet points should be removed, and a statement added that the same principles should be applied to kits as for new assays. The requirements for kits listed are not specifically different.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: Remove line 1242 and lines 1243-1261. Replace with "**Kits are subject to the same validation standards as required for newly developed assays**."
1243-1247	3	**Comments:** Add *Justification: As described in this guideline, the same principles of validation apply to the use of kits and validation should be performed under actual conditions of use in the facility conducting the sample analysis.* **Proposed change:** Kits components should be considered as a source of critical reagents (refer to Section 4.1.2)
1243-1247	4	**Comments:** Add *Justification: As described in this guideline, the same principles of validation apply to the use of kits and validation should be performed under actual conditions of use in the facility conducting the sample analysis.* **Proposed change:** Kits components should be considered as a source of critical reagents (refer to Section 4.1.2)
1246-1247	2	**Comments:** Ambiguous wording, the whole method should be validated, not only the modifications **Proposed change:** Please change "Modifications from kit processing instructions should be completely validated to "***Final assay procedure*** should be completely validated".
1248-1250	2	**Comments:** Why do we not allow to show that the one- or two point calibration is sufficient to provide accurate data? In the end the quality of

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		data counts, not the number of calibrators. **Comments:** Here more flexibility is given -> justified use of alternative matrices! **Proposed change:** Please use the same wording as in section 4.2.3
1248-1250	3	**Comments:** remove
1248-1250	4	**Comments:** remove
1254-1256	3	**Comments:** Suggest to allow same flexibility in use of surrogate/alternative matrix for preparation of calibration standards and QCs for standard PK assays (see section 4.2.3) as it is allowed for kits described in section 7.5 as long as the use is verified and justified.
1254-1256	4	**Comments:** Suggest to allow same flexibility in use of surrogate/alternative matrix for preparation of calibration standards and QCs for standard PK assays (see section 4.2.3) as it is allowed for kits described in section 7.5 as long as the use is verified and justified.
1259-1261	3	**Comments:** remove
1259-1261	4	**Comments:** remove

Line no.	Stakeholder no.	Comment and rationale; proposed changes
1263-1275	2	**Comments:** Here the guideline is inconsistent. If a method is fully validated for a given analyte, there should be no difference when another technology is used for the identical analyte. This could be made clear by introducing that the guideline covers "total" drug quantification only. Differences between assays or assay technologies will be observed if one assay determines e.g. total drug and another "free" drug.....but correct free drug quantification is not in the scope of the current document (unfortunately). If the assay detects different analyte "species" (e.g. free and total) a cross validation will never be successful. **Proposed change:** Consider x-validation only ***if the same analyte species*** are to be analysed by different methods or technologies
1265-1273	3	**Comments:** Replace the sentence describing "cross validation" to reflect that the two techniques/technologies should be compared to understand and establish the correlation between the two measurements while being mindful that the two measurements can give different values.
1265-1273	4	**Comments:** Replace the sentence describing "cross validation" to reflect that the two techniques/technologies should be compared to understand and establish the correlation between the two measurements while being mindful that the two measurements can give different values.
1265-1293	6	**Comments:** A new platform/technology is already defined in partial and cross validation and the use of cross validation should be removed from this section. It needs to be clearer that the intention is for new methods that are not covered earlier in the document and that novel or repurposed technologies may have different requirements. The DMM is an example of when you need to consider what is novel about the technology and should not be a prescriptive list for the DMM technology itself. Proposed change: Rewrite this section and only use DMM as an example rather than a separate sub-section.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
1266	1	**Comments:** In the column "Validation report" per the 4th bullet point, in a table of within- and between- run QC results, values outside should be clearly marked. We welcome the requirement to clearly mark the values outside of the acceptance criteria, and propose to widen it. **Proposed change:** widen the requirement for clearly marking the values outside of acceptance criteria to cover both QC and Calibration Standards results, in both validation report and bioanalytical report.
1267 (Table 1)	1	**Comments:** For comparative BA/BE studies, IS response plots for each analytical run, including failed runs should be include in the report. IS response plots are generating for each analytical run and this data is part of the raw data. Is it required to mention in the report? **Proposed change:** We suggest, it is not required to mention in the report since IS response plots are generating for each analytical run and this data is part of the raw data and also IS is unit concentrations as long as batch is accepted based on the QC acceptance.
1267 (Table 1)	1	**Comments:** For comparative BA/BE studies, original and reintegrated chromatograms and initial and repeat integration results. The reintegration is performed before saving the result table with the pre-set integration parameters and therefore original integrated chromatograms are not available. **Proposed change:** We are proposing to modify the statement as "rationale for re integration should be clearly described and documented. Audit trials

<table>
<tr><th>Line no.</th><th>Stakeholder no.</th><th>Comment and rationale; proposed changes</th></tr>
<tr><td></td><td></td><td>should be maintained”</td></tr>
<tr><td>1267 (Table 1)</td><td>1</td><td>Comments:

In the column “Bioanalytical report”, first bullet point: “For and comparative BA/BE studies, 100% of chromatograms” - typo mistake

Proposed change:

change “For and comparative BA/BE studies” into “For comparative BA/BE studies”

Comments:

Columns “Validation report” and “Bioanalytical report”, third and first bullet point, respectively - 100% of chromatograms are required to be included in both validation report and bioanalytical report.

We appreciate the need for data transparency, however considering that many sponsors are yet to upgrade their archiving systems from paper to validated electronic ones, we see the requirement for 100% of chromatograms to be routinely included in the validation report and bioanalytical report as excessive.

Proposed change:

Bioanalytical report: For comparative BA/BE studies, all chromatograms from the runs which include 20% of the subjects, including the corresponding QC samples and calibration standards. Additional chromatograms should be available on request.

Validation report: For comparative BA/BE studies, all chromatograms should be available on request.</td></tr>
<tr><td>Table 1: Documentation at Analytical Site</td><td>6</td><td>Proposed change: (see inserted table)
<table><tr><th>Items</th><th>Documentation at the Analytical Site</th></tr><tr><td>Reference Standards</td><td>Comment: There is a difference between the interpretation of a reference standard between CC and LBA - Vials are not numbered for LBA and therefore usage</td></tr></table></td></tr>
</table>

Line no.	Stakeholder no.	Comment and rationale; proposed changes	
			is not tracked in the same way for LBA. Clarification is needed on the level of tracking required. The term "log records" can be ambiguous. Proposed change: "~~Log records~~ **Records** of …"
		Internal Standard	Proposed change: "~~Log records~~ **Records** of …"
		Critical Reagents	Add: **If expired or bridged, documentation supporting use**
		Calibration Standards and QCs	Comment: Single use aliquots are typically used and makes this redundant. For laboratories without LIMS this becomes a resource intensive requirement. Proposed change: delete text in brackets on 2nd bullet.
		Analysis	Comment: Instrument use can be tracked within the system and therefore the requirement for instrument logs should be removed. Given that many labs are going electronic for raw purposes and sustainability reasons, additional paper logs should be discouraged. Additionally, the requirement should be limited to instrumentation that generates analytical data and LIMS, if applicable. Proposed change: Bullet points on last bullet can be removed as redundant - change to Validation information,

<table>
<tr><th>Line no.</th><th>Stakeholder no.</th><th>Comment and rationale; proposed changes</th></tr>
<tr><td></td><td></td><td>
<table>
<tr><td></td><td>documentation and data.</td></tr>
<tr><td>Deviations from Procedures</td><td>Comment: Change bullet 2 to “investigations when conducted”. "Unexpected events" is ambiguous and may be interpreted differently.

Proposed change: “Investigation(s) <s>of unexpected events</s> when conducted”</td></tr>
</table>
</td></tr>
<tr><td>Table 1: Validation Report</td><td>6</td><td>
Proposed change: (see inserted table)
<table>
<tr><th>Items</th><th>Validation Report*</th></tr>
<tr><td>Internal Standard</td><td>Proposed change: Origin and source are the same. For consistency of the terminology used, “origin” should be changed to “source”</td></tr>
<tr><td>Critical Reagents</td><td>Proposed change: Add “Concentration, if applicable”</td></tr>
<tr><td>Calibration Standards and QCs</td><td>Stability period for standards and QCs (long term) is not yet established during validation. Suggest removing this requirement.

Proposed change: Remove text in bracket on bullet 3 and dates of preparation from bullet 2 (held at site)</td></tr>
<tr><td>SOPs</td><td>Proposed change: Change to a description and overview of the method rather than detailed description of the method</td></tr>
</table>
</td></tr>
</table>

<table>
<tr><th>Line no.</th><th>Stakeholder no.</th><th>Comment and rationale; proposed changes</th></tr>
<tr><td></td><td></td><td>
<table>
<tr><td>Analysis</td><td>Analysis dates are not needed in the Table of all runs - remove. Appending a report should not be mandatory, some activities for validation may exist as a stand-alone report and would be included in a submission.

Proposed change: Remove response functions (calibration curve parameters) from bullet 3; a representative curve fit can be used in the report. 4th bullet - Values outside acceptance criteria should be clearly marked. 6th bullet - remove the list of parameters (e.g. recovery is an additional consideration so this list adds no value).</td></tr>
<tr><td>Chromatograms and Reintegration</td><td>Proposed change: Remove “reintegration” from bullet 1 and bullet 2. "Run summary sheet" needs to be further defined.</td></tr>
<tr><td>Deviations from Procedures</td><td>Proposed change: Remove supporting data as there should be the option to take the method into an investigation and maybe further development. A summary should suffice, and data kept at the analytical site.</td></tr>
</table>
</td></tr>
<tr><td>Table 1: Bioanalytical Report</td><td>6</td><td>Proposed change: (see inserted table)
<table>
<tr><th>Items</th><th>Bioanalytical Report*</th></tr>
<tr><td>Internal Standard</td><td>Proposed change: Origin and source are the same. For consistency of the terminology used, origin should be changed to “source”</td></tr>
</table>
</td></tr>
</table>

Line no.	Stakeholder no.	Comment and rationale; proposed changes

Critical Reagents	Proposed change: Add “Concentration, if applicable”
Calibration Standards and QCs	Proposed change: Remove stability and preparation dates - stability may not be known - may have a separate stability study on-going
Sample Tracking	Proposed change: Remove requirement for actual sample dates and shipping dates, a range should suffice for shipping dates. A statement stating that all samples were measured within established stability limits is sufficient.
Analysis	Proposed change: Remove response functions (calibration curve parameters) from bullet 3; a representative curve fit can be used in the report. Acceptance criteria of correlation coefficient for chromatography is usually provided. Change "study concentration" to "sample concentration".
Chromatograms and Reintegration	Comment: This statement is confusing if BioA reports are written stand-alone for each study how does the applicant know how many studies are being submitted? My assumption is the expectation that a bioA report is required for each study, pre-clin or clin study. Excessive requirements for preclinical. Proposed change: “For other clinical studies, randomly selected chromatograms from 5% of subject/patient samples representing concentrations across the validated assay range should be provided in each study's bioanalytical report.”

<table>
<tr><th>Line no.</th><th>Stakeholder no.</th><th>Comment and rationale; proposed changes</th></tr>
<tr><td></td><td></td><td>
<table>
<tr><td>Deviations from Procedures</td><td>Proposed change: Remove supporting data. A summary should suffice, and data kept at the analytical site.</td></tr>
<tr><td>Repeat Analysis</td><td>Proposed change: Remove reanalysis SOP - details are likely within the study plan</td></tr>
<tr><td>ISR</td><td>Comment: ISR may be conducted as a stand-alone study or equally reported in the BioA report or appended to the validation report. The flexibility for all approaches should be allowed.</td></tr>
</table>
</td></tr>
<tr><td>1276-1300</td><td>3</td><td>Comments:

This section is too specific and relates only to dried blood spot sampling. The suggested revisions - below- enables the inclusion of all/other dried matrix sampling techniques/technologies</td></tr>
<tr><td>1276-1300</td><td>4</td><td>Comments:

This section is too specific and relates only to dried blood spot sampling. The suggested revisions - below- enables the inclusion of all/other dried matrix sampling techniques/technologies</td></tr>
<tr><td>1277-1281</td><td>3</td><td>Comments:

delete

Proposed change:

Dried matrix methods (DMM) is a sampling methodology that offers benefits such as collection of reduced blood sample volumes <s>as a microsampling technique</s>. In addition to the typical methodological validation for LC-MS or LBA, use of DMM necessitates further assessment of this sampling approach before using DMM in studies that support a regulatory application, such as:</td></tr>
<tr><td>1277-1281</td><td>4</td><td>Comments:</td></tr>
</table>

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		delete **Proposed change:** Dried matrix methods (DMM) is a sampling methodology that offers benefits such as collection of reduced blood sample volumes ~~as a microsampling technique~~. In addition to the typical methodological validation for LC-MS or LBA, use of DMM necessitates further assessment of this sampling approach before using DMM in studies that support a regulatory application, such as:
1282	3	**Comments:** delete **Proposed change:** Haematocrit ~~(especially for spotting of whole blood into cards~~
1282	4	**Comments:** delete Proposed change: Haematocrit ~~(especially for spotting of whole blood into cards)~~
1282-1285	5	**Comments:** Correlating results obtained with dried blood spots to traditional sampling may be problematic in the case of variable drug plasma to blood partition ratio (see R.Z. Hahn et al. http://dx.doi.org/10.1016/j.jpba.2017.11.079). **Proposed change:** add "drug plasma to blood partition ratio"
1283-1284	3	**Comments:** delete

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: Sample homogeneity ~~(especially for sub-punch of the sample on the card/device)~~
1283-1284	4	**Comments:** delete **Proposed change:** Sample homogeneity ~~(especially for sub-punch of the sample on the card/device)~~
1285	3	**Comments:** rephrase Proposed change: ~~DMM sample collection for ISR~~ Consideration for being able to conduct ISR
1285	4	**Comments:** rephrase Proposed change: ~~DMM sample collection for ISR~~ Consideration for being able to conduct ISR
1286-1287	3	**Comments:** remove
1286-1287	4	**Comments:** remove
1288-1289	3	**Comments:** remove

Line no.	Stakeholder no.	Comment and rationale; proposed changes
1288-1289	4	**Comments:** remove
1290-1293	3	**Comments:** rephrase **Proposed change:** When DMM is used for clinical or nonclinical studies in addition to typical liquid approaches (e.g., liquid plasma samples) ~~in the same studies, these two methods should be cross validated as described (Refer to Section 6.2). For nonclinical~~ the comparability of the two methods should be determined using a priori defined correlation approach. In addition, for nonclinical TK studies, refer ~~to Section 4.1 of~~ ICH S3A Q&A. Feedback from the appropriate regulatory authorities is encouraged in early drug development.
1290-1293	4	**Comments:** rephrase **Proposed change:** When DMM is used for clinical or nonclinical studies in addition to typical liquid approaches (e.g., liquid plasma samples) ~~in the same studies, these two methods should be cross validated as described (Refer to Section 6.2). For nonclinical~~ the comparability of the two methods should be determined using a priori defined correlation approach. In addition, for nonclinical TK studies, refer ~~to Section 4.1 of~~ ICH S3A Q&A. Feedback from the appropriate regulatory authorities is encouraged in early drug development.
1294	3	**Comments:** As per our general comment on page 2, we propose to add an additional column, specifically for BA/BE studies. The requirement for reporting for the other studies could be simplified and focus on 'documentation at the analytical site' rather than intensive reporting in Bioanalytical reports
1294	4	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		As per our general comment on page 2, we propose to add an additional column, specifically for BA/BE studies. The requirement for reporting for the other studies could be simplified and focus on 'documentation at the analytical site' rather than intensive reporting in Bioanalytical reports
1300-1301	5	**Comments:** The time of documentation storage should be defined. According to GLP principles 10 years after completion of bioanalytical study is proposed. Proposed change: This documentation may be stored at the analytical site or at another secure location. The documentation should be readily available when requested within 10 years after the study was completed.
1330	6	**Comments:** The documentation requires listed in Table 1 as extensive. For BA/BE studies a higher level of documentation and data are required. The document may benefit from removing the extra requirement for all studies in scope and have a separate column for BA/BE studies. Detailed changes to Table 1 are included in the subsequent comments and proposed changes.
1333	2	**Comments:** **Blank matrix:** Is there no expiry date required? Why is a receipt date required, if an expiry date is not needed?
1334	2	**Comments:** **Sample Tracking in column Bioanalytical Report**: Could it be clarified if sample condition on receipt and total duration from sample collection to analysis is needed to be reported for each single sample or if a summarized description is sufficient. E.g. All sample were receipt frozen in good conditions. All samples were analysed within the stability period. **Proposed change:** Detailed information about receipt of shipments is available in the raw data folders, should not be part of the BAR.
1340	2	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Please add "Sample", "Aliquot", "Replicate" and "Replicate analysis" to the glossary
1340-1549	5	**Comments:** Definitions may start at the same line as term, blank lines are not necessary because terms are in bold. Reducing number of pages of final guideline (which will be printed in thousands copies all over the world) would decrease negative impact to the environment.
1340-1549	5	**Comments:** To facilitate understanding of definitions and to standardise calculations, specific equations should support definitions of validation parameters. Positive example is definition of accuracy supported with equation (line 1345). **Proposed change:** Equations should be added to the following parameters: Matrix effect, Recovery, Reproducibility
1345	3	**Comments:** add **Proposed change:** Accuracy (%) as defined by relative error = Measured Value/Nominal Value x 100
1345	4	**Comments:** add **Proposed change:** Accuracy (%) as defined by relative error = Measured Value/Nominal Value x 100
1345	6	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Accuracy (%) = ((Measured Value-Nominal Value)/Nominal Value) × 100 is incorrect for accuracy. **Proposed change:** "~~Accuracy (%) = ((Measured Value-Nominal Value)/Nominal Value) × 100~~ **Accuracy (%) = (Measured Value/Nominal Value) × 100**"
1351-1353	6	**Comments:** It is a biological rather than biologic matrix **Proposed change:** "A specific chemical moiety being measured, including an intact drug, a biomolecule or its derivative or a metabolite in a biologic**al** matrix."
1363-1365	6	**Comments:** Suggest rewording to be similar to EMA, "Calibration standard points set at ……." remove "analysed" as the anchor points have no acceptance criteria **Proposed change:** "Anchor Calibration Standards/Anchor Points: ~~Spiked samples~~ **Calibration standards** set at concentrations below the LLOQ or above the ULOQ of the calibration curve, **used** ~~and analysed~~ to improve curve fitting in LBAs."
1367-1369	6	**Proposed change:** Add a sentence "**An analytical run may contain multiple distinct batches.**"
1371-1373	6	**Proposed change:** "Batch (for Reference Standards and Reagents):

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		A specific quantity of material produced in a process or series of processes so that it is expected to be homogeneous ~~within specified limits~~. Also referred to as "Lot"."
1382-1383	6	**Comments:** Critical reagents may not only be binding agents **Proposed change:** "Binding Reagent: A reagent that ~~directly~~ binds to the analyte in LBA-based bioanalytical methods."
1385-1386	6	**Comments:** Add "if applicable" to cover LBA methods **Proposed change:** "Blank Sample: A sample of a biological matrix to which no analyte and no IS **(if applicable)** has been added"
1387-1390	6	**Comments:** Concentrations are calculated from a curve. This language is confused by using the term "sample". **Proposed change:** "Calibration Curve: The relationship between the instrument response (e.g., peak area, height or signal) and the concentration (amount) of analyte in the ~~sample~~ **calibration standard** within a given range. Also referred to as Standard Curve."
1392-1396	6	**Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		"Calibration Range: The calibration range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the ~~sample~~ **calibration standard** (including these concentrations) for which it has been demonstrated that the analytical procedure meets the requirements for precision, accuracy and response function."
1393-1396	4	**Comments:** new definition **Proposed change:** The calibration range of an analytical procedure is the interval between the LLOQ and ULOQ of the calibration curve (excluding any anchor point samples) for which it has been demonstrated that the analytical procedure meets the requirements for precision, accuracy and response function.
1405-1406	6	**Comments:** Not all chemically synthesised drugs are small molecules, e.g. chemically synthesised peptides that are submitted as BLA rather than NDA (based on amino acid number). **Proposed change:** "Chemical Drugs: Chemically synthesised drugs. ~~Also referred to as small molecule drugs."~~
1408-1410	3	**Comments:** new definition **Proposed change:** Reagents, including binding reagents (e.g. binding proteins, aptamers, antibodies or conjugated antibodies), that have direct

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		impact on the results of the assay and therefore their quality must be assured.
1408-1410	4	**Comments:** new definition **Proposed change:** Reagents, including binding reagents (e.g. binding proteins, aptamers, antibodies or conjugated antibodies), that have direct impact on the results of the assay and therefore their quality must be assured.
1408-1410	6	**Proposed change:** "Critical Reagent: ~~Critical~~ **Requisite** reagents for LBAs ~~include~~ **including, but not limited to,** binding reagents (e.g., antibodies, binding proteins, peptides) and those containing enzymatic moieties **or are conjugated** that have a direct impact on the results of the assay."
1411-1413	6	**Comments:** To align with the text in the Cross Validation section, the definition should include the understanding of bias rather than direct comparability. **Proposed change:** "Cross Validation: ~~Comparison~~ **Assessment of potential bias between** ~~of~~ two bioanalytical methods or the same bioanalytical method in different laboratories in order to **determine** ~~demonstrate~~ ~~that~~ **whether** the reported data are comparable."
1419-1422	3	**Comments:** new definition **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		A parameter demonstrating that the method can appropriately analyse samples at a concentration exceeding the ULOQ of the calibration curve when diluted within the calibration range in LBAs.
1419-1422	4	**Comments:** new definition **Proposed change:** A parameter demonstrating that the method can appropriately analyse samples at a concentration exceeding the ULOQ of the calibration curve when diluted within the calibration range in LBAs.
1424-1426, 1476-1477	5	**Comments:** Definitions of "Full validation" and "partial validation" should be consistent with definition of "Validation" **Proposed change:** **Full validation** – validation based on evaluation of all validation parameters. **Partial validation** – validation based on evaluation of selected validation parameters. Applicable to methods that were changed after full validation.
1433-1434	6	**Comments:** Only dosed animals or subjects should under-go ISR **Proposed change:** "Incurred Sample: A sample obtained from study subjects or animals **that have received drug**"
1443	5	**Comments:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Analogue is by definitions structurally similar. **Proposed change:** A ~~structurally similar~~ analogue
1452-1454	6	**Comments:** Definition indicates LLOQ is a sample parameter rather than a method parameter **Proposed change:** "The lowest amount of an analyte in a sample that can be quantitatively determined **using a method** with predefined precision and accuracy."
1462-1466	6	**Comments:** Implies that MRD will be performed first before any other dilutions. Propose that flexibility is provided by changing "initial" to "minimum" and changing further to more open language. **Proposed change:** "Minimum Required Dilution (MRD): The ~~initial~~ **minimum** dilution factor by which biological samples are diluted with buffer solution for the analysis by LBAs. The MRD may not necessarily be the ultimate dilution but should be identical for all samples including calibration standards and QCs. However, samples may **undergo or have been subjected to** ~~require further~~ additional dilution."
1479-1483	6	**Proposed change:** "Precision: The closeness of agreement (i.e., degree of scatter) among a series of measurements. Precision is expressed as the coefficient of variation (CV) or the relative standard deviation (RSD) expressed as a percentage.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		~~Precision~~ **CV or RSD** (%) = (Standard Deviation / Mean) x 100”
1488-1491	6	**Proposed change:** “Quality Control Sample (QC): A ~~sample~~ **biological matrix** spiked with a known quantity of analyte that is used to monitor the performance of a bioanalytical method and assess the integrity and validity of the results of the unknown samples analysed in an individual batch or run.”
1500-1503	6	**Proposed change:** “Response Function: ~~A function~~ **A mathematical expression** which adequately describes the relationship between instrument response (e.g., peak area or height ratio or signal) and the concentration (amount) of analyte in the ~~sample~~ **calibration standard**. Response function is defined within a given range. See also Calibration Curve.”
1508-1510	5	**Comments:** Assessment of sensitivity was not described in the main body of the guideline. It was mentioned in the Glossary section only. However, the definition should be supplemented with information regarding signal-to-noise ratio. **Proposed change:** The lowest analyte concentration that can be measured with acceptable accuracy and precision (i.e., LLOQ). The analyte response at the LLOQ should be ≥ five times the analyte response of the blank sample.
1517-1520	6	**Proposed change:** “Standard Curve: The relationship between the instrument response (e.g., peak area, height or signal) and the concentration (amount) of analyte in the ~~sample~~ **calibration standard** within a given range. Also referred to as ~~c~~**C**alibration Curve.”

Line no.	Stakeholder no.	Comment and rationale; proposed changes
1523	5	**Comments:** SOP definition may be supplied with "process". **Proposed change:** Detailed written instructions to achieve uniformity of the performance of a specific function and processes.
1536-1538	6	**Proposed change:** "Upper Limit of Quantification (ULOQ): The upper limit of quantification of an individual analytical procedure is the highest amount of analyte in a sample that can be quantitatively determined **using a method** with pre-defined precision and accuracy."
New to glossary	3	**Comments:** add individual definitions to unambiguously define "sample" " aliquot" and "Replicate Analysis or Measurement" **Proposed change:** suggestions given • Sample = a quantity (of something) from which the general quality (of the whole) may be inferred • Aliquot = any representative portion of the sample • Replicate Analysis or Measurement = The repeated analysis or measurement of the variable of interest performed as identically as possible.
New to glossary	3	**Comments:** add individual definitions to unambiguously define "fresh" **Proposed change:**

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		suggestion given • Prepared on the day of analysis or analysed within stability and the using intermediates which are within known stability (or to be proven stability).'
New to glossary	3	**Comments:** add individual definitions to unambiguously define "initial integration" and "reintegration", which are quite distinct processes and need clear definition and control. Proposed change: suggestions given • Initial integration is defined as the process by which the area (or height) of a chromatographic peak is adequately defined by trained personnel using the most appropriate parameters prior to regression. (consider GBC S1-3 recommendation paper) • Reintegration: any changes, either automatic or manual, applied to individual chromatograms after having established the integration parameters for the run. Re-integration is applied prior to regression and/or calculation of concentrations to ensure non-bias of the process.
New to glossary	3	**Comments:** add individual definition to unambiguously define "geometric mean" Proposed change: suggestion given • Geometric mean is defined as the square root of product of LLOQ and ULOQ
New to glossary	3	**Comments:** add individual definition to unambiguously define "primary matrix" Typically, a study has one primary matrix. Additional matrices should be considered as non-primary matrix. Only in rare cases a study can have multiple primary matrices. It is recommended the primary matrix(ces) is/are clearly defined in the protocol.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
New to glossary	3	**Comments:** add individual definition to define "Reference Standard" **Proposed change:** suggestion given • A well-characterised substance used to prepare calibration and quality control samples. A reference standard should be accompanied by a certificate of analysis or equivalent documentation to prove identity, purity and stability (expiration or retest date)
New to glossary	3	**Comments:** add individual definition to define "Dilution ratio (or factor)" **Proposed change:** suggestion given • The ratio of sample to diluent used to dilute the sample. Also referred to as a dilution factor.
New to glossary	4	**Comments:** add individual definitions to unambiguously define "sample" " aliquot" and "Replicate Analysis or Measurement" Proposed change: suggestions given • Sample = a quantity (of something) from which the general quality (of the whole) may be inferred • Aliquot = any representative portion of the sample • Replicate Analysis or Measurement = The repeated analysis or measurement of the variable of interest performed as identically as possible.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
New to glossary	4	**Comments:** add individual definitions to unambiguously define "fresh" **Proposed change:** suggestion given • Prepared on the day of analysis or analysed within stability and the using intermediates which are within known stability (or to be proven stability).'
New to glossary	4	**Comments:** add individual definitions to unambiguously define "initial integration" and "reintegration", which are quite distinct processes and need clear definition and control. **Proposed change:** suggestions given • Initial integration is defined as the process by which the area (or height) of a chromatographic peak is adequately defined by trained personnel using the most appropriate parameters prior to regression. (consider GBC S1-3 recommendation paper) • Reintegration: any changes, either automatic or manual, applied to individual chromatograms after having established the integration parameters for the run. Re-integration is applied prior to regression and/or calculation of concentrations to ensure non-bias of the process.
New to glossary	4	**Comments:** add individual definition to unambiguously define "geometric mean" **Proposed change:** suggestion given

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		• Geometric mean is defined as the square root of product of LLOQ and ULOQ
New to glossary	4	**Comments:** add individual definition to unambiguously define "primary matrix" Typically, a study has one primary matrix. Additional matrices should be considered as non-primary matrix. Only in rare cases a study can have multiple primary matrices. It is recommended the primary matrix(ces) is/are clearly defined in the protocol.
New to glossary	4	**Comments:** add individual definition to define "Reference Standard" **Proposed change:** suggestion given • A well-characterised substance used to prepare calibration and quality control samples. A reference standard should be accompanied by a certificate of analysis or equivalent documentation to prove identity, purity and stability (expiration or retest date)
New to glossary	4	**Comments:** add individual definition to define "Dilution ratio (or factor)" **Proposed change:** suggestion given • The ratio of sample to diluent used to dilute the sample. Also referred to as a dilution factor.
Additions to glossary terms	6	**Comments:** Add individual definitions to unambiguously define the following:

<table>
<tr><th>Line no.</th><th>Stakeholder no.</th><th>Comment and rationale; proposed changes</th></tr>
<tr><td></td><td></td><td>8. Aliquot
9. Geometric Mean
10. Fresh (to remove ambiguous of fresh and freshly frozen)
11. Pivotal Study (this term is unclear to industry as to which studies are in scope)
12. Primary Matri(ces) (by definition primary = 1 but in the guideline the term is used also in the plural)
13. Purity
14. Replicate (to distinguish between replicate measurement in chromatography and replicate wells used in LBA (e.g. duplicate))
15. Sample
16. Strength
Proposed change:
Add suggested additions (as detailed above) to the glossary</td></tr>
<tr><td>Documentation table 1</td><td>3</td><td>Comments:
Proposed changes to table 1, section “Documentation at the Analytical Site” applicable for BA/BE studies
<table>
<tr><th>Items</th><th>Documentation at the Analytical Site</th><th>Proposed changes or comments</th></tr>
<tr><td>Blank Matrix</td><td>• Records of matrix descriptions, lot numbers, receipt dates, storage conditions, and source/supplier</td><td>Change to – “Description”</td></tr>
<tr><td>Sample Tracking</td><td>• Records that indicate how samples were transported and received. Sample inventory and reasons for missing samples</td><td>Add: Sample inventory and, where available, reasons for missing samples</td></tr>
</table></td></tr>
</table>

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<tr><th>Line no.</th><th>Stakeholder no.</th><th>Comment and rationale; proposed changes</th></tr>
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<tr><td>Audits and Inspections</td><td>• Audit and inspection report</td><td>QA audit reports are not shared</td></tr>
</table>
</td></tr>
<tr><td>Documentation table 1</td><td>3</td><td>Proposed changes to table 1, section “Validation Report” applicable for BA/BE studies
<table>
<tr><th>Items</th><th>Validation Report*</th><th>Proposed changes or comments</th></tr>
<tr><td>Calibration Standards and QCs</td><td>• Batch number, preparation dates and stability period</td><td>Change to – “A list of analytical procedure(s)”</td></tr>
<tr><td>Analysis</td><td>• Instrument ID for each run in comparative BA/BE studies</td><td>Delete</td></tr>
<tr><td>Analysis</td><td>• Table of calibration standard concentration and response functions results (calibration curve parameters) of all accepted runs with accuracy and precision.</td><td>Change to – “Table of calibration standard concentration and response functions results (~~all~~ applicable calibration curve parameters) of all runs, with accuracy and precision of accepted runs”</td></tr>
<tr><td>Analysis</td><td>• 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</td><td>change to: Bench-top, freeze-thaw, long-term, extract, and stored working solution stability</td></tr>
<tr><td>Chromatograms and Reintegration</td><td>• For comparative BA/BE studies, 100% chromatograms of original and reintegration from accepted and fail runs.</td><td>Does not belong in the validation report</td></tr>
<tr><td>Chromatograms and Reintegration</td><td>• Chromatograms may be submitted as a supplement</td><td>Does not belong in the validation report</td></tr>
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<tr><td>Chromatograms and Reintegration</td><td>• 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.</td><td>Provide example of run summary sheet</td></tr>
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<tr><td>Documentation table 1</td><td>3</td><td>
Proposed changes to table 1, section “Bioanalytical Report” applicable for BA/BE studies
<table>
<tr><th>Items</th><th>Bioanalytical Report*</th><th>Proposed changes or comments</th></tr>
<tr><td>Blank Matrix</td><td>• Description, lot number, receipt dates[††]</td><td>remove receipt dates; kept at the analytical site</td></tr>
<tr><td>Sample Tracking</td><td>• Dates of receipt of shipments number of samples, and for comparative BA/BE studies the subject ID</td><td>Change to – “For comparative BA/BE studies, dates of receipt of shipments, number of samples and subject ID”</td></tr>
<tr><td>Sample Tracking</td><td>• Analytical site storage condition and location</td><td>Change to – “Analytical site storage condition”</td></tr>
<tr><td>Sample Tracking</td><td>• List of any deviations from planned storage conditions, and potential impact</td><td>Change to – “List of any deviations from planned storage conditions that impacted on study results”</td></tr>
<tr><td>Analysis</td><td>• Instrument ID for each run in comparative BA/BE studies</td><td>Delete</td></tr>
<tr><td>Analysis</td><td>• 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.</td><td>Change to – “Table of QCs results of all runs with accuracy and precision results of the QCs and between-run accuracy and precision results from accepted runs.”</td></tr>
<tr><td>Chromatograms and Reintegration</td><td>• For comparative BA/BE studies, 100% of run summary sheets of accepted and failed runs, including calibration curve, regression, weighting function, analyte</td><td>Provide example of run summary sheet</td></tr>
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Line no.	Stakeholder no.	Comment and rationale; proposed changes

Analysis	• 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	change to: Bench-top, freeze-thaw, long-term, extract, and stored working solution stability
Chromatograms and Reintegration	• For comparative BA/BE studies, 100% chromatograms of original and reintegration from accepted and fail runs.	Does not belong in the validation report
Chromatograms and Reintegration	• Chromatograms may be submitted as a supplement	Does not belong in the validation report
Chromatograms and Reintegration	• 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.	Provide example of run summary sheet

Documentation table 1 | 4

Proposed changes to table 1, section "Bioanalytical Report" applicable for BA/BE studies

Items	**Bioanalytical Report***	**Proposed changes or comments**
Blank Matrix	• Description, lot number, receipt dates††	remove receipt dates; kept at the analytical site
Sample Tracking	• Dates of receipt of shipments number of samples, and for comparative BA/BE studies the subject ID	Change to – "For comparative BA/BE studies, dates of receipt of shipments, number of samples and subject ID"
Sample Tracking	• Analytical site storage condition and location	Change to – "Analytical site storage condition"
Sample Tracking	• List of any deviations from planned storage conditions, and potential impact	Change to – "List of any deviations from planned storage conditions that impacted on study results"

Line no.	Stakeholder no.	Comment and rationale; proposed changes

Analysis	• Instrument ID for each run in comparative BA/BE studies	Delete
Analysis	• 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.	**Change to –** "Table of QCs results of all runs with accuracy and precision results of the QCs and between-run accuracy and precision results from accepted runs."
Chromatograms and Reintegration	• 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.	Provide example of run summary sheet

Table 1 page 45	5	**Comments:** Row "Analysis" / Column "Bioanalytical report" – text is difficult to understand **Proposed change:** "Table of QCs results of all accepted runs with accuracy and precision results of the QCs and between-run accuracy and precision results from accepted runs." => "Table of QCs results of all accepted runs with between-run accuracy and precision."
Table 1 page 45	5	**Comments:** Row "Analysis" / Column "Bioanalytical report": "QCs graphs trend analysis encouraged" – this should be supported with some recommendations or suggestions in the body of the guideline.
Table 1 page 45	5	**Comments:** Row "Analysis" / Column "Bioanalytical report": "Study concentration results table" – could be also presented in the PK/TK report.

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		Proposed change: Study concentration results table (if not presented in PK/TK report).
Table 1 page 46	5	**Comments:** Row "Chromatograms and Reintegration" / Column "Validation report": "For comparative BA/BE studies, 100% chromatograms of original and reintegration from accepted and fail runs." and "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." – treating validation reports dedicated to BA/BE studies (usually printed in few copies) in different way than other studies is not necessary and is not environmentally friendly. Respective chromatograms for validation report should be enough. In case of any doubts all chromatograms and run summary sheets are available at analytical site and may be send immediately upon request. **Proposed change:** Delete: "For comparative BA/BE studies, 100% chromatograms of original and reintegration from accepted and fail runs." and "For comparative BA/BE studies,100% of run summary sheets of accepted and failed runs, including calibration curve, regression, weighting function, analyte and IS responses and retention times and dilution factor if applicable."
Table 1 page 46	5	**Comments:** Row "Chromatograms and Reintegration" / Column "Bioanalytical report": "For and comparative BA/BE studies, 100% of chromatograms" – printing 100% of chromatograms from reports of BA/BE studies (usually prepared in few copies) is not necessary and is not environmentally friendly. Current practice of 20% chromatograms (i.e. 4 times more than for any other studies) should be continued. In case of any doubts all chromatograms are available at analytical site and may be send immediately upon request. **Proposed change:** For and comparative BA/BE studies, 100% of chromatograms => For and comparative BA/BE studies, 20% of chromatograms
Table 1 page 47	5	**Comments:** Row "Repeat Analysis" / Column "Bioanalytical report": "Reanalysis SOP, if requested" – all data may be send to regulatory

Line no.	Stakeholder no.	Comment and rationale; proposed changes
		authority upon request. It is not necessary to put it in the table. **Proposed change:** Delete: "Reanalysis SOP, if requested"
Table 1 page 47	5	**Comments:** Row "Repeat Analysis" / Column "Bioanalytical report": "SOP for ISR†† (if requested)" – all data may be send to regulatory authority upon request. It is not necessary to put it in the table. **Proposed change:** Delete: "SOP for ISR†† (if requested)"